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Régulation de l'activité et de la connectivité synaptique par les cellules gliales au cours du développement de la jonction neuromusculaire de mammifères

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Résumé

Le système nerveux est composé de milliards de connexions synaptiques qui forment des réseaux complexes à la base de la communication dans le cerveau. Dès lors, contrôler la localisation, le type et le nombre des synapses est un défi considérable au cours du développement du système nerveux. Étonnamment, la production de connexions synaptiques est démesurée de façon à ce que beaucoup plus de synapses soient formées au cours du développement que ce qui est maintenu chez l'adulte. Ces connexions surnuméraires sont en compétition pour l'innervation d'une même cellule cible ce qui mène au maintien de certaines terminaisons nerveuses et à l'élimination de d'autres. Ces processus de compétition et d'élimination sont grandement façonnés par l'activité du système nerveux et l'expérience sensorielle de manière à ce que les terminaisons qui montrent la meilleure activité sont favorisées alors que les synapses mal adaptées sont éliminées.

Jusqu'à récemment, les mécanismes et les types cellulaires responsables de l'élimination synaptique étaient inconnus. Les études de la dernière décennie montrent que les cellules gliales jouent un rôle clé dans l'élimination de synapses. Cependant, il demeure inconnu si les cellules gliales peuvent décoder les niveaux d'activité des terminaisons en compétition, ce qui est un déterminant majeur de l'issue de la compétition synaptique. De plus, il n'est pas connu si les cellules gliales sont capables de réguler l'activité synaptique des terminaisons, ce qui pourrait influencer l'issue de l'élimination synaptique. Ceci est d'un intérêt particulier puisqu'il est connu que les cellules gliales interagissent activement avec les neurones, détectent et modulent leur activité dans plusieurs régions du système nerveux mature.

Par conséquent, l'objectif de cette thèse était d'étudier la capacité des cellules gliales à interagir avec les terminaisons nerveuses en compétition pour l'innervation d'une même cellule cible. Nous avons donc analysé la capacité des cellules gliales à décoder l'activité des terminaisons, à réguler leur activité synaptique et à influencer le processus de l'élimination synaptique au cours du développement du système nerveux. Pour cette fin, nous avons profité

de la jonction neuromusculaire, un modèle simple et le bien caractérisé, et nous avons combiné l'imagerie Ca^{2+} des cellules gliales, un rapporteur fiable de leur activité avec des enregistrements synaptiques de jonctions neuromusculaires poly-innervées de souriceaux.

Dans la première étude, nous montrons que les cellules gliales détectent et décodent l'efficacité synaptique des terminaisons nerveuses en compétition. L'activité des cellules gliales reflète la force synaptique de chaque terminaison nerveuse et l'état de la compétition synaptique. Ce décodage est médié par des récepteurs purinergiques gliaux fonctionnellement distincts et les propriétés intrinsèques des cellules gliales. Nos résultats indiquent que les cellules gliales décodent la compétition synaptique et, par conséquent, sont favorablement positionnées pour influencer son issue.

Dans la seconde étude, nous montrons que les cellules gliales régulent différemment la plasticité synaptique de terminaisons en compétition. De manière dépendante du Ca^{2+}, les cellules gliales induisent une potentialisation persistante de l'activité de la terminaison forte alors qu'elles n'ont que peu d'effets sur la terminaison faible. Bloquer l'activité gliale altère la plasticité des terminaisons *in situ* et se traduit par un retard de l'élimination des synapses *in vivo*. Ainsi, nous décrivons un nouveau mécanisme par lequel les cellules gliales, non seulement renforcent activement la terminaison forte, mais influencent aussi la compétition et l'élimination.

Dans l'ensemble, ces études sont les premières à démontrer que les cellules gliales sont activement impliquées dans la modulation de l'activité synaptique des terminaisons en compétition ainsi que dans la régulation de l'élimination synaptique et la connectivité neuronale.

Mots-clés : Synapse, synaptogenèse, compétition synaptique, élimination synaptique, plasticité synaptique, cellule gliale, cellule de Schwann périsynaptique, jonction neuromusculaire.

Abstract

The nervous system is composed of billions of synaptic connections forming complex networks that define the basis of neuronal communication in the brain. The control of the localization, type and number of synapses is a considerable challenge during development of the nervous system. Surprisingly, there is an excessive production of synaptic connections so that many more synapses are formed during developmental stages than what is maintained in the adult. A process of competition and elimination then occurs during which connections are in competition for the innervation of the same target cell. These processes of competition and elimination are greatly shaped by activity and sensory experience. Nerve terminals that show the best activity are favoured, while weak and poorly adapted synapses are eliminated.

Until recently, the mechanisms and the cell types responsible for the elimination of supernumerary connections were unknown. Studies from the last decade identified glial cells as major players in synapse elimination. However, it remains unknown whether glial cells are able to decode the levels of synaptic activity of competing terminals, which is a major determinant of the outcome of synaptic competition. Moreover, it is unknown whether glial cells are able to regulate synaptic activity, which could influence the outcome of synapse elimination. This is especially relevant because it is known that glial cells actively interact with neurons, detect and modulate their activity in many regions of the nervous system.

Therefore, the goal of this thesis was to study the ability of glial cells to interact with terminals competing for the innervation of the same target cell. We tested the ability of glial cells to decode the activity nerve terminals, regulate their synaptic activity and influence the process of synapse elimination during development of the nervous system. For this purpose, we took advantage of the neuromuscular junction, a simple and well-characterized model, and used simultaneous Ca^{2+}-imaging of glial cells, a reliable reporter of their activity and synaptic recordings of dually-innervated neuromuscular junctions from newborn mice.

In the first study, we report that single glial cells detect and decode the synaptic efficacy of competing nerve terminals. Activity of single glial cells reflects the synaptic strength of each competing nerve terminal and the state of synaptic competition. This deciphering is mediated by functionally segregated purinergic receptors and intrinsic properties of glial cells. Our results indicate that glial cells decode ongoing synaptic competition and, hence, are poised to influence its outcome.

In the second study, we show that glial cells differentially regulate the synaptic plasticity of competing terminals. In a Ca^{2+}-dependent manner, glial cells induce a long lasting synaptic potentiation of strong but not weak terminals. Preventing glial activity alters the plasticity of terminals *in situ* and delays synapse elimination *in vivo*. Thus, we describe a novel mechanism by which glial cells, not only actively reinforce the strong input but regulate synapse competition and elimination.

As a whole, these studies are the first to demonstrate that glial cells are actively involved in the modulation of synaptic activity of competing terminals as well as in the regulation of synapse elimination and neuronal connectivity.

Keywords : Synapse, synaptogenesis, synaptic competition, synapse elimination, synaptic plasticity, glial cells, neuromuscular junction.

Table des matières

Liste des tableaux

Tableaux de l'introduction

Tableaux de l'article de revue dans l'introduction

Liste des figures

Liste des figures de l'introduction

Liste des figures de l'article de revue dans l'introduction

Liste des figures du premier article de recherche

Liste des figures du deuxième article de recherche

Liste des figures de la discussion générale

Liste des abréviations

A1R : Récepteur à l'adénosine de type A1

A2AR : Récepteur à l'adénosine de type A2A

ACh : Acétylcholine

AChE : Acetylcholinesterase

ADO : Adénosine

ATP : Adénosine triphosphate

BDNF : Brain derived neurotrophic factor

ChAT : Choline acétyltransferase

CSP : Cellule de Schwann périsynaptique

DHS : Dépression hétérosynaptique

DLT : Dépression à long terme

EDL : Extensor digitorum longus

EPP : End plate potential

Ex : Par exemple

GABA : Gamma-Aminobutyric acid

GDNF : Glial derived neurotrophic factor

GFAP : Glial fibrillary acidic protein

GTPγS : Analogue non-hydrolysable du guanosine triphosphate

IP3 : Inositol 1,4,5-trisphosphate

IP3R2 : Récepteurs à l'IP3 de type R2

JNM : Jonction neuromusculaire

mAChR : Récepteur muscarinique à l'acétylcholine

nAChR : Récepteur nicotinique à l'acétylcholine

NMDAR : N-methyl-D-aspartate receptor

P2Y1R : Récepteur purinergique de type 2Y1

PLT : Potentialisation à long terme

PPM : Potentiel de plaque motrice

SNARE : Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

SNC : Système nerveux central

SNP : Système nerveux périphérique

SOL : Soleus

TNFα : Tumor necrosis factor alpha

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1. Préambule

Le fonctionnement du système nerveux repose sur la communication entre les neurones au niveau d'une structure spécialisée nommée synapse. La synapse permet la transformation de l'information électrique présynaptique en information chimique qui active une cellule postsynaptique. Ceci est à la base de toute tâche cognitive, quelle que soit sa nature.

Le système nerveux est composé de milliards de connexions synaptiques qui forment des réseaux complexes. Dès lors, contrôler la localisation, le type et le nombre des synapses est un défi considérable. Une grande partie de ce contrôle se ferait lors du développement du système nerveux. Étonnamment, la production de connexions synaptiques lors du développement est démesurée de façon à ce que beaucoup plus de synapses soient formées que ce qui est maintenu chez l'adulte. Il s'ensuit alors un processus de compétition et d'élimination durant lequel des connexions excédentaires luttent pour l'innervation de la même cellule cible. Ces processus de compétition et d'élimination sont grandement façonnés par l'activité du système nerveux et l'expérience sensorielle. Les terminaisons qui montrent la meilleure activité seront favorisées pour gagner cette compétition alors que les synapses mal adaptées seront éliminées. Le choix des synapses à maintenir ou à éliminer est crucial puisqu'il va directement influencer le fonctionnement du système nerveux et la survie de l'organisme.

Jusqu'à récemment, les mécanismes responsables de l'élimination synaptique et les types cellulaires impliqués étaient inconnus. De manière intéressante, les études des dernières années montrent que les cellules gliales jouent un rôle clé dans l'élimination de synapses. Cependant, la manière dont cette élimination est dirigée spécifiquement vers des synapses particulières est mal comprise. De plus, il n'est pas connu si les cellules gliales sont capables de réguler l'activité synaptique des terminaisons, ce qui pourrait influencer l'issue de l'élimination synaptique. Ceci est d'un intérêt particulier puisqu'il est connu que les cellules gliales modulent l'activité des neurones dans le système nerveux mature.

Dans cette thèse, j'ai exploré le rôle des cellules gliales dans la régulation de l'activité des terminaisons et leur influence sur l'issue de l'élimination synaptique. Pour ce faire, j'ai tiré avantage de la simplicité de la jonction neuromusculaire (JNM), synapse formée entre un motoneurone et une fibre musculaire qui permet le contrôle du mouvement volontaire, pour étudier la compétition synaptique et la contribution gliale. C'est un modèle particulièrement intéressant puisqu'il permet la manipulation de chacune des terminaisons en compétition à une même jonction neuromusculaire, d'étudier et de perturber l'activité des cellules gliales et d'observer l'impact sur la compétition et l'élimination synaptique.

Cette thèse comprend principalement deux articles de recherche dont je suis le premier auteur. Le premier article a été publié dans « *The Journal of Neuroscience* ». Le deuxième article à été soumis au journal « *Neuron* ».

Dans l'introduction, je vais présenter le fonctionnement et l'organisation de la synapse, particulièrement de la JNM. Je vais aussi élaborer sur le rôle et la contribution des cellules gliales dans le système nerveux. Ensuite, je vais présenter les mécanismes impliqués dans la formation et la maturation synaptique. Une attention particulière sera portée sur les processus de compétition et d'élimination synaptique ainsi que sur la contribution des cellules gliales à ces phénomènes. Une partie de la section sur la formation et la maturation synaptique est sous forme d'un article de revue publié dans « *Nature Reviews Neuroscience* » dont je suis le premier auteur.

Finalement, la discussion générale va porter sur les aspects importants des études présentées tout en mettant en perspective nos données avec celles dans la littérature. Deux articles de recherches auxquels j'ai participé durant mon doctorat et qui contribuent à la compréhension de la présente thèse se trouvent dans l'annexe I et l'annexe II. L'annexe III contient les persmissions nécessaires pour la reproduction du Tableau 1 ainsi que les figures utilisées qui sont sous droits d'auteurs.

2. Introduction

Le fonctionnement du système nerveux repose sur la communication synaptique. La synapse est à la base de toute fonction nerveuse allant d'un simple mouvement volontaire à des tâches cognitives complexes. De manière simplifiée, elle permet la transformation de l'information neuronale électrique en information chimique qui active une cellule cible. Ceci est possible par la libération de neurotransmetteurs par la terminaison axonale, ce qui active les récepteurs présents sur la cellule postsynaptique.

La communication entre les éléments pré- et postsynaptiques est hautement régulée et montre une plasticité impressionnante en réponse à des changements de l'activité neuronale. La synapse possède une organisation très spécifique de manière à ce que la terminaisonaxonale soit dotée de la machinerie nécessaire pour assurer une libération adéquate et régulée de neurotransmetteurs et un arrangement précis de l'appareil postsynaptique pour assurer une communication efficace. Pour aboutir à cette organisation, la synapse doit subir un long processus de formation et de maturation durant le développement. Une étape cruciale de la maturation synaptique est la compétition et l'élimination synaptique durant laquelle les connexions nerveuses sont remodelées et raffinées. De plus, le fonctionnement et la maturation adéquate des synapses ne dépendent pas uniquement des neurones, mais requièrent une grande contribution des cellules gliales qui accomplissent plusieurs fonctions. Cependant, leur rôle dans la régulation de l'activité synaptique au cours du développement du système nerveux est inconnu. Ainsi, le but de cette thèse était d'étudier le rôle des cellules gliales dans la régulation de l'activité synaptique au cours de la compétition et l'élimination synaptique.

Une des synapses qui a permis de mieux comprendre les phénomènes de communication et de plasticité synaptique ainsi que la synaptogenèse est la jonction neuromusculaire (JNM). En effet, en raison de sa grande taille, son accessibilité et son organisation simple, la JNM a été largement utilisée comme modèle synaptique pour étudier le fonctionnement du système nerveux. Cette synapse est formée entre un motoneurone et une fibre musculaire et permet le contrôle du mouvement volontaire.

Cette introduction va d'abord traiter du fonctionnement de la synapse, particulièrement de la JNM. Ensuite, la contribution des cellules gliales dans le système nerveux sera détaillée. Finalement, les mécanismes impliqués dans la formation et la maturation synaptique seront abordés.

2.1. Le fonctionnement de la synapse

La communication synaptique est un processus complexe qui peut se diviser en 5 étapes principales :

1) La synthèse des neurotransmetteurs dans le neurone présynaptique. Ceci nécessite souvent l'action d'enzymes et de cofacteurs pour la synthèse de neurotransmetteurs à partir de précurseurs.

2) Le stockage des neurotransmetteurs dans la terminaison axonale. Souvent, les neurotransmetteurs sont stockés dans des vésicules synaptiques dans lesquelles ils y sont confinés, protégés d'une potentielle dégradation enzymatique et prêts à être rapidement libérés. Les neurotransmetteurs classiques tels que l'acétylcholine (ACh) et le glutamate sont stockés dans de petites vésicules. Ces vésicules sont concentrées près des zones actives de la membrane présynaptique : sites préférentiels de la libération de neurotransmetteurs.

3) La relâche de neurotransmetteurs dans la fente synaptique. Ceci se produit principalement au niveau des zones actives qui contiennent la machinerie nécessaire pour libérer le contenu des vésicules. Cette libération est induite par une augmentation de la concentration du Ca^{2+} intracellulaire dans la terminaison suite à l'arrivée d'un influx nerveux. La relâche vésiculaire est hautement régulée et requièrt la contribution de complexes moléculaires pour permettre le transport, l'amorçage et la fusion des vésicules synaptiques. Parmi les mieux étudiées, les molécules qui composent le complexe SNARE (*soluble N-ethylmaleimide-sensitive-factor attachment protein receptor*) sont à la base du mécanisme qui contrôle la libération de neurotransmetteurs (voir revue récente Sudhof, 2013). Ce complexe est le lien entre le potentiel d'action, l'entrée de calcium dans la terminaison axonale et la libération vésiculaire. Naturellement, les mécanismes qui assurent la libération de neurotransmetteurs sont hautement régulés.

4) Liaison des neurotransmetteurs aux récepteurs cibles. Une fois libérés dans la fente synaptique, les neurotransmetteurs se lient aux récepteurs postsynaptiques. Ces récepteurs font face aux zones actives et sont maintenus en place par des protéines d'échafaudage telles que « postsynaptic density protein 95 (PSD-95)» (Sheng and Sala, 2001) dans le système nerveux central (SNC) ou Rapsyn (Gautam et al., 1995), à la JNM. À ces récepteurs et molécules d'échafaudage s'ajoutent les protéines responsables de la transduction du signal et de la genèse de la réponse postsynaptique.

5) Cessation de l'action des neurotransmetteurs libérés. Cette étape assure la fin de la réponse synaptique. Selon le neurotransmetteur, l'action de ces derniers peut être terminée de plusieurs façons incluant la diffusion passive des neurotransmetteurs, leur recapture et leur dégradation enzymatique.

La synapse type est celle qui permet la communication entre deux neurones. Cependant, tel que mentionné plutôt, l'une des synapses les mieux étudiées est la JNM qui est formée entre un motoneurone et une fibre musculaire (Figure 2.1).

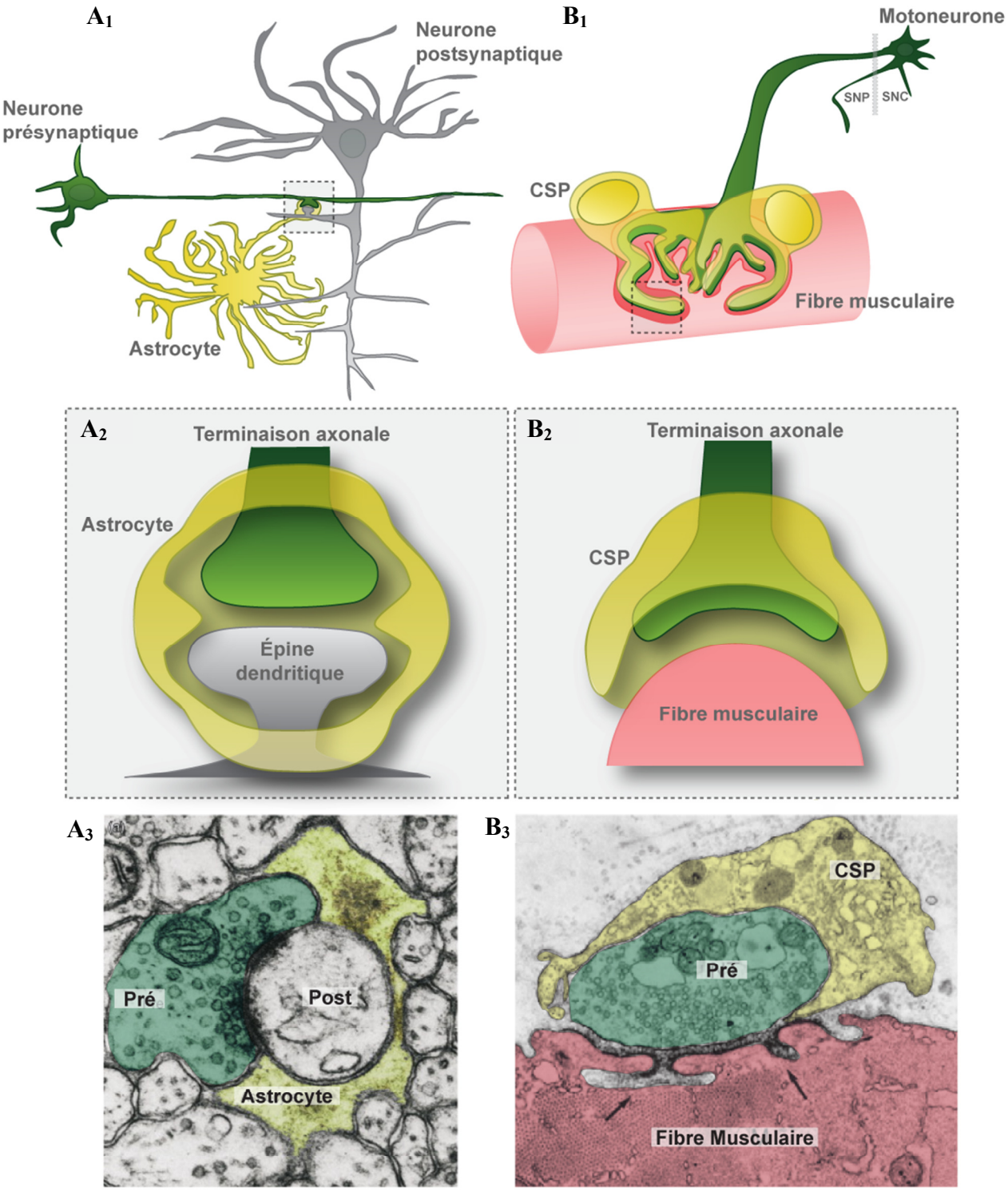

Figure 2.1. Communication synaptique dans le SNC et à la JNM.

A1. Communication entre un neurone présynaptique (vert) avec un neurone postsynaptique (gris) ainsi que la présence d'un astrocyte (jaune) qui contacte la synapse dans le SNC. **A2.**

Au niveau de la synapse, une terminaison axonale est en contact avec une épine dendtritique. Cette synapse est enveloppée par un prolongement astrocytaire qui est à proximité des éléments pré- et postsynaptiques. **A3.** Image en microscopie électronique montrant l'organisation de la synapse tripartie et la proximité des éléments pré- (terminaison axonale en vert), post- (épine dendritique en blanc), et périsynaptique (prolongement d'un astrocyte en jaune). **B1.** Communication entre un motoneurone (vert) et une fibre musculaire (rouge) ainsi que la présence de CSPs (jaune) à la JNM. Notez que le corps cellulaire du motoneurone est dans le SNC alors que son axone et la JNM sont dans le SNP. **B2.** Au niveau de la JNM, une terminaison axonale est en contact avec la fibre musculaire. Cette synapse montre la présence de CSPs qui envoient des prolongements à proximité de la fente synaptique. **B3.** Image en microscopie électronique montrant l'organisation de la synapse tripartite à la JNM et la proximité des éléments pré- (terminaison nerveuse en vert), post- (fibre musculaire en rouge) et périsynaptique (prolongement d'une CSP en jaune). La figure A3 a été modifiée de (Halassa et al., 2007), la figure B3 a été modifiée de Fundamental Neuroscience (4th edition; 2013). P385-403.

2.2. La jonction neuromusculaire

La JNM a largement été utilisée en tant que modèle synaptique. En plus de partager plusieurs similitudes avec les synapses du SNC, la JNM a permis de mieux comprendre les mécanismes impliqués dans la libération de neurotransmetteurs, l'aspect quantique des potentiels synaptiques (Del Castillo and Katz, 1954), la présence de différents réserves de vésicules (Richards et al., 2000; Rizzoli and Betz, 2004) et les mécanismes de plasticité synaptique (Betz, 1970; Magleby and Zengel, 1976). De plus, elle est souvent utilisée pour étudier les interactions neurone-glie (Jahromi et al., 1992; Robitaille, 1998; Rochon et al., 2001; Todd et al., 2010), la synaptogènese (Gautam et al., 1996; Gautam et al., 1995; Miner and Sanes, 1994; Noakes et al., 1995; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001) ainsi que la régénérescence et les maladies neurodégénératives (Arbour et al., 2015; Higuchi et al., 2011; Hoch et al., 2001; Huijbers et al., 2013; Maselli et al., 2012; Miledi and Slater, 1968, 1970; Plomp et al., 2012). Les références citées ne sont que quelques exemples montrant l'importance et l'utilité d'étudier la JNM pour mieux comprendre le fonctionnement du système nerveux.

2.2.1. Organisation de la JNM

La JNM mature est composée d'une seule terminaison nerveuse qui innerve la fibre musculaire. À ces éléments pré- et postsynaptiques s'ajoutent les cellules de Schwann perisynaptiques (CSPs), parfois appelées cellules de Schwann terminales. Tout comme les synapses du SNC, la JNM est dite tripartite et montre la présence d'un élément glial nécessaire à sa fonction (Figure 2.1). En effet, les CSPs, cellules gliales non-myélinisantes à la JNM, sont nécessaires pour la maturation, la communication et le maintien de l'intégrité synaptique. Le nombre de CSPs par JNM varie selon l'espèce et le muscle, mais, de façon générale, il se situe entre 4 et 5 aux JNMs du muscle *Soleus* (SOL) de souris adultes (Hirata et al., 1997; Love and Thompson, 1998).

Chaque terminaison présynaptique montre un haut niveau de branchement et de ramification qui correspond à la forme de la plaque motrice délimitée par la distribution d'une forte densité de récepteurs postsynaptiques. La terminaison axonale est organisée en différentes zones actives qui contiennent une haute densité de vésicules (Figure 2.2). La terminaison contient aussi les enzymes nécessaires à la synthèse des neurotransmetteurs, les transporteurs requis pour leur stockage dans les vésicules synaptiques et la machinerie pour la libération et la recapture de ces neurotransmetteurs. De plus, la terminaison nerveuse montre un grand nombre d'organelles tel qu'un large réseau de réticulum endoplasmique et des mitochondries.

La fibre musculaire montre aussi une certaine spécialisation au niveau de la synapse puisqu'elle contient de nombreux replis ainsi qu'une grande densité de récepteurs cholinergiques pour la détection des neurotransmetteurs libérés. Ces récepteurs se situent aux crêtes des replis et font directement face aux zones actives présynaptiques (Figure 2.2).

Quant aux CSPs, elles couvrent entièrement la JNM mature et montrent plusieurs ramifications et une empreinte territoriale comparable à celle de la terminaison axonale et à la plaque motrice. À la JNM de souris, les prolongements des CSPs n'envahissent pas la fente synaptique, mais sont situés à proximité des sites de libérations de neurotransmetteurs (Figure 2.2; Zuo and Bishop, 2008).

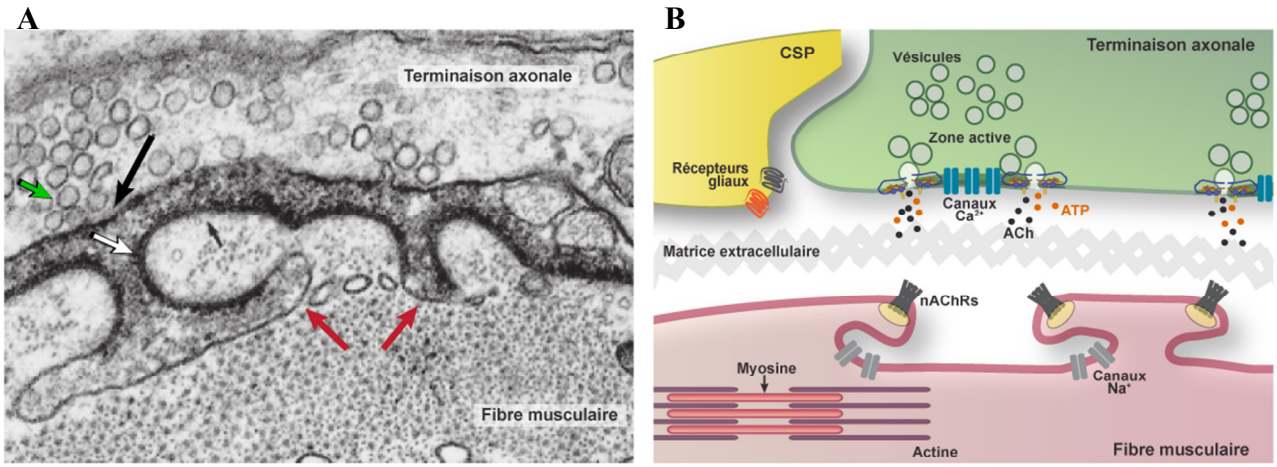

Figure 2.2. Organisation de la JNM.

A. Image en microscopie électronique montrant l'organisation de la terminaison axonale, incluant des zones actives (flèche noire). La zone active qui contient la machinerie nécessaire pour la libération du contenu des vésicules synaptiques (ronds). Notez que les vésicules sont concentrées (flèche verte) près de la zone active, site préférentiel d'exocytose. La fibre musculaire montre aussi une spécialisation au niveau de la synapse. Elle présente de nombreuses invaginations de la membrane (flèches rouges) et une grande densité de récepteurs postsynaptiques (flèche blanche) qui font face aux zones actives. **B.** Représentation schématique de l'organisation de la JNM. Dans la terminaison axonale (vert), les neurotransmetteurs, principalement l'ACh et l'ATP sont stockés dans les vésicules synaptiques qui sont concentrées au niveau des zones actives. La zone active comprend la machinerie pour l'exocytose de neurotransmetteurs incluant les canaux Ca^{2+} responsables de l'entrée de Ca^{2+} dans la terminaison ainsi que le complexe SNARE responsable de la fusion des vésicules. La fibre musculaire (rouge) contient plusieurs invaginations. Une haute densité de nAChRs est présente sur les crêtes des replis de la fibre musculaire faisant face à la zone active. Des canaux Na^{+} voltage-dépendants sont présents au fond des replis de la fibre. Les filaments d'actine et myosine, responsables de la contraction musculaire, sont aussi présents dans la fibre musculaire. Les CSP envoient des prolongements (jaune) à proximité de la fente synaptique. Les CSPs expriment des récepteurs pour détecter les neurotransmetteurs. La figure A a été modifiée de Fundamental Neuroscience (4th edition; 2013). P385-403).

2.2.2. Communication synaptique à la JNM

La communication synaptique à la JNM se fait principalement par la libération d'ACh. L'ACh est synthétisée dans le cytoplasme des terminaisons axonale par la choline-acétyle-transférase (ChAT) à partir de l'acétyl-CoA et de la choline. Grâce aux pompes à protons présentes sur la membrane vésiculaire et le gradient de protons qui en résulte, l'ACh synthétisée est transférée du cytoplasme vers les vésicules. De cette façon, l'ACh est emmagasinée dans les vésicules. La terminaison axonale est organisée en de multiples zones actives. Même si chaque terminaison axonale peut contenir des centaines de milliers de vésicules (Cano et al., 2013), une très petite proportion serait arrimée à chacune des zones actives (~40 à chaque zone active de grenouille et ~2 à chaque zone active de souris; Cano et al., 2013; Nagwaney et al., 2009; Rizzoli and Betz, 2005; Ruiz et al., 2011) . Le reste des vésicules forme un ensemble regroupé dans la terminaison nerveuse (Richards et al., 2000; Rizzoli and Betz, 2004; Ruiz et al., 2011). Le premier groupe de vésicules serait rapidement mobilisable « *readily releasable pool* » alors que le deuxième formerait les vésicules de réserve « *reserve pool* » (Richards et al., 2000).

La relâche de vésicules est déclenchée par une augmentation de la concentration du calcium intracellulaire dans la terminaison nerveuse. Ceci est la conséquence de l'ouverture des canaux calciques voltage-dépendants, situés près des zones actives, suite à l'arrivée de l'influx nerveux dépolarisant (Robitaille et al., 1990; Wood and Slater, 2001). Ces canaux calciques seraient principalement de type P/Q à la JNM de souris et auraient une faible probabilité d'ouverture (Urbano et al., 2003; Xu and Atchison, 1996). Un très grand nombre de ces canaux calciques voltage-dépendants sont présents à la terminaison nerveuse, ce qui pourrait favoriser une grande efficacité de la neurotransmission. L'augmentation de la concentration du Ca^{2+} intracellulaire permet l'enclenchement d'une série d'évènements qui mènent à la relâche de neurotransmetteurs. Parmi les mécanismes dépendants du calcium et responsables de la relâche de vésicules, le complexe SNARE est le mieux étudié. Il est principalement composé des protéines SNAP-25 et la syntaxine, présentes principalement sur la membrane de la terminaison nerveuse. Ces dernières peuvent interagir avec la

synaptobrévine, présente sur la membrane vésiculaire. Alors que SNAP-25 et la syntaxine sont liés aux canaux calciques (Jarvis et al., 2002; Keith et al., 2007; Leveque et al., 1994; Martin-Moutot et al., 1996; Sheng et al., 1994), le Ca^{2+} peut se lier directement à la synaptotagmine, autre protéine sur la surface vésiculaire, et enclencher la fusion des vésicules (Bennett et al., 1992; Geppert et al., 1994; Nishiki and Augustine, 2004). De manière simplifiée, le complexe SNARE permet la fusion des vésicules synaptiques avec la membrane cellulaire et la relâche de leur contenu (Figure 2.3).

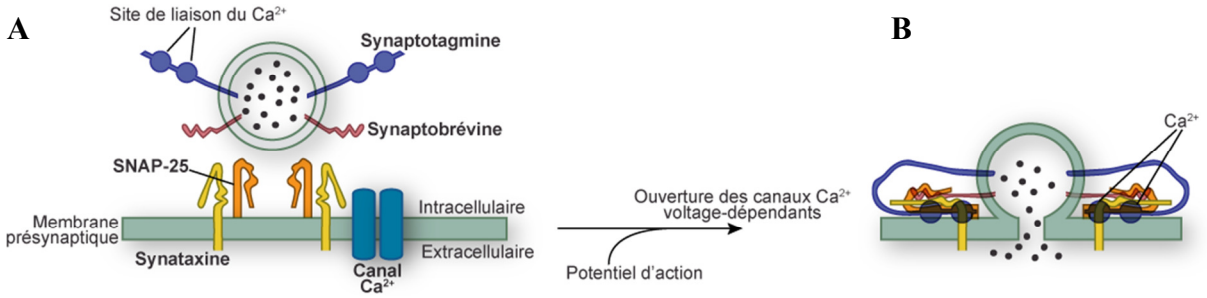

Figure 2.3. Complexe SNARE et exocytose.

Parmi les mécanismes dépendants du calcium et responsables de la relâche vésiculaire, le complexe SNARE est le mieux étudié. **A.** SNAP-25 et syntaxine sont des protéines présentes principalement sur la membrane de la terminaison nerveuse et la synaptobrévine sur la surface de la membrane vésiculaire. SNAP-25 et syntaxine seraient liés aux canaux calciques. La synaptotagmine, autre protéine sur la surface vésiculaire, contient des sites de liaison au Ca^{2+}. **B.** L'entrée de calcium dans la terminaison permet l'interaction entre les protéines qui composent le complexe SNARE pour enclencher la fusion des vésicules et ainsi relâcher leur contenu.

Naturellement, les mécanismes qui assurent la libération de neurotransmetteurs sont hautement régulés. Par exemple, la distribution spatiale des canaux calciques aux zones actives permet de restreindre les élévations calciques intracellulaires à des sites précis (Dodge and Rahamimoff, 1967; Schneggenburger and Neher, 2000; Van der Kloot, 1988). Entre autres, des protéines d'échafaudage, telles que Bassoon et Piccolo, s'assurent de maintenir les canaux calciques à proximité des zones actives (Cases-Langhoff et al., 1996; Kittel et al., 2006; Nishimune et al., 2004; tom Dieck et al., 1998). De cette façon, le calcium est restreint à un domaine précis nommé domaine calcique. En parallèle, d'autres mécanismes assurent la fin de libération de neurotransmetteurs. Par exemple, plusieurs types de canaux potassiques sont présent à la terminaison nerveuse incluant des canaux potassium voltage- et Ca^{2+}-dépendants. Ces canaux seraient importants pour limiter la durée de la dépolarisation nerveuse et, par conséquent, l'entrée de calcium et la libération de neurotransmetteurs (Robitaille et al., 1993a; Robitaille and Charlton, 1992; Robitaille et al., 1993b).

L'acétylcholine (ACh) et l'adénosine triphosphate (ATP) sont les principaux neurotransmetteurs libérés par la terminaison axonale à la JNM (Redman and Silinsky, 1994; Smith, 1991). Alors que l'ATP aurait un rôle modulateur qui sera discuté dans la section 2.3, l'ACh active principalement les récepteurs nicotiniques (nAChRs) situés sur les crêtes des replis de la fibre musculaire. La liaison de l'ACh avec les nAChRs entraîne l'ouverture du canal qui est perméable aux cations. L'augmentation de la concentration cationique (Na^+, K^+, Ca^{2+}) dans la fibre musculaire mène à la dépolarisation et à l'apparition d'un potentiel de plaque motrice (PPM). Si ce potentiel dépasse un certain seuil, il entraînera l'ouverture des canaux Na^+ voltage-dépendants, situés au fond des replis de la fibre musculaire (Figure 2.2), et un potentiel d'action postsynaptique sera alors généré (Flucher and Daniels, 1989). Ce potentiel d'action peut se propager et, lorsque la dépolarisation arrive au niveau du réticulum sarcoplasmique (réserves calciques des muscles striés), elle déclenche l'ouverture des canaux Ca^{2+} et donc l'augmentation de la concentration du Ca^{2+} dans le cytoplasme. Cette augmentation du Ca^{2+} est à la base du mouvement des filaments d'actine par la myosine qui génère la contraction musculaire (Figure 2.2). En parallèle, l'ACh et l'ATP libérés peuvent activer respectivement les récepteurs muscariniques (mAChRs) et purinergiques situés sur les CSPs (Jahromi et al., 1992; Robitaille, 1995; Robitaille et al., 1997; Rochon et al., 2001). Cet

aspect important de l'implication des cellules gliales dans l'activité synaptique sera abordé dans la section interactions neurone-glie (section 2.4).

Plusieurs mécanismes régulent l'activation des nAChRs par l'ACh. Entre autres, cette régulation est définie par la sensibilité des nAChRs ainsi que le type et les sous-unités qui les composent (Lindstrom, 2003). L'action de l'ACh est aussi régulée par la présence de l'acétylcholinestérase (AChE). L'AChE est présente dans la lame basale synaptique et elle est responsable de l'hydrolyse de l'ACh en choline et acétate, ce qui permet de contrôler l'action et la diffusion de l'ACh (Leuzinger, 1969; Leuzinger et al., 1969).

2.2.3. Activité endogène de la JNM

La fonction principale de la JNM est d'assurer une contraction efficace et fiable de la fibre musculaire. Cependant, son activité endogène peut être différente selon le muscle innervé ou même d'une fibre musculaire à l'autre. Ceci est défini à la fois par les propriétés contractiles de la fibre ainsi que par les propriétés fonctionnelles de la terminaison nerveuse.

Le type de fibre musculaire est souvent caractérisé par l'expression d'isoformes de la chaîne lourde de la myosine. Quatre isoformes ont été identifiées, I, IIa, IIx et IIb qui dictent la vitesse de contraction et la fatigue des fibres musculaires (Peter et al., 1972; Pette and Staron, 2000). Par exemple, une fibre musculaire de type I aurait une contraction lente, mais résistante à la fatigue. Une fibre musculaire de type IIb aurait une contraction rapide, mais peu résistante à la fatigue. Une fibre musculaire de type IIa aurait des propriétés intermédiaires et serait à fois rapide et résistante à la fatigue.

Un seul motoneurone envoie des terminaisons nerveuses vers plusieurs JNMs. L'ensemble des fibres musculaires innervées par un seul motoneurone est appelé unité motrice. L'organisation en unité motrice de chaque muscle serait importante pour la coordination de la contraction musculaire (Sears and Stagg, 1976). Les terminaisons nerveuses

de chaque unité motrice montrent des propriétés similaires (ex : le nombre de vésicules disponible et relâché lors de l'activité synaptique; Connor et al., 1997; Reid et al., 1999) et innervent des fibres de même type. Les unités motrices se regroupent en trois principaux types (S : *slow;* FR : *fast resistant;* FF : *fast fatigable*) basées sur les propriétés électrophysiologiques du motoneurone et les propriétés physiologiques de la vitesse de contraction, la force générée et la résistance à la fatigue. Les unités motrices de type S montrent une faible contraction et force générée, mais une grande résistance à la fatigue. Le type FR est défini par une contraction rapide ainsi qu'une force et résistance à la fatigue relativement élevées. Les unités motrices de type FF montrent une contraction rapide, une grande force générée, mais une faible résistance à la fatigue.

Dès lors, à chaque NMJ, la terminaison axonale montre des propriétés fonctionnelles qui sont compatibles à celles de la fibre musculaire innervée. Par exemple, les motoneurones de type S innervent des fibres musculaire de type I, les motoneurones de type FR innervent les fibres IIa et les motoneurones de types FF innervent les fibres musculaires de type IIb. Donc chaque NMJ peut être considérée comme « forte » (ex : motoneurone FF - fibre musculaire IIb) et donc adaptée à une activité robuste, mais de courte durée ou elle peut être « faible » (ex : motoneurone S - fibre musculaire I) avec une activité moins robuste, mais soutenue.

L'identité et la proportion de chaque type d'unité motrice peuvent varier d'un muscle à un autre (Reid et al., 1999). La proportion de chaque type d'unités motrices correspond à la fonction physiologique du muscle dans la genèse du mouvement volontaire. Tout comme les autres éléments de cette synapse, les CSPs ont aussi des propriétés spécifiques selon le type de terminaisons nerveuses et de fibres musculaires (Rousse et al., 2010).

Chez les mammifères, quelques types d'unités motrices semblent être définis très tôt après la naissance de sorte que des fibres musculaires sont innervées par des motoneurones qui ont des propriétés équivalentes (Thompson et al., 1990). Par contre, l'identité finale de chaque muscle et la proportion de chaque type d'unité motrice ne sont définies qu'à la fin du développement (Cramer and Van Essen, 1995). Ceci suggère qu'il y a un appariement du type de motoneurones au type de fibre musculaire au moment de l'innervation, mais aussi un

mécanisme de conversion du type de fibre musculaire en fonction du motoneurone qui l'innerve suite au processus d'élimination synaptique (Cramer and Van Essen, 1995; Sanes and Lichtman, 1999). Ce processus sera discuté à la section 2.6.

De manière générale, l'activité endogène des JNMs dans un muscle particulier peut être soutenue ou phasique en fonction des propriétés de l'unité motrice qui l'innerve. Par exemple, le muscle *Extensor Digitorum Longus* (EDL), considéré comme un muscle « fort », n'est activé que durant de courts épisodes d'activité de manière phasique et à de hautes fréquences de décharge (~80 Hz), mais montre rapidement de la fatigue (Hennig and Lomo, 1985). Par contre, le muscle SOL est considéré comme un muscle « faible ». Dans ce muscle, moins de fatigue est observée et l'activité est de longue durée, mais à de plus basses fréquences de décharge (~20 Hz) (Eken et al., 2008; Hennig and Lomo, 1985). Ces différences entre types de muscles peuvent s'expliquer entre autres, par de plus grosses terminaisons nerveuses et une plus grande réserve de vésicules synaptique, mais un contenu plus faible en neurotransmetteurs au niveau du SOL par rapport à l'EDL (Reid et al., 1999; Waerhaug, 1992; Wood and Slater, 1997). Ces propriétés sont compatibles avec la fonction de chaque muscle. Par exemple, le muscle SOL joue un rôle important dans le maintien de la posture (activité soutenue) alors que l'EDL est plus actif lors de la locomotion (activité phasique) (Hennig and Lomo, 1985).

L'activité endogène des unités motrices des muscles matures diffère de celle des muscles en développement. En effet, des études du muscle SOL de rats montrent que, durant l'activité tonique *in vivo*, la fréquence de décharge d'unités motrices uniques est très élevée peu de temps après la naissance et diminue graduellement avec l'âge (Eken et al., 2008). À quelques jours après la naissance, âge où le processus de compétition et d'élimination synaptique est bien en place, les motoneurones qui innervent le SOL déchargent de manière synchrones (Favero et al., 2012; Personius and Balice-Gordon, 2001) et à des fréquences élevées autour de 50 Hz. À un âge mature où la compétition et l'élimination sont déjà terminées (~ 21 jours), les unités motrices du SOL déchargent de manière asynchrone (Favero et al., 2012; Personius and Balice-Gordon, 2001) à des fréquences plus basse autour de 20 Hz (Eken et al., 2008). Cette fréquence est ensuite stable jusqu'à un âge adulte plus avancé (~ 100

jours; Eken et al., 2008) . Quoique ceci n'ait pas été directement démontré, ce changement d'activité a certainement une importance physiologique et dépend de la maturation de la JNM, des motoneurones qui l'innervent ainsi que des circuits spinaux. Par exemple, la maturation de la JNM résulte en une meilleure efficacité synaptique et énergétique tout en développant la résistance musculaire à la fatigue. Ceci expliquerait la diminution de la fréquence de décharge, qui permet à la fois de maintenir la contraction musculaire ainsi qu'une activité soutenue de longue durée du muscle SOL, telles que nécessaire pour le support postural (Eken et al., 2008). De plus, il semble que le mode de décharge lui aussi change et, en plus de l'activité tonique, une activité de type « phasique » se développe graduellement lors de la maturation ce qui serait compatible avec une contribution du muscle à l'activité locomotrice (Gorassini et al., 2000; Hennig and Lomo, 1985).

2.2.4. Plasticité synaptique à la JNM

Bien que le but principal de la JNM soit d'assurer la contraction musculaire, son activité n'est pas fixe. En effet, la transmission synaptique est variable dans différentes conditions et la libération de neurotransmetteurs peut être modifiée en réponse à des changements d'activité. Ceci est une propriété généralisée du système nerveux pour permettre une adaptation à l'environnement ou à des stimuli externes. Cette plasticité synaptique peut être exprimée sous différentes formes et échelles de temps pouvant aller de quelques millisecondes (court-terme) à plusieurs heures (long-terme). De manière générale, tout mécanisme qui peut faciliter la libération de neurotransmetteurs peut être catégorisé comme mécanisme de potentialisation synaptique alors qu'un mécanisme qui réduit l'activité serait responsable de la dépression synaptique. Ces deux familles de plasticité ont été décrites à la JNM mature.

2.2.4.1. Potentialisation synaptique à la JNM

L'augmentation de la transmission synaptique peut être transitoire et ne durer que quelques millisecondes ou soutenu et durer plusieurs minutes ou heures. De manière générale, différents mécanismes sont mis en place pour assurer cette plasticité. Principalement, tout mécanisme qui permettrait d'augmenter la concentration du Ca^{2+} dans la terminaison axonale, ou la durée de son action, entrainera une augmentation de la libération de neurotransmetteurs et de la réponse synaptique. Par exemple, la facilitation synaptique, qui est une augmentation de la transmission synaptique de quelques millisecondes, est le résultat de la présence de Ca^{2+} résiduel, suite à un premier influx nerveux, qui s'additionne à l'entrée de Ca^{2+} au niveau de la terminaison synaptique en réponse à un deuxième influx (Cooper et al., 1996; Katz and Miledi, 1968). Ceci provoque une augmentation du nombre de vésicules libérées et donc une plus grande réponse synaptique.

D’autres formes de potentialisation de plus longues durées nécessitent une augmentation du calcium présynaptique pour une plus longue période. Ceci pourrait être possible par des mécanismes qui réduisent l’élimination du Ca^{2+} par les pompes et échangeurs calciques (Parnas et al., 1982; Wojtowicz and Atwood, 1985) ou par la libération lente, mais soutenue, du Ca^{2+} accumulé par les mitochondries durant une activité synaptique robuste (David and Barrett, 2000; David et al., 1998; Tang and Zucker, 1997). Il est aussi connu que les mécanismes de potentialisation synaptique de plus longue durée impliqueraient l’action de neuromodulateurs qui peuvent réguler la fonction de certains canaux calciques ou activer d’autres types de canaux calciques plus propices à une plus grande entrée de Ca^{2+}. Par exemple, les purines (l'ATP et ces dérivés) sont des neuromodulateurs libérés à plusieurs synapses du système nerveux qui peuvent réguler la neurotransmission à la JNM en activant ou inhibant différents types de canaux calciques présynaptiques (Correia-de-Sa et al., 1996; Cunha, 2008; De Lorenzo et al., 2004; Oliveira et al., 2004; Silinsky, 2005). Ceci sera discuté dans une section dédiée aux rôles des purines dans la plasticité synaptique (section 2.3).

2.2.4.2. Dépression synaptique à la JNM

D’autres mécanismes sont responsables de diminuer la neurotransmission. La dépression synaptique peut être de courte ou de longue durée. Par exemple, une diminution de la libération de neurotransmetteurs a été observée lors d’une activité soutenue à haute fréquence et a souvent été attribuée à une déplétion en vésicules (Betz, 1970; Newman et al., 2007; Robitaille, 1998; Rousse et al., 2010). En ce qui concerne la dépression à long terme, ceci nécessite des modifications persistantes de la neurotransmission incluant l’action de neuromodulateurs. Par exemple, à la JNM d’amphibien, le glutamate, l’oxyde nitrique et les endocannabinoïdes peuvent induire une réduction persistance de l’activité synaptique en diminuant la libération de neurotransmetteurs (Lindgren et al., 2013; Newman et al., 2007; Pinard et al., 2003; Thomas and Robitaille, 2001).

2.2.4.3. Implications de la plasticité synaptique à la JNM

Puisque le but principal de la neurotransmission à la JNM est d'assurer une contraction musculaire fiable, tout mécanisme qui régule et adapte l'activité synaptique serait bénéfique pour l'organisme.

2.2.4.3.1. Implication physiologique de la plasticité synaptique à la JNM

Qu'il s'agisse de la potentialisation ou la dépression à long terme, ces formes de plasticité peuvent avoir d'importantes implications. L'implication demeure incertaine dans le contexte de la JNM, mais pourrait avoir des conséquences considérables. Par exemple, la dépression synaptique pourrait avoir un rôle protecteur. En diminuant la neurotransmission, ceci permettrait d'éviter une hyperactivité non nécessaire et d'économiser neurotransmetteurs et énergie pour s'assurer d'une transmission de plus longue durée (Newman et al., 2007). En contrepartie, augmenter la neurotransmission peut servir de mécanisme facilitateur qui permet une libération suffisante de neurotransmetteurs pour, encore une fois, assurer la contraction musculaire. À la JNM, la quantité de neurotransmetteurs libérée est plus importante que ce qui est nécessaire pour induire un potentiel d'action postsynaptique et une contraction musculaire. Cet excès de neurotransmetteurs, nommé « facteur de sécurité », permet à la JNM de demeurer efficace dans diverses conditions (Wood and Slater, 2001). Le facteur de sécurité de situe entre 2 et 6 aux JNM de souris (Wood and Slater, 2001) ce qui suggère qu'au moins 2 fois plus de neurotransmetteurs sont libérés que ce qui est nécessaire. Ainsi, la plasticité synaptique pourrait aussi permettre de réguler le facteur de sécurité pour assurer une contraction musculaire durable en réponse à des changements d'activité.

D'un autre côté, ces formes de plasticité peuvent faire partie d'un mécanisme d'adaptation en réponse à des conditions particulières et être impliquées dans des processus physiologiques importants. Par exemple, dans le SNC, la potentialisation à long terme (PLT)

permet d'améliorer l'efficacité synaptique et elle est considérée comme étant la base physiologique de l'apprentissage et la mémoire (Whitlock et al., 2006).

2.2.4.3.2. Implication structurale de la plasticité synaptique à la JNM

En plus d'une importance physiologique, la plasticité synaptique peut avoir un impact direct sur l'organisation de la synapse. Par exemple, dans le SNC, les synapses qui sont potentialisées sont renforcées et stabilisées (Matsuzaki et al., 2004; Nagerl et al., 2004) alors que les synapses qui subissent une dépression synaptique peuvent être affaiblies et éliminées (Bastrikova et al., 2008; Oh et al., 2013). À la JNM, il est possible d'imaginer un processus capable de renforcer les synapses potentialisées en accroissant le territoire synaptique ou en ajoutant de nouvelles branches présynaptiques. De la même façon, il est possible qu'une JNM subissant une dépression synaptique puisse provoquer une rétraction de branches présynaptiques de manière analogue au SNC (Bastrikova et al., 2008; Oh et al., 2013). De manière intéressante, une augmentation transitoire, mais robuste de l'activité synaptique induit la formation de nouveaux boutons synaptiques et mène à l'expansion de la synapse à la JNM de drosophiles (Ataman et al., 2008; Fuentes-Medel et al., 2009). Ainsi, il semble y avoir un lien direct entre l'activité, la plasticité et l'organisation synaptique.

2.3. Modulation purinergique de la transmission synaptique à la JNM

Les purines, principalement l'ATP et l'adénosine, sont connues comme d'importants neuromodulateurs dans le système nerveux, incluant la JNM. La modulation purinergique de la JNM peut être bidirectionnelle et induire soit une potentialisation, soit une dépression de la relâche de neurotransmetteurs (Correia-de-Sa et al., 1996; Todd et al., 2010). Cette potentialisation et dépression de l'activité résultent respectivement de l'activation des récepteurs présynaptiques A2A ou A1 et dépendent de la fréquence et du patron de stimulation de la terminaison nerveuse (Correia-de-Sa et al., 1996; Todd et al., 2010). Ces récepteurs sont activés par l'adénosine, généralement produite suite à la dégradation de l'ATP par l'action d'ectonucleotidases (Dunwiddie et al., 1997; Rebola et al., 2008). La dépression dépendante des récepteurs A1 est prédominante en réponse à l'activité de basse fréquence alors qu'une activité de plus haute intensité entraine une potentialisation dépendante des récepteurs A2A (Correia-de-Sa et al., 1996).

La signalisation purinergique semble être régulée durant le développement. En effet, il semble que les récepteurs A2A seraient plus abondants au cours du développement de la JNM alors que les récepteurs A1 sont plus abondants à la JNM mature (Garcia et al., 2013). Ceci est compatible avec d'anciennes études qui ont montré que, durant le développement de la JNM du têtard, les purines potentialisent la neurotransmission (Fu and Poo, 1991) alors que, chez la grenouille adulte, un effet opposé est observé (Giniatullin and Sokolova, 1998).

L'ATP, sans être dégradée en adénosine, peut aussi affecter la neurotransmission à la JNM et peut ainsi potentialiser ou déprimer la relâche de neurotransmetteurs en fonction des récepteurs activés (Giniatullin et al., 2005; Giniatullin and Sokolova, 1998; Grishin et al., 2005; Moores et al., 2005). L'ATP peut agir via les récepteurs ionotropes purinergiques de type P2X (P2XRs) ou les récepteurs metabotropes purinergiques de type P2Y (P2YRs) (Ralevic and Burnstock, 1998). Par exemple, l'activation de certains sous-types des P2XRs peut faciliter la relâche de neurotransmetteurs (Hong and Chang, 1998; Moores et al., 2005).

En parallèle, il a été montré que l'activation de certains P2YRs entraine la production d'acide arachidonique ce qui causerait une réduction de l'activité synaptique en inhibant certains canaux Ca^{2+} dont l'identité n'est pas claire, et affecterait aussi les mécanismes agissant en amont de l'entrée de Ca^{2+} incluant les canaux potassiques (Grishin et al., 2005). Un résumé simplifié de la modulation purinergique de la neurotransmission à la JNM est présenté à la Figure 2.4.

2.3.1. Rôle des récepteurs A1 dans la dépression présynaptique

L'activation des récepteurs A1 présynaptiques par l'adénosine semble inhiber les canaux calciques synaptiques de type P/Q, ce qui réduit la relâche de neurotransmetteurs (Silinsky, 2004). Ces canaux sont impliqués dans l'entrée de calcium durant la libération de neurotransmetteurs à la JNM de souris (Urbano et al., 2003; Xu and Atchison, 1996). Les A1Rs sont des récepteurs couplés aux protéines G. Leur activation mènerait à une interaction avec la syntaxine, protéine du complexe SNARE, qui contient un site de liaison avec les sous-unités β/γ des protéines G couplées aux récepteurs A1. Cette interaction du A1R avec la machinerie d'exocytose expliquerait la réduction de la relâche de neurotransmetteurs (Silinsky, 2005).

2.3.2. Rôle des récepteurs A2A dans la potentialisation présynaptique

En plus de l'activation des récepteurs A1, l'adénosine peut aussi moduler l'activité synaptique à travers les récepteurs à l'adénosine A2A. En effet, l'activation des récepteurs A2A permet de potentialiser la neurotransmission (Oliveira et al., 2004). Par exemple, durant une stimulation continue et à haute fréquence de la terminaison nerveuse, il y a une augmentation de la relâche de neurotransmetteurs à travers l'activation des récepteurs A2A présynaptiques qui mène au recrutement de canaux calciques de type L (Oliveira et al., 2004).

Ces canaux ne participent pas d'habitude à la relâche de neurotransmetteurs, mais leur recrutement dans ces conditions augmenterait l'influx calcique dans la terminaison axonale et faciliterait la libération de neurotransmetteurs (Robitaille et al., 1990; Tsien et al., 1988).

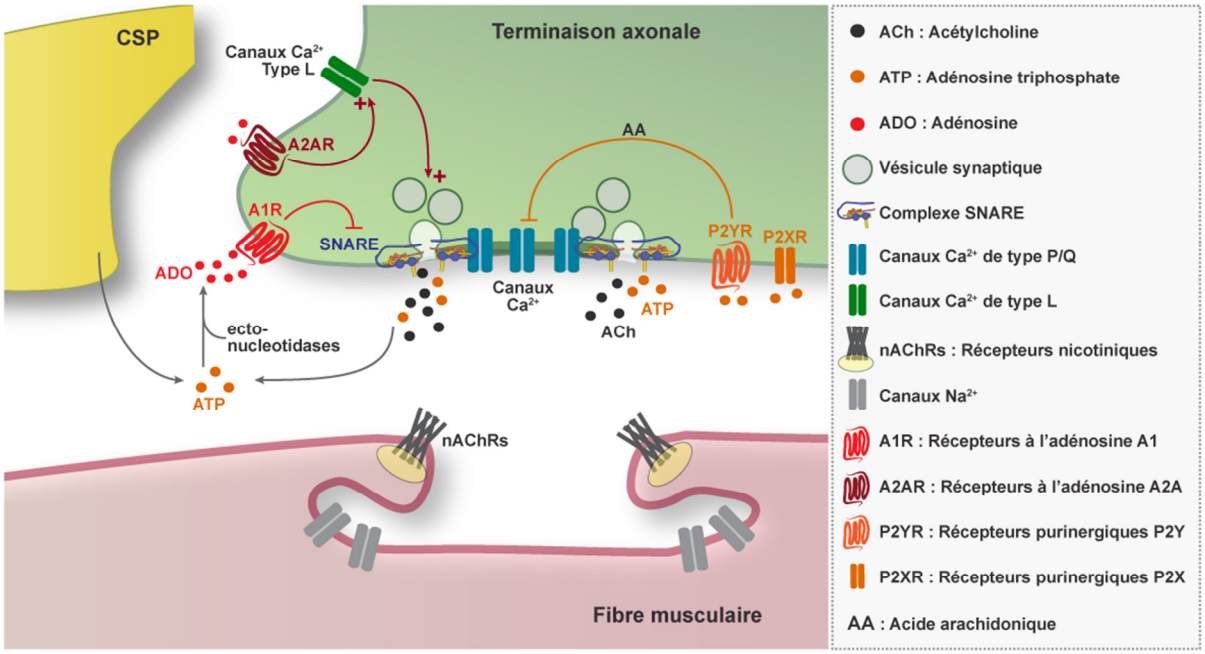

Figure 2.4. Modulation purinergique de la relâche de neurotransmetteurs à la JNM.

Les sources de purines incluent la libération par la terminaison axonale et les CSPs. L'ATP peut activer les récepteurs présynaptiques P2Y qui diminuent la relâche de neurotransmetteurs par la production d'acide arachidonique qui mène à l'inhibition des canaux Ca^{2+}. L'activation de récepteurs P2X augmenterait la relâche de neurotransmetteur. L'ATP serait dégradée rapidement en adénosine par des ectonucleosidases. L'adénosine, en activant les récepteurs présynaptiques A2A, augmente la relâche de neurotransmetteur. L'activation des récepteurs A2A entrainerait le recrutement des canaux Ca^{2+} de type L. À l'inverse, l'activation des récepteurs A1 entraine une diminution de la neurotransmission. Les récepteurs A1 causeraient l'inhibition des canaux Ca^{2+} de type P/Q en interagissant avec le complexe SNARE.

2.3.3. Origine des purines à la JNM

L'adénosine serait principalement produite suite à la dégradation rapide de l'ATP. Il est bien connu que l'ATP est un neurotransmetteur à la JNM libéré avec l'ACh par la terminaison nerveuse (Redman and Silinsky, 1994; Smith, 1991). Cependant, il est aussi connu que l'ATP peut être libérée par les cellules gliales dans le SNC (Lalo et al., 2014; Newman and Zahs, 1997; Panatier et al., 2011; Pascual et al., 2005; Serrano et al., 2006) et à la JNM (Todd et al., 2010). Une étude de notre laboratoire montre que la balance entre la potentialisation, dépendante de A2ARs, et la dépression, dépendante de A1Rs, pourrait être contrôlée par les cellules gliales à la JNM (Annexe I; Todd et al., 2010). En effet, les CSPs régulent l'activité et la plasticité synaptique en libérant des purines capables de changer la relâche de neurotransmetteurs (Todd et al., 2010).

À la JNM, tout comme dans plusieurs régions du SNC, l'adaptabilité et la plasticité synaptique ne dépendent pas uniquement des éléments pré- et postsynaptiques, mais requièrent la contribution de l'élément périsynaptique : les cellules gliales. En effet, il a été montré que différentes formes de plasticité dépendent de l'activité des cellules gliales. De plus, les cellules gliales interagissent activement avec les neurones et accomplissent diverses fonctions fondamentales pour le fonctionnement du système nerveux. Dans le contexte de la présente thèse, nous voulions étudier l'implication des cellules gliales à la fois dans la régulation de l'activité synaptique, mais aussi dans l'élimination synaptique lors de la maturation de la JNM. Par conséquent, la section suivante va traiter du rôle des cellules gliales dans la fonction du système nerveux. Celle-ci sera suivie d'une section sur la synaptogenèse et la maturation de la JNM ainsi que le rôle des cellules gliales dans ces processus.

2.4. Interactions neurone-glie

Initialement, les cellules gliales ont étées associées à un rôle accessoire dans le système nerveux. Leur but principal était de soutenir les neurones en leur fournissant les facteurs trophiques et en régulant le milieu extracellulaire. Cependant, plusieurs études ont démontré l'importance cruciale des cellules gliales dans diverses fonctions du système nerveux incluant la régulation de l'activité synaptique et la synaptogenèse.

Dans le système nerveux, il y a 5 principaux types de cellules gliales : astrocytes, microglies, oligodendrocytes, les cellules $NG2^{+}$ et les cellules de Schwann (Barres, 2008). Les quatre premiers types se trouvent dans le SNC. Contrairement aux neurones, la majorité des cellules gliales n'émettent pas de potentiel d'action.

- **Les astrocytes** sont les cellules gliales les plus abondantes du SNC. Elles sont associées aux synapses et jouent plusieurs rôles en conditions physiologiques incluant la régulation de l'homéostasie, l'apport énergétique, la formation de la barrière hématoencéphalique ainsi que la régulation de l'activité synaptique et la synaptogenèse (Eroglu and Barres, 2010). Ces rôles ont un lien direct ou indirect avec l'activité neuronale et seront détaillés dans la section suivante.

- **Les microglies** sont les seules cellules gliales dérivées du développement du mésoderme et sont les cellules immunitaires du SNC. Elles constituent la population de macrophages résidents du SNC et s'occupent entre autres d'éliminer les neurones endommagés et de prévenir l'inflammation du cerveau (Barres, 2008; Streit et al., 1999). Les microglies scrutent constamment le tissu et peuvent passer à un état réactif en situation pathologique. L'activation des microglies peut avoir des conséquences fonctionnelles et pourrait avoir un rôle neuroprotecteur ou neurotoxique dans diverses conditions (Hanisch and Kettenmann, 2007). Les microglies peuvent aussi réguler le développement du système nerveux en éliminant certains contacts synaptiques et ainsi raffiner les circuits neuronaux (Schafer et al., 2012; Stevens et al., 2007). Cet aspect est particulièrement important pour la

présente thèse et sera détaillé dans une section ultérieure (section 2.8.2). Plus récemment, il a été décrit que les microglies peuvent aussi influencer l'activité synaptique chez le poisson zébré. En effet, les microglies peuvent réagir à l'activité neuronale physiologique en envoyant des prolongements aux neurones hautement actifs pour réguler leur excitabilité (Li et al., 2012).

- **Les oligodendrocytes** forment la myéline qui enveloppe les axones qui assure une propagation efficiente de l'influx nerveux ainsi que la conduction saltatoire (Pfrieger, 2009). Chaque oligodendrocyte peut contacter et myéliniser différents neurones (Chvatal et al., 1995).

- **Les cellules NG^{2+}** peuvent servir de précurseurs d'oligodendrocytes dans le SNC mature, mais peuvent aussi générer des cellules de Schwann remyelénisantes et possiblement des astrocytes suite à des atteintes du SNC (Nishiyama et al., 2002a; Richardson et al., 2011). De manière intéressante, une proportion des cellules de la famille NG^{2+} peuvent recevoir des connexions de différents neurones et même exprimer des potentiels d'action (Karadottir et al., 2008). Cette propriété pourrait être utile pour initier la différentiation en oligodendrocyte et la myélinisation neuronale (Karadottir et al., 2008).

- **Les cellules de Schwann** sont le seul type de cellules gliales dans le SNP. Ces cellules peuvent envelopper des faisceaux d'axones et former la myéline (Feng and Ko, 2007). À la JNM, les cellules Schwann les plus distales sont associées à la synapse et sont nommées cellules de Schwann périsynaptiques (ou terminales; Darabid et al., 2014; Feng and Ko, 2007) . De manière similaire aux astrocytes, ces cellules sont maintenant connues pour réguler l'activité synaptique, maintenir l'intégrité synaptique et sont aussi impliquées dans la formation et la maturation de la JNM (Darabid et al., 2014; Feng and Ko, 2007; Reddy et al., 2003; Robitaille, 1998; Todd et al., 2010).

Étant donné que les astrocytes et les CSPs sont parmi les seuls types de cellules gliales en contact avec les synapses (Pfrieger, 2009), les sections suivantes traiteront principalement

de leur rôle dans la fonction du système nerveux. Sauf si autrement spécifié, le terme « cellule gliale » fera référence principalement aux astrocytes et CSPs.

2.4.1. Rôle des cellules gliales

Les cellules gliales sont présentes dans l'intégralité du système nerveux et accomplissent diverses fonctions nécessaires pour le développement, le maintien et la régulation de celui-ci. Pendant longtemps, les cellules gliales ont été considérées comme des cellules de soutien qui maintiennent les neurones dans un environnement optimal. Cependant, les études au cours des dernières années ont permis d'identifier des fonctions dynamiques et actives des cellules gliales. En effet, ces cellules peuvent détecter la neurotransmission et influencer directement l'activité et la structure synaptique. Les rôles des cellules gliales dans le support neuronal et la régulation de l'activité ne sont pas mutuellement exclusifs et sont tout aussi importants puisqu'un maintien inadéquat de l'environnement neuronal entraînerait des déficits de l'activité synaptique. Donc, je vais commencer par présenter le rôle des cellules gliales dans le support neuronal (section 2.4.1.1). Ensuite, je présenterai le rôle des cellules gliales dans la communication synaptique (section 2.4.1.2).

2.4.1.1. Rôle des cellules gliales dans le support neuronal

2.4.1.1.1. Homéostasie ionique extracellulaire

Un des rôles classiques des cellules gliales, plus spécifiquement des astrocytes, est le contrôle de l'homéostasie ionique extracellulaire. Ceci permet d'optimiser l'environnement neuronal pour permettre une transmission synaptique adéquate. Par exemple, les astrocytes s'occupent de la clairance du potassium extracellulaire, ce qui peut influencer l'excitabilité et l'efficacité neuronale (Djukic et al., 2007). Les astrocytes contrôlent aussi l'homéostasie du

Ca^{2+} en régulant sa concentration extracellulaire par la relâche de chélateur endogène du calcium (Morquette et al., 2015). De plus, les astrocytes sont aussi connus pour tamponner le proton H^+ et donc réguler le pH (Deitmer and Rose, 1996).

2.4.1.1.2. Apport énergétique

Le système nerveux est très énergivore et requiert un apport soutenu en métabolites pour assurer son fonctionnement. Le glucose est le substrat énergétique nécessaire pour le cerveau et permet la production d'énergie principalement sous forme d'ATP. Dans le SNC, les astrocytes sont à la base du métabolisme du glucose (Belanger et al., 2011). De plus, l'entreposage de glucose, sous forme de glycogène, est exclusivement retrouvé dans les astrocytes (Belanger et al., 2011). De cette façon, les astrocytes entreposent et fournissent l'énergie nécessaire pour l'activité neuronale.

Le lactate, formé du glucose ou importé de la circulation sanguine dans le cerveau est aussi un substrat énergétique important (Schurr, 2006). De manière intéressante, le lactate est libéré par les astrocytes et il est utilisé par les neurones pour la production d'énergie (Pellerin and Magistretti, 1994). Il va sans dire que le transport du lactate des astrocytes vers les neurones est nécessaire pour les fonctions cognitives telles que la formation à long terme de la mémoire (Suzuki et al., 2011).

2.4.1.1.3. Contrôle du débit sanguin

L'apport énergétique par les astrocytes est variable et peut être modifié en réponse à des changements d'activité neuronale (Pellerin and Magistretti, 1994). Vu le rôle des astrocytes dans l'apport énergétique et la proximité des prolongements astrocytaires (*endfeet*) des vaisseaux sanguins, il a été démontré que les astrocytes, non seulement régulent l'apport

énergétique en réponse à l'activité nerveuse (Pellerin and Magistretti, 1994), mais contrôlent le débit sanguin (Attwell et al., 2010; Duffy and MacVicar, 1995; Mulligan and MacVicar, 2004; Zonta et al., 2003).

Les *endfeets* d'astrocytes sont en contact avec les vaisseaux sanguins et contribuent à maintenir la barrière hématoencéphalique qui limite les échanges entre le domaine vasculaire et le domaine cérébral (Simard et al., 2003). En accord avec cette organisation, les astrocytes jouent un rôle important dans le contrôle de la neuro-vascularisation, le débit sanguin et l'apport énergétique. L'activité neuronale peut déclencher la libération de substances vasoactives par les astrocytes, tels que les prostanoïdes, qui permettent d'ajuster le débit sanguin à la demande énergétique du système (Gordon et al., 2007; Iadecola and Nedergaard, 2007).

La capacité des astrocytes à ajuster le débit sanguin en réponse à des changements de l'activité neuronale sous-tend que ces cellules ont la capacité de monitorer l'activité synaptique et neuronale. De manière intéressante, il est maintenant connu que les cellules gliales peuvent détecter l'activité synaptique (voir la section 2.4.1.2). La détection de l'activité par les cellules gliales permettrait d'ajuster la vascularisation proportionnellement aux besoins du système et, en retour, maintenir une activité synaptique appropriée. La capacité des cellules gliales à détecter l'activité synaptique leur permettrait de fournir un feedback adéquat aux neurones et ainsi réguler la communication synaptique. D'ailleurs, l'implication des cellules gliales dans la communication synaptique est un domaine hautement actif dont les principaux aspects sont traités dans la section suivante.

2.4.1.2. Rôle des cellules gliales dans la communication synaptique

Les cellules gliales ont une cytoarchitecture unique et sont idéalement positionnées au niveau des synapses pour détecter et répondre de manière dynamique aux changements dans leur microenvironnement. En effet, tel qu'illustré dans la Figure 2.1, les prolongements des

cellules gliales, plus spécifiquement des astrocytes dans le CNS et des CSPs à la JNM, sont à proximité de la fente synaptique et forment des domaines spécialisés (Ventura and Harris, 1999; Witcher et al., 2007; Zuo and Bishop, 2008). De manière intéressante, ces cellules gliales expriment plusieurs récepteurs capables de détecter les neurotransmetteurs relâchés (Perea et al., 2009), ce qui les placent dans une position favorable pour interagir avec les synapses. En effet, les cellules gliales ont la capacité de détecter leur environnement synaptique et d'agir de façon adaptée en régulant la neurotransmission. Dès lors, cette section décrit le rôle des cellules gliales dans la régulation de l'activité synaptique. Pour que cette régulation soit adaptée au contexte synaptique, il faut que les cellules gliales soient capables de 1) détecter l'activité synaptique, 2) décoder et traiter l'information synaptique pour finalement 3) réguler l'activité synaptique.

2.4.1.2.1. Détection de la transmission synaptique

Pour que les cellules gliales soient impliquées dans la régulation de la neurotransmission, elles doivent d'abord être compétentes à détecter l'activité neuronale. Plusieurs études montrent que les cellules gliales détectent les principaux neurotransmetteurs libérés dans différents contextes synaptiques et diverses régions du système nerveux. Dans le SNC, les astrocytes sont capables de détecter la libération de différents neurotransmetteurs incluant le glutamate (neurotransmetteur principal des synapses excitatrices; Panatier et al., 2011; Wang et al., 2006) le GABA (neurotransmetteurs aux synapses inhibitrices; Serrano et al., 2006) , l'ATP (neurotransmetteur co-libéré à certaines synapses; Di Castro et al., 2011; Jourdain et al., 2007), l'ACh (neurotransmetteur libéré dans le SNC aux synapses cholinergiques; Perea and Araque, 2005; Takata et al., 2011) , la dopamine dans les ganglions de la base (Miyazaki et al., 2004) et les endocannabinoïdes (neuromodulateur rétrograde impliqué dans diverses fonctions physiologiques; Han et al., 2012; Navarrete and Araque, 2008). De par leur capacité à détecter la libération de neurotransmetteurs, les cellules gliales ressembleraient aux cellules postsynaptiques. Ceci est basé sur le fait que les astrocytes expriment une variété de récepteurs ayant une grande affinité aux neurotransmetteurs. La

majorité des récepteurs décrits sont des récepteurs couplés aux protéines G. Parmi ceux-ci, plusieurs sont des récepteurs couplés aux protéines G de type q (G_q) tel que le groupe I des récepteurs metabotropes au glutamate (mGluRs; particulièrement mGluR 1 et mGluR 5) ou les récepteurs purinergiques de type P2Y. L'activation des récepteurs couplés aux protéines Gq entraine l'activation de la phospholipase C. En retour, cette dernière hydrolyse le phosphatidylinositol bisphosphate en diacyl glycerol et inositol trisphosphate (IP3). De manière générale, l'augmentation de la concentration intracellulaire de Ca^{2+} dans les cellules gliales dépendrait de l'IP3 qui cause la libération du calcium des réserves internes et enclencherait l'activation de plusieurs mécanismes dépendants du Ca^{2+} (voir les revues Araque et al., 2014 et Perea et al., 2009).

À la JNM, les CSPs détectent la libération présynaptique de l'ACh par l'activation des mAChRs (Arbour et al., 2015; Rochon et al., 2001). Les CSPs détectent aussi la relâche d'ATP par l'activation des récepteurs purinergiques à l'ATP, particulièrement P2YRs, et à l'adénosine, particulièrement les A1Rs (Robitaille, 1995; Rochon et al., 2001; Todd et al., 2007).

La capacité des cellules gliales à détecter l'activité synaptique a aussi été confirmée *in vivo*. En effet, les astrocytes montrent des élévations calciques en réponse à la libération endogène de neurotransmetteurs (Bekar et al., 2008; Hirase et al., 2004; Navarrete et al., 2012; Wang et al., 2006) et aux stimuli sensoriels physiologiques tels que la stimulation de vibrisses de souris (Takata et al., 2011; Wang et al., 2006), une stimulation aversive périphérique (Navarrete et al., 2012), un stimulus brusque et menaçant (Dombeck et al., 2007; Srinivasan et al., 2015) ou des stimuli visuels (Schummers et al., 2008).

La présence d'une diversité de récepteurs gliaux et modes de signalisation calciques suggère une importance dans la détection de l'activité synaptique par les cellules gliales. De cette façon, cette détection serait spécifique au contexte synaptique défini par les différents neurotransmetteurs libérés et l'activation des récepteurs gliaux appropriés. Dès lors, la cellule gliale aurait la capacité d'intégrer et décoder avec précision les propriétés de l'activité synaptique.

2.4.1.2.2. Décodage de l'activité synaptique

Il devient de plus en plus évident que chaque signalisation calcique gliale est unique et véhicule une information importante sur l'activité synaptique (Araque et al., 2014). L'activation de différents récepteurs des cellules gliales permettrait de mieux analyser et intégrer les subtilités de l'activité synaptique (Perea and Araque, 2005). De plus, la réponse Ca^{2+} gliale est en soi un code qui reflète le décodage et le traitement de l'information synaptique (Araque et al., 2014; Perea and Araque, 2005; Todd et al., 2010).

Dans l'hippocampe, un même astrocyte peut détecter l'activité cholinergique des connexions de l'alveus ainsi que l'activité glutamatergique des collatérales de Schaffer en montrant une activité calcique unique à chacune de ces connexions synaptiques (Perea and Araque, 2005). De plus, lorsque les terminaisons cholinergiques et glutamatergiques sont activées simultanément, la réponse calcique astrocytaire n'est pas une simple sommation, mais est unique à cette nouvelle condition ce qui suggère une intégration de l'information synaptique par l'astrocyte (Perea and Araque, 2005).

À la JNM, la présence d'une signalisation purinergique et cholinergique au niveau des CSPs permettrait de mieux détecter la neurotransmission (Rochon et al., 2001). Il est donc possible que les types de récepteurs activés puissent entraîner des réponses calciques gliales distinctes et une rétroaction spécifique. De plus, les différents aspects de la réponse calcique des CSPs, incluant son amplitude, sa durée et sa cinétique témoignent du décodage de l'activité synaptique (Todd et al., 2010). Par exemple, les CSPs sont capables de détecter différents niveaux d'activité et de décoder l'activité neuronale en discriminant les patrons d'activité synaptique (Todd et al., 2010). En effet, un patron de stimulation de terminaison nerveuse de type continu induit une réponse calcique monophasique de courte durée, mais d'une forte amplitude. En parallèle, un patron de stimulation de type phasique induit une réponse calcique oscillatoire des CSPs de faible amplitude, mais de longue durée (Todd et al., 2010). Ces données indiquent que les cellules gliales distinguent les différents patrons d'activité synaptique. Ceci suggère que les CSPs décodent avec fiabilité les subtilités de

l'activité synaptique et intègrent cette information, ce qui est reflété par une diversité de signalisations calciques.

D'ailleurs, il existe une grande hétérogénéité de l'activité calcique gliale. Différents groupes rapportent des réponses calciques ayant différentes caractéristiques (à savoir amplitude, durée et cinétique) dans différentes structures et conditions (Volterra et al., 2014; Zorec et al., 2012). Dès lors, il devient évident que les cellules gliales sont équipées de la machinerie nécessaire pour détecter l'activité synaptique. De plus, l'activité calcique gliale est un code bien précis qui inclut le décodage et le traitement de l'activité synaptique (Araque et al., 2014; Volterra et al., 2014). Ceci serait un préalable pour une régulation appropriée de la neurotransmission par les cellules gliales.

2.4.1.2.3. Régulation de l'activité synaptique

Le Ca^{2+} est un messager second ubiquitaire qui sous-tend plusieurs fonctions cellulaires et peut activer différentes voies de signalisations intracellulaires. De la même manière, l'augmentation de la concentration du Ca^{2+}intracellulaire dans les cellules gliales, en réponse à l'activation de leurs récepteurs par les neurotransmetteurs, peut enclencher une variété de signaux intracellulaires et de conséquences. Une des implications potentielles de l'activité calcique des cellules gliales qui suscite un grand intérêt est la libération de gliotransmetteurs (Bezzi and Volterra, 2001; Newman, 2003b). Les gliotransmetteurs sont des molécules neuroactives, les mêmes que celles libérées par les neurones à différentes synapses, et sont appelées de la sorte en raison de leur origine gliale (Bezzi and Volterra, 2001; Newman, 2003b). Ces gliotransmetteurs seraient libérés par les cellules gliales dans l'espace extracellulaire, où ils peuvent diffuser et promouvoir la régulation de la neurotransmission (Araque et al., 2014). Un modèle simplifié de la régulation de l'activité synaptique par les cellules gliales est présenté à la Figure 2.5.

2.4.1.2.3.1. Gliotransmission

Plusieurs gliotransmetteurs tels que le glutamate, l'ATP, le GABA, la D-serine et le facteur de nécrose tumorale alpha (TNFα) ont été identifiés (Araque et al., 2014). D'ordinaire, ce sont des agonistes de récepteurs neuronaux ou des co-agonistes qui facilitent l'activation de certains types de récepteurs (Araque et al., 2014). De plus, certains gliotransmetteurs permettraient le changement d'expression de récepteurs alors que d'autres influenceraient directement l'homéostasie ionique extracellulaire (Morquette et al., 2015; Stellwagen and Malenka, 2006). Le tableau I montre une liste de gliotransmetteurs connus, l'effet qui leur est attribué et la région du système nerveux dans laquelle ils ont été identifiés.

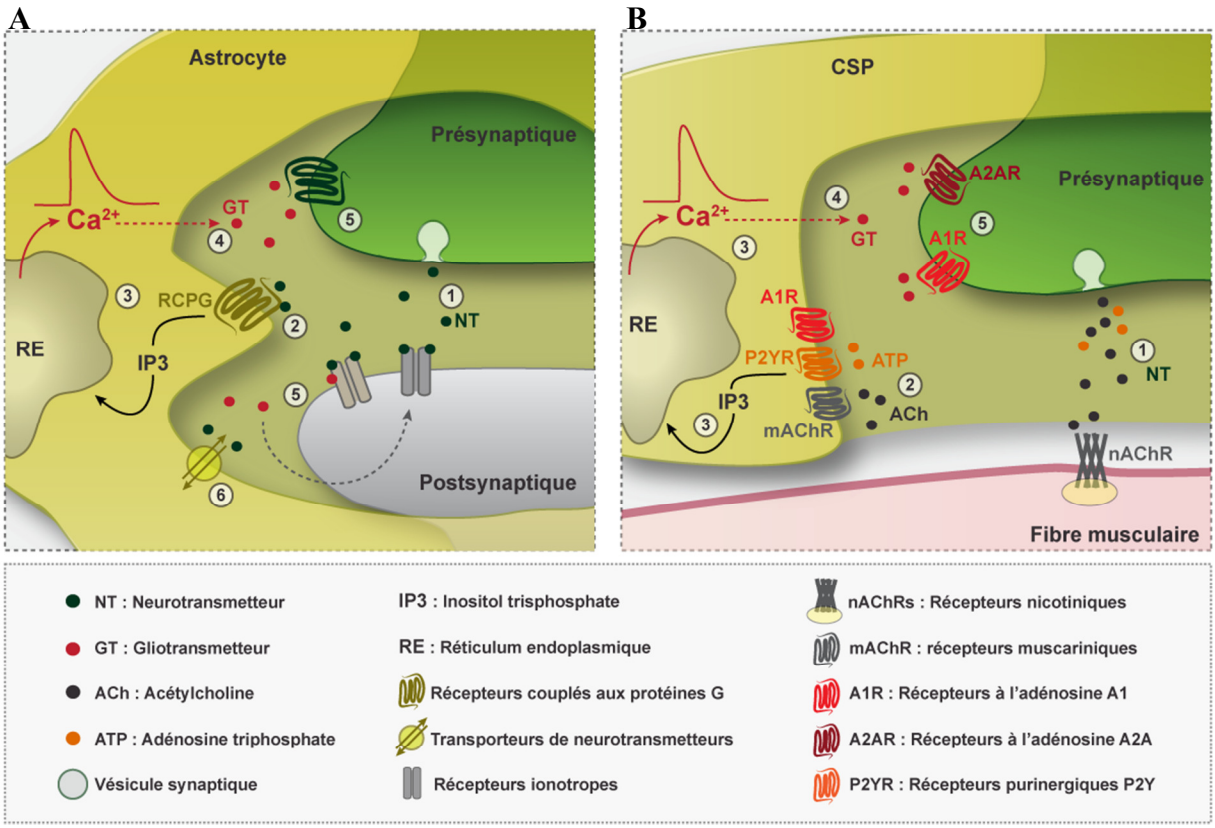

Figure 2.5. Modèle simplifié de la régulation de l'activité synaptique par les cellules gliales.

A. Interaction astrocyte-neurone aux synapses du SNC. 1. L'élément présynaptique libère des neurotransmetteurs. 2. Ces neurotransmetteurs sont détectés par l'astrocyte par l'activation de récepteurs gliaux (principalement des récepteurs couplés aux protéines G). 3. Par l'entremise de l'IP3, il y a une libération du Ca^{2+} du réticulum endoplasmique. 4. Cette élévation de la concentration intracellulaire de Ca^{2+} peut mener à la relâche de gliotransmetteurs. 5. Ces gliotransmetteurs, en activant des récepteurs neuronaux ou en modifiant la fonction de certains récepteurs, régulent l'activité synaptique. 6. Les astrocytes peuvent aussi réguler l'activité synaptique en contrôlant la concentration ambiante de neurotransmetteurs par l'entremise de transporteurs. **B.** Régulation de l'activité synaptique à la JNM. 1. La terminaison axonale libère des neurotransmetteurs (principalement l'ACh et l'ATP). 2. Ces neurotransmetteurs sont détectés par la CSP par l'activation de récepteurs gliaux (principalement des récepteurs couplés aux protéines G). L'ACh serait détecté par l'activation de mAChRs gliaux et l'ATP par des récepteurs P2YRs. Les récepteurs A1Rs contribueraient aussi à la détection de

l'activité synaptique. 3. Par l'entremise de l'IP3, il y a une libération du Ca^{2+} du réticulum endoplasmique. 4. Cette élévation de la concentration intracellulaire de Ca^{2+} peut mener à la relâche de gliotransmetteurs. 5. Ces gliotransmetteurs, principalement des purines, peuvent activer des récepteurs présynaptiques (ex : A1Rs et A2ARs) et ainsi réguler l'activité synaptique.

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2.4.1.2.3.1.1. Mécanismes de libération des gliotransmetteurs

Les mécanismes de relâche de gliotransmetteurs semblent être variés. Parmi ceux-ci, l'hypothèse de relâche vésiculaire est très attrayante puisqu'il y a évidence de présence de vésicules astrocytaires ainsi que des protéines du complexe SNARE (Bezzi et al., 2004; Zhang et al., 2004). De plus, interférer avec les mécanismes d'exocytose vésiculaire semble altérer la libération de gliotransmetteurs *in vitro* et *in vivo* (Panatier et al., 2011; Pascual et al., 2005; Slezak et al., 2012). En effet, l'expression de la portion cytosolique de la synaptobrevine 2 du complexe SNARE spécifiquement dans les astrocytes agit comme dominant négatif qui empêcherait la formation du complexe SNARE et l'exocytose vésiculaire (Pascual et al., 2005). Cette approche a été utilisée pour perturber la gliotransmission et a entrainé une diminution de la libération d'ATP par les astrocytes (Pascual et al., 2005). De la même manière, exprimer génétiquement la chaîne légère du sérotype B de la neurotoxine botulique dans les cellules gliales entraîne une baisse de libération de gliotransmetteurs (Slezak et al., 2012). Cette toxine cliverait la synaptobrevine 2 associée aux vésicules ce qui empêcherait l'exocytose (Schiavo et al., 1992). Finalement, introduire la chaîne légère de la toxine tétanique, aussi connue pour interférer avec le complexe SNARE, directement dans un astrocyte a permis également de bloquer la gliotransmission (Panatier et al., 2011). Dès lors, il semble que la gliotransmission serait en partie dépendante du complexe SNARE.

Il n'en demeure pas moins que la gliotransmission par voie d'exocytose est hautement controversée (Nedergaard and Verkhratsky, 2012; Wang et al., 2013). De plus, différents mécanismes de libération de neurotransmetteurs ont été proposés incluant l'inversion des transporteurs du glutamate, les hémicanaux type pannexines/connexines et les récepteurs pore-canaux (Perea et al., 2009). De manière générale, même si les mécanismes détaillés de la relâche de gliotransmetteurs ne sont pas bien compris, cela mène à la régulation de l'activité synaptique par les cellules gliales.

2.4.1.2.3.1.2. Gliotransmetteurs : agoniste de récepteurs neuronaux

2.4.1.2.3.1.2.1. Les purines

L'ATP et l'adénosine, produite à la suite de la dégradation de l'ATP, sont des purines connues pour médier l'effet de la régulation de la neurotransmission par les cellules gliales. L'ATP serait un agoniste des récepteurs purinergiques de type P2 (Gordon et al., 2005; Gordon et al., 2009; Guthrie et al., 1999; Haas et al., 2006) alors que l'adénosine activerait principalement les récepteurs A1 (Newman, 2003a; Serrano et al., 2006; Todd et al., 2010) ou A2A (Panatier et al., 2011; Todd et al., 2010).

L'ATP relâchée par les astrocytes permet entre autres la propagation de l'activité calcique à travers un réseau d'astrocytes (Haas et al., 2006; Newman and Zahs, 1997). De plus, l'ATP est impliquée dans l'amélioration de l'efficacité synaptique par les astrocytes dans le noyau paraventriculaire de l'hypothalamus (Gordon et al., 2005; Gordon et al., 2009).

Les purines d'origine gliale sont impliquées dans différents types de régulation de l'activité synaptique. Ceci inclut la dépression de l'activité excitatrice évoquée dans l'hippocampe (Martin et al., 2007), la diminution de l'activité spontanée dans le cervelet (Brockhaus and Deitmer, 2002), la régulation de l'inhibition dans le cortex (Lalo et al., 2014) ainsi que la régulation de l'activité neuronale induite par la lumière dans la rétine (Newman,

2003a; Newman and Zahs, 1998). De plus, ces purines ont une importance particulière dans l'expression de différentes formes de plasticité dans le SNC (Chen et al., 2012; Pascual et al., 2005; Serrano et al., 2006) et à la JNM mature (Todd et al., 2010) ainsi que dans la régulation de l'activité de synapses uniques (Panatier et al., 2011). Ces rôles sont discutés dans les sections 2.4.1.2.3.2 et 2.4.1.2.3.3.

2.4.1.2.3.1.2.3. Le glutamate

Les astrocytes pourraient aussi moduler les synapses du SNC par la relâche de glutamate (Angulo et al., 2004; Di Castro et al., 2011; Fellin et al., 2004; Parpura et al., 1994; Perea and Araque, 2007). Ceci inclut la régulation de l'activité excitatrice et inhibitrice évoquée (Araque et al., 1998; Bezzi et al., 1998; Liu et al., 2004), l'activité spontanée (Jourdain et al., 2007; Liu et al., 2004), la modulation de la PLT (Navarrete et al., 2012), la modulation de la dépression à long terme (DLT) (Han et al., 2012; Min and Nevian, 2012) ainsi que la dépression hétérosynaptique (Andersson et al., 2007).

La modulation gliale par la relâche de glutamate reste d'une certaine façon controversée (Nedergaard and Verkhratsky, 2012; Wang et al., 2013). Ceci est peut-être dû à la nature du glutamate qui est le neurotransmetteur principal des synapses excitatrices et qui est présent en milieu extracellulaire à des niveaux variables. Dès lors, il pourrait être difficile de dissocier l'action du glutamate ambiant, neuronale et gliale. Une étude récente montre que les cellules gliales, du moins à la rétine, ont la capacité de libérer le glutamate. Une manipulation génétique qui interfère avec le complexe SNARE, spécifiquement dans les cellules gliales, a entraîné une baisse de la libération du glutamate par les cellules gliales de la rétine (Slezak et al., 2012).

2.4.1.2.3.1.2.4. Le GABA

Le GABA, principal neurotransmetteur aux synapses inhibitrices du SNC, serait aussi libéré par les astrocytes dans certaines conditions. Par exemple, dans le bulbe olfactif, les astrocytes libéreraient le GABA ce qui causerait une inhibition persistante de différents types de neurones dans cette structure (Kozlov et al., 2006). Dans le cervelet, le GABA d'origine astrocytaire permettrait l'inhibition tonique de la neurotransmission (Duguid et al., 2012; Lee et al., 2010). Finalement, dans l'hippocampe, les astrocytes ont aussi la capacité de libérer le GABA (Le Meur et al., 2012). La libération du GABA par les astrocytes est aussi controversée puisque son importance physiologique n'a pas été révélée de manière directe.

2.4.1.2.3.1.3. Gliotransmetteurs : co-agoniste de récepteurs neuronaux

L'un des gliotransmetteurs qui a sollicité beaucoup d'intérêt dans les dernières années est la D-serine. La D-serine est synthétisée dans les astrocytes (Stevens et al., 2003; Wolosker et al., 1999) et elle serait libérée par des mécanismes dépendants du calcium et du complexe SNARE (Mothet et al., 2005) suite à l'activation des récepteurs glutamatergiques gliaux. La D-serine est principalement un co-agoniste des récepteurs N-methyl-D-aspartate (NMDARs) qui jouent plusieurs rôles importants dans le système nerveux incluant la transmission glutamatergique, la transduction de l'activité synaptique, l'induction de la plasticité synaptique ainsi que les fonctions cognitives complexes telles que la mémoire et l'apprentissage (Feldman et al., 1999; Malenka and Nicoll, 1999; Mothet et al., 2000; Whitlock et al., 2006).

Étant un co-agoniste nécessaire pour l'activité des récepteurs NMDAR, la D-serine d'origine astrocytaire a été associée à la régulation de la transmission des NMDARs ainsi que dans la régulation de PLT et la DLT dans le CNS (Fossat et al., 2012; Henneberger et al., 2010; Panatier et al., 2006; Stevens et al., 2003; Takata et al., 2011; Yang et al., 2003; Zhang et al., 2008)

2.4.1.2.3.1.4. Diversité des gliotransmetteurs

Tel que montré plus haut, il y a une diversité de gliotransmetteurs qui a été identifiée (voir Tableau I). Dès lors, plusieurs questions importantes s'imposent. Dans quelles conditions un gliotransmetteur spécifique est-il relâché? Est-ce qu'une même cellule gliale peut libérer plusieurs gliotransmetteurs? La majorité des études se concentrent sur un seul type de gliotransmetteur à la fois. Tel qu'il a été discuté par d'autres, si la relâche d'un gliotransmetteur est détectée, ceci n'exclut pas que d'autres gliotransmetteurs soient libérés en même temps (Sahlender et al., 2014). Il est donc possible que certains gliotransmetteurs soient libérés en même temps en réponse à un contexte synaptique particulier. En parallèle la libération d'une diversité de gliotransmetteurs pourrait faciliter la régulation par les cellules gliales. En effet, il semble que les cellules gliales pourraient réguler la balance entre l'excitation et l'inhibition par la relâche du glutamate et du GABA (Kozlov et al., 2006). Cette balance pourrait aussi être contrôlée par la libération de l'ATP en raison des différents rôles facilitateurs et inhibiteurs des purines (Araque et al., 2014). Il est donc possible qu'une même cellule gliale puisse libérer différents gliotransmetteurs selon le contexte. Le décodage du contexte synaptique permettrait la relâche appropriée de gliotransmetteurs spécifiques pour réguler adéquatement la transmission synaptique. L'un des phénomènes qui supporte cette hypothèse est la plasticité synaptique dans laquelle les cellules gliales sont activement impliquées en libérant différents gliotransmetteurs dépendamment du type de la plasticité et de la région du système nerveux étudiée.

Tableau 1 : Effets des gliotransmetteurs dans différentes régions du système nerveux.

Région du système nerveux	Effet neuromodulateur
Glutamate	
Hippocampe	Dépression des courants postsynaptiques inhibiteurs et excitateurs évoqués
Hippocampe	Augmentation de la fréquence des miniatures postsynaptiques
Hippocampe	Augmentation de la fréquence des courants miniatures inhibiteurs
Hippocampe	Augmentation de la fréquence des courants spontanés excitateurs
Hippocampe	Augmentation de la fréquence des courants spontanés inhibiteurs
Hippocampe	Courants entrants postsynaptiques lents
Hippocampe	Augmentation de l'excitabilité neuronale
Hippocampe	Dépression héterosynaptique
Hippocampe	Modulation de la DLT
Hippocampe	Modulation de la PLT
Hippocampe	Potentialisation synaptique
Hippocampe	Modulation des potentiels d'action
Hippocampe	Régulation de l'activité de synapses uniques
Hippocampe	Régulation de la cinétique des courants miniatures excitateurs évoqués
Cortex	Courants entrants postsynaptiques lents
Cortex	Modulation de la DLT
Thalamus ventro-basal	Courants entrants postsynaptiques lents
Corne dorsale de la moelle épinière	Courants entrants postsynaptiques lents
Noyau médial du corps trapézoïde	Courants entrants postsynaptiques lents
ATP/Adénosine	
Hippocampe	Dépression héterosynaptique
Hippocampe	Modulation de la PLT
Hippocampe	Dépression de l'activité synaptique basale
Hippocampe	Régulation de l'activité de synapses uniques
Hippocampe	Dépression de l'activité excitatrice évoquée
Cortex	Modulation de l'activité de synapses uniques
Cortex	Régulation des oscillations corticales lentes
Cervelet	Dépression de l'activité spontanée excitatrice
Rétine	Activité neuronale induite par la lumière
Rétine	Dépression de l'activité excitatrice induite par la lumière
Nucleus accumbens	Courants entrants postsynaptiques lents
Noyau paraventriculaire de l'hypothalamus	Augmentation de l'amplitude des courants excitateurs
Jonction neuromusculaire	Potentialisation et dépression persistante

Medulla oblongata	Activation des neurones chémorécepteurs
D-Serine	
Hippocampe	Modulation de la PLT
Cortex	Modulation de la PLT/DLT
Rétine	Potentialisation de la transmission NMDARs
Noyau supraoptique de l'hypothalamus	Modulation de la PLT/DLT
Amygdale	Modulation des NMDARs
Amygdale	Augmentation de la plasticité homéosynaptique
TNF	
Hippocampe	Insertion de récepteurs AMPA
Hippocampe	Augmentation de la plasticité homéosynaptique
GABA	
Hippocampe	Courants sortants postsynaptiques lents
Cervelet	Courants toniques
Bulbe olfactif	Courants sortants postsynaptiques lents
S100β	
Tronc cérébral	Régulation du mode de décharge des neurones
Gliotransmetteurs inconnu	
Cortex	Régulation de l'état d'éveil cortical
Jonction neuromusculaire	Dépression synaptique
Jonction neuromusculaire	Potentialisation synaptique

**Tableau modifié de Araque et al., 2014*

2.4.1.2.3.2. Régulation de la plasticité synaptique dans le SNC

L'un des rôles des cellules gliales dans la fonction synaptique est la régulation de la plasticité synaptique. L'activité gliale a été jugée importante pour au moins 4 types de plasticité synaptique inculant la potentialisation à long terme (PLT), la dépression à long terme (DLT), la dépression hétérosynaptique (DHS) et la plasticité homéostatique. Les sections suivantes montrent quelques exemples choisis pour illustrer le rôle crucial qu'occupent les cellules gliales dans la régulation de la plasticité synaptique.

2.4.1.2.3.2.1. Potentialisation à long terme

L'une des formes de plasticité des mieux étudiées est la potentialisation à long terme (PLT). Cette plasticité se traduit par une augmentation persistante et de longue durée des réponses synaptiques et dépend souvent de l'activation des récepteurs NMDA (Escobar and Derrick, 2007; Luscher and Malenka, 2012; Malenka and Nicoll, 1999; Whitlock et al., 2006). Dans le SNC, la PLT serait la base physiologique de plusieurs fonctions incluant l'apprentissage et la formation de mémoires persistantes (Escobar and Derrick, 2007; Malenka and Nicoll, 1999; Whitlock et al., 2006). La PLT peut être induite *in vivo* lors de tâche d'apprentissage ou lors du traitement de l'information sensorielle (Han et al., 2015; Navarrete et al., 2012; Takata et al., 2011; Whitlock et al., 2006). Elle peut aussi être induite *in situ* suite à des stimulations à haute fréquence des terminaisons présynaptiques (Henneberger et al., 2010; Navarrete et al., 2012). Ces méthodes d'induction de la PLT *in vivo* et *in situ* sont aussi connues pour activer les astrocytes et induire des élévations du calcium intracellulaire (Chen et al., 2012; Henneberger et al., 2010; Navarrete et al., 2012; Takata et al., 2011).

Dans différentes régions du SNC, la PLT dépendante des NMDARs requiert la libération astrocytaire de D-serine, co-agoniste nécessaire des NMDARs pour l'induction de la PLT (Henneberger et al., 2010; Panatier et al., 2006; Yang et al., 2003). Ceci a été décrit pour l'hippocampe (Henneberger et al., 2010; Yang et al., 2003; Zhang et al., 2008), le cortex

(Takata et al., 2011) ainsi que le noyau supraoptique de l'hypothalamus (Panatier et al., 2006). De plus, cette libération de D-sérine est dépendante de l'activité Ca^{2+} des astrocytes pusique le blocage des élévations calciques gliales réduit la libération de D-sérine (Henneberger et al., 2010; Takata et al., 2011).

La PLT peut aussi être modulée par des gliotransmetteurs autres que la D-serine. En effet, l'adénosine d'origine astrocytaire régule le seuil et la fenêtre d'induction de la PLT (Pascual et al., 2005; Schmitt et al., 2012). De manière similaire, le glutamate d'origine astrocytaire participerait à la PLT induite dans l'hippocampe par la stimulation sensorielle ou électrique des connexions cholinergiques (Navarrete et al., 2012).

L'implication des astrocytes dans la plasticité a récemment été décrite *in vivo*. Par exemple, l'induction de la PLT par stimulation sensorielle requiert une augmentation de la concentration du Ca^{2+} intracellulaire des astrocytes et nécessite la relâche de gliotransmetteurs (Chen et al., 2012; Navarrete et al., 2012; Takata et al., 2011).

Le rôle des astrocytes dans la régulation de la plasticité synaptique reste hautement controversé. Par exemple, les souris qui montrent un déficit d'activité calcique dépendante de l'IP3, en raison de l'absence génétique des récepteurs à l'IP3 de type R2 (IP3R2) préférentiellement exprimés par les astrocytes (Petravicz et al., 2008), ne montrent pas de déficit de PLT induite *in situ* (Agulhon et al., 2010). Paradoxalement, ces mêmes souris montrent des déficits de la PLT *in vivo*, dans l'hippocampe (Navarrete et al., 2012) ainsi que le cortex (Chen et al., 2012; Takata et al., 2011). Ces observations suggèrent que les astrocytes peuvent avoir un rôle diffèrent selon la méthode d'induction de la PLT et leurs propriétés spécifiques à chaque structure. De plus, il est possible que l'activité des astrocytes ne soit pas entièrement dépendante de l'IP3R2 et que d'autres récepteurs à l'IP3 ou d'autres voies de signalisation calciques soient impliqués. En effet, une récente étude montre que les réponses calciques des astrocytes ne sont pas toutes bloquées en l'absence de l'IP3R2 (Srinivasan et al., 2015). En réalité, certaines variations calciques, dépendantes des protéines G, sont observées dans les prolongements astrocytaires ce qui suggère que l'IP3R2 n'est pas responsable de l'ensemble de l'activité calcique des astrocytes (Srinivasan et al., 2015).

2.4.1.2.3.2.2. Dépression à long terme

Tout comme la PLT, la DLT serait aussi impliquée dans la mémoire et l'apprentissage (Escobar and Derrick, 2007). Dans le cortex, il semblerait que la libération de glutamate par les astrocytes régule la dépression à long terme (DLT) à travers l'activation de récepteurs NMDA présynaptiques (Han et al., 2012; Min and Nevian, 2012). Puisque certaines formes de la DLT dépendent aussi des NMDARs (Luscher and Malenka, 2012). Il est possible que la D-serine en tant que gliotransmetteur affecte cette plasticité. En effet, la modulation de la DLT dans le cortex par la D-serine a aussi été décrite (Fossat et al., 2012). Dès lors, la D-serine, tout comme d'autres gliotransmetteurs (les purines particulièrement), peut avoir un double rôle dans la régulation à la fois de la potentialisation ainsi que la dépression. Ce rôle des cellules gliales dans la régulation du sens de la plasticité a été clairement démontré à la JNM où la relâche d'un même gliotransmetteur permet l'induction d'une potentialisation ou d'une dépression persistante de l'activité synaptique (Todd et al., 2010). La régulation gliale de la plasticité synaptique à la JNM est présentée à la section 2.4.1.2.3.5.

2.4.1.2.3.2.3. Régulation de la dépression hétérosynaptique

Les astrocytes participent aussi à d'autres formes de plasticité telles que la dépression héterosynaptique (DHS). Cette plasticité est la conséquence de la potentialisation d'une synapse précise qui entraîne la dépression d'une autre synapse avoisinante. Ceci permettrait d'augmenter le contraste entre les synapses et dépendrait de l'activité astrocytaire. Une synapse hautement active (p.ex. potentialisée par l'induction de la PLT) peut stimuler les astrocytes par l'intermédiaire d'interneurones GABAergiques (Serrano et al., 2006). Une fois activé, l'astrocyte libérerait de l'ATP. L'adénosine, produit de l'hydrolyse de l'ATP, réduirait la neurotransmission d'une autre synapse avoisinante par l'activation de récepteurs à l'adénosine de type A1 (A1Rs) (Pascual et al., 2005; Serrano et al., 2006). De manière similaire, une dépression hétérosynaptique transitoire a été décrite dans l'hippocampe où l'activation d'un astrocyte par un interneurone GABAergiques, entraineraît la libération

astrocytaire de glutamate. Ce gliotransmetteur provoquerait une réduction transitoire de la neurotransmission d'une autre synapse avoisinante par l'activation de récepteurs mGluRs présynaptiques (Andersson et al., 2007).

2.4.1.2.3.2.4. Régulation de la plasticité homéostatique

La plasticité homéostatique est un type de plasticité qui peut influencer une population de synapses et de neurones pour maintenir des niveaux propices d'excitabilité et de connectivité en réponse à des changements de l'environnement des neurones (Turrigiano, 2011). Par exemple, en ajustant le nombre de récepteurs postsynaptiques, il serait possible de pallier à des manques d'activité synaptique et de rectifier l'activité neuronale globale (Turrigiano et al., 1998). Ce phénomène est crucial pour la fonction des circuits neuronaux (Turrigiano, 2011) et n'est pas purement neuronal puisqu'une implication gliale y a été associée. Dans l'hippocampe, de longues périodes de privation d'activité entraînent une régulation à la hausse de l'activité synaptique. Ceci est médié par une augmentation extracellulaire du TNFα dérivé des astrocytes (Beattie et al., 2002; Stellwagen and Malenka, 2006). Ce TNFα régule la mobilisation des récepteurs postsynaptiques (ex : insertion de récepteurs ionotropes) ce qui permet le renforcement des synapses excitatrices (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Le TNFα promeut aussi l'affaiblissement des synapses inhibitrices (Pribiag and Stellwagen, 2013; Stellwagen et al., 2005). De cette façon, l'excitabilité globale des circuits neuronaux est améliorée, ce qui peut compenser pour la privation de l'activité. De façon similaire, la norépinephrine peut provoquer la relâche d'ATP par les astrocytes ce qui permet d'améliorer l'efficacité synaptique par l'insertion de récepteurs postsynaptiques dans l'hypothalamus (Gordon et al., 2005).

2.4.1.2.3.2.5. Plasticité synaptique et protéines gliales

Les cellules gliales expriment une variété de protéines telles que S100β (commun à la majorité des cellules de la lignée gliale; Reynolds and Woolf, 1992; Van Eldik et al., 1984) et la protéine acide fibrillaire gliale « Glial fibrillary acidic protein (GFAP) » (spécifique aux astrocytes et leurs analogues incluant les CSPs; Georgiou et al., 1999; Jessen et al., 1990; Kisseberth, 1994) . Ces protéines ont diverses fonctions et pourraient aussi affecter l'activité gliale et neuronale.

Le S100β est une protéine intracellulaire capable de lier le Ca^{2+}. Étonnamment, les souris transgéniques dépourvues de S100β montrent une augmentation de la PLT est une meilleure performance à diverses tâches liées à la mémoire et l'apprentissage (Nishiyama et al., 2002b). Ceci suggère que le S100β serait un modulateur de l'activité neuronale et de la plasticité synaptique. De manière intéressante, une nouvelle étude montre que le S100β serait un gliotransmetteur qui pourrait être relâché par les astrocytes dans certaines conditions (Morquette et al., 2015). Ce S100β permettrait la chélation du calcium extracellulaire et l'activation de conductances sensibles au calcium (Morquette et al., 2015). Un tel mécanisme permet la modulation de l'activité neuronale et a été lié à des fonctions importantes du système nerveux dans la genèse de mouvements rythmiques qu'impliquent certaines tâches comme la locomotion ou la mastication (Kadala et al., 2015; Morquette et al., 2015).

Le GFAP est un filament intermédiaire du cytosquelette présent dans les astrocytes et les CSPs. La délétion génétique du GFAP entraine des déficits de plasticité majeure incluant une diminution de la DLT dans le cervelet (Shibuki et al., 1996) ainsi qu'une augmentation de la PLT dans l'hippocampe (McCall et al., 1996). Ceci suggère que le GFAP serait important pour les interactions neurones-glies. Puisqu'il s'agit d'un filament du cytosquelette important dans la croissance des prolongements astrocytaires (Chen and Liem, 1994; Rutka and Smith, 1993; Weinstein et al., 1991), ceci pourrait renforcer l'importance des prolongements gliaux et leur couverture de la synapse.

2.4.1.2.3.3. Régulation de l'activité de synapses uniques dans le SNC

Malgré la proximité des prolongements astrocytaires des fentes synaptiques, l'activité des astrocytes a longtemps été considérée trop lente par rapport à la transmission synaptique pour être impliquée dans la modulation locale de la neurotransmission (Araque et al., 2014). Cependant, deux nouvelles études montrent que les domaines astrocytaires, associées à une seule synapse, détectent l'activité locale en montrant des réponses calciques isolées d'une résolution temporelle compatible avec la relâche de neurotransmetteurs (Di Castro et al., 2011; Panatier et al., 2011). En retour, les astrocytes régulent l'efficacité de synapses uniques (Di Castro et al., 2011; Panatier et al., 2011). Ceci suggère que les domaines de la cellule gliale seraient équipés de la machinerie nécessaire pour détecter et influencer l'activité de synapses individuelles. Il est donc possible que la base des interactions neurone-glie et la régulation de l'activité synaptique par les cellules gliales soient une combinaison de la modulation spécifique de chaque synapse selon le contexte et les besoins du système.

2.4.1.2.3.4. Recapture de neurotransmetteurs

Une autre façon, indépendante de la relâche de gliotransmission, qui permet aux cellules gliales de contrôler l'activité synaptique, serait la recapture de neurotransmetteurs et le contrôle de leur concentration ambiante.

Les neurotransmetteurs relâchés dans la fente synaptique sont éliminés rapidement par différents mécanismes pour limiter leur action. Dans le SNC, le glutamate libéré est rapidement éliminé par l'action de transporteurs. Les astrocytes participant activement à la recapture du glutamate par l'intermédiaire de leurs transporteurs glutamatergique : « glutamate transporter 1 » (GLT1) et « glutamate aspartate transporter » (GLAST) (Bergles et al., 1999; Bergles and Jahr, 1998; Danbolt, 2001). Ceci est facilité par la proximité des prolongements gliaux de la synapse ce qui permet de créer une isolation physique qui peut contrer la diffusion du glutamate (Oliet et al., 2001; Piet et al., 2004). Ceci est compatible avec une nouvelle étude

qui montre que le transporteur GLT-1 n'est pas exprimé n'importe où sur la membrane astrocytaire, mais qu'il est spatialement confiné aux sites synaptiques (Murphy-Royal et al., 2015). Le glutamate transporté est transformé par les astrocytes en glutamine qui est rendue disponible pour la synthèse du glutamate (voir revue Bak et al., 2006). Cette recapture est importante puisque l'absence de GLT-1 ou GLAST au niveau des astrocytes résulte en une augmentation du glutamate extracellulaire, de l'excitotoxicité et de phases épileptiques (Huang and Bergles, 2004; Peghini et al., 1997).

De plus, les astrocytes sont impliqués dans la recapture du transmetteur inhibiteur GABA par les transporteurs GABAergiques (Schousboe, 2003). C'est un mécanisme régulateur important puisque l'élimination et la dégradation du GABA des synapses est critique pour la cessation de la signalisation GABAergique. En effet, la délétion des transporteurs GABA des astrocytes entraîne une augmentation de la concentration extracellulaire du GABA et une augmentation de l'inhibition (Kersante et al., 2013; Shigetomi et al., 2012).

Dès lors, le contrôle de la recapture de neurotransmetteurs par les astrocytes, en réponse à l'activité neuronale, permet de contrôler l'activité synaptique et la balance excitation-inhibition pour ainsi éviter des effets néfastes et des altérations de l'activité nerveuse.

2.4.1.2.3.5. Régulation de la plasticité synaptique à la JNM

À la JNM de souris, la potentialisation persistante de la neurotransmission dépend aussi de la libération d'ATP par les CSPs qui est dégradée en adénosine. L'adénosine en activant les récepteurs à l'adénosine de type A2A (A2ARs) permet une augmentation persistante de la neurotransmission (Annexe I; Todd et al., 2010). Cette potentialisation se produit à la suite d'une activité neuronale soutenue qui induit une réponse calcique gliale de type monophasique. Cette activité calcique monophasique entraîne la libération d'ATP par les

CSPs et l'activation des récepteurs A2ARs présynaptiques (Todd et al., 2010). Une potentialisation similaire de la neurotransmission a été décrite à la JNM de grenouilles et dépendrait de l'augmentation de la concentration du Ca^{2+} intracellulaire des CSPs suite à sa relâche des réserves internes (Castonguay and Robitaille, 2001). Non seulement ces CSPs régulent la potentialisation synaptique, mais elles contrôlent aussi la dépression persistante de la neurotransmission à la JNM de souris. En effet, tel que mentionné plus haut, une stimulation de type phasique produit une réponse calcique des CSPs de type oscillatoire. En retour, les CSPs induisent une dépression synaptique en activant les récepteurs présynaptiques A1Rs (Todd et al., 2010). L'activité calcique des CSPs est à la fois nécessaire et suffisante pour induire la plasticité de l'activité synaptique. En effet, activer directement les CSPs, en induisant une réponse calcique monophasique ou oscillatoire par la photoactivation de molécules de Ca^{2+}-encagé, permet d'induire directement une potentialisation ou une dépression selon le type de la réponse Ca^{2+} gliale (Todd et al., 2010). De manière générale, ceci suggère que les cellules gliales contrôlent la modulation purinergique et la balance entre potentialisation et dépression de la neurotransmission en relation avec l'activité synaptique. Ceci renforce le lien entre le décodage de l'activité synaptique par les cellules gliales et la régulation appropriée de la neurotransmission.

Les CSPs sont aussi impliquées dans la dépression de l'activité synaptique durant une activité à haute fréquence (Robitaille, 1998). L'activation des protéines G des CSPs, par l'injection intracellulaire de la forme non hydrolysable du Guanosine triphosphate (GTPγS) cause une dépression de la neurotransmission. L'activation des protéines G des CSPs serait responsable de la dépression de la neurotransmission lors d'une activité neuronale endogène intense. En effet, le blocage des protéines G des CSPs réduit la dépression synaptique alors que leur activation occlut cette dépression endogène (Robitaille, 1998).

En conclusion, il semble que les cellules gliales régulent différentes formes de plasticités et que cette régulation dépend de la capacité des cellules gliales à décoder l'activité synaptique.

2.4.2. Plasticité des cellules gliales

Tout comme les neurones, les astrocytes montrent une plasticité et une capacité d'adaptation à des changements de leur environnement. Les astrocytes peuvent remodeler leurs prolongements pour réguler leur organisation autour des synapses (Haber et al., 2006). De cette façon, les mécanismes de détection, de recapture de neurotransmetteurs et de régulation peuvent être adaptés aux besoins des synapses. Dans le noyau supraoptique de l'hypothalamus d'animaux en lactation, il y a une rétraction des prolongements astrocytaires des synapses, ce qui affecte directement la régulation de l'activité synaptique (Oliet et al., 2001; Panatier et al., 2006; Piet et al., 2004). Ceci permettrait par exemple d'adapter la fonction des astrocytes à ce contexte précis et de permettre le fonctionnement adéquat du système nerveux. Dans le même ordre d'idées, la couverture des synapses par les astrocytes augmente dans le cortex somatosensoriel suite à une stimulation sensorielle (Genoud et al., 2006). De plus, une récente étude montre que l'induction de la PLT dans l'hippocampe change l'interaction des prolongements astrocytaires avec la synapse (Perez-Alvarez et al., 2014).

Non seulement la structure des cellules gliales est variable, mais son excitabilité aussi s'adapte en réponse à des changements de leur environnement. À la JNM, l'excitabilité calcique des CSPs n'est pas identique à chaque JNM, mais varie selon les propriétés synaptiques et la fonction musculaire (Annexe II; Rousse et al., 2010). De cette manière, la réponse des CSPs est adaptée à leur environnement et à l'efficacité des synapses et dépend tant des propriétés synaptiques que des propriétés intrinsèques des CSPs (Rousse et al., 2010). De manière intéressante, une adaptation inadéquate des propriétés gliales à leur environnement a été décrite dans la sclérose latérale amyotrophique (Arbour et al., 2015). De plus, les propriétés des CSPs peuvent être activement modifiées en réponse à des changements chroniques de l'activité synaptique de manière à ce que les récepteurs gliaux, impliqués dans la détection de neurotransmetteurs, et leur contribution à l'activité calcique, puissent changer (Belair et al., 2010).

Bref, il devient évident que la structure et l'activité gliale peuvent s'adapter à la fonction, la condition et le contexte synaptique, ce qui permettrait une détection et une régulation appropriée de l'activité synaptique.

2.5. Le développement du système nerveux

Comme décrit dans les parties précédentes, la synapse montre une organisation spécifique, nécessite une machinerie sophistiquée et spécialisée dans la communication synaptique qui est hautement régulée. D'ailleurs, plusieurs mécanismes endogènes régulent l'activité neuronale qui montre une grande plasticité et capacité d'adaptation aux changements. De plus, cette activité neuronale est grandement régulée par les cellules gliales qui contrôlent plusieurs aspects du fonctionnement du système nerveux. Le développement de toutes ces capacités nécessite un long processus de synaptogenèse qui assure la formation d'une synapse équipée de la machinerie nécessaire pour permettre le fonctionnement du système nerveux. Dès lors, la formation synaptique nécessite une coordination d'évènements pour assurer un assemblage synaptique précis, suivi d'un long processus de maturation. Là aussi, la formation et la maturation ne sont pas des mécanismes purement neuronaux, mais requièrent la contribution des cellules gliales.

Le développement du système nerveux est un processus complexe qui nécessite la mise en marche de plusieurs mécanismes et l'interaction de différents types cellulaires. Le type et le nombre de cellules qui composent le système nerveux doivent être contrôlés. Ces cellules doivent migrer au bon endroit et envoyer des projections vers des cibles précises. Ces projections doivent naviguer sur de longues distances pour atteindre leur cible appropriée et former des connexions synaptiques. Par la suite, le développement du système nerveux nécessite un long processus de maturation où les connexions synaptiques sont remodelées, raffinées et renforcées. Cette phase de maturation nécessite la collaboration de plusieurs types cellulaires et l'action de différentes molécules ayant diverses origines et cibles. L'étape de maturation est influencée par l'expérience du monde externe et elle est grandement façonnée par l'activité synaptique. Tout cela pour former des contacts synaptiques dotés de toutes les propriétés nécessaires pour contrôler différentes fonctions.

Le développement du système nerveux peut se diviser principalement en 3 étapes : 1. Formation et différenciation cellulaire, 2. Guidance axonale et formation synaptique, 3.

Maturation et raffinement synaptique. Étant donné que cette thèse traite principalement du rôle des cellules gliales dans la compétition et l'élimination synaptique qui sont des étapes cruciales pour la maturation synaptique, la première étape sera présentée brièvement. Plus d'emphase sera mise sur la deuxième, mais surtout sur l'étape de maturation et raffinement synaptique en présentant la synaptogenèse à la JNM.

2.5.1. Formation et différenciation cellulaire du système nerveux

Le système nerveux est formé de cellules qui proviennent du développement de l'ectoderme, couche de cellules épithéliales qui couvre l'extérieure de l'embryon durant le développement embryonnaire précoce. La partie dorsale de l'ectoderme s'épaissit pour former la plaque neurale. Cette plaque neurale, à la suite d'un processus complexe de neurulation, s'invagine pour former le tube neural qui est à l'origine du système nerveux central (Fundamental Neuroscience (4th edition; 2013). P287-308) .

À la limite dorsale du tube neural, sur les bords de l'invagination de la plaque neurale, des cellules précurseurs forment les crêtes neurales. Au moment de l'invagination et de la fermeture du tube neural, les cellules de la crête se détachent et migrent. Celles-ci sont à l'origine de la formation d'une partie importante du système nerveux périphérique (Fundamental Neuroscience (4th edition; 2013). P287-308).

Le développement du système nerveux s'en suit et les différentes parties sont graduellement formées. Ce n'est qu'une fois que l'encéphale et la moelle sont formés par le tube neural que la différenciation des cellules du système nerveux peut commencer (Fundamental Neuroscience (4th edition; 2013). P287-308).

Le système nerveux est composé principalement de neurones et de cellules gliales formés suite aux processus de neurogenèse et gliogenèse. Ces deux types cellulaires

proviennent d'un progéniteur commun : les cellules souches neurales. Ces cellules ont la capacité de se reproduire et se différencier en neurones et en cellules gliales. L'identité cellulaire est définie par un processus complexe de différenciation qui dépend de signaux de transduction et l'expression de marqueurs spécifiques qui permet, subséquemment, la genèse de neurones et cellules gliales matures (voir revue Paridaen and Huttner, 2014).

2.5.2. La synaptogenèse

Une fois les neurones formés, ils envoient des projections vers des cibles bien spécifiques. Ces projections doivent parcourir de longues distances et sont guidées vers leur cible par des molécules et mécanismes de guidance tout au long du trajet (Bashaw and Klein, 2010; Tessier-Lavigne and Goodman, 1996). Une fois que les axones atteignent leurs cibles, les contacts synaptiques sont formés et l'assemblage de la synapse peut commencer. Au moment de l'initiation de la formation de synapses, un nombre important de mécanismes sont responsables de la formation, la solidification et le maintien des contacts synaptiques. D'ailleurs, il y a une coordination d'événements qui nécessitent divers facteurs et signaux chimiques pour assurer un assemblage synaptique précis. En effet, un ensemble de molécules régulent à la fois l'organisation pré- et postsynaptique. De manière intéressante, le processus de formation des connexions synaptiques est démesuré et mène à la production excessive de synapses. De manière transitoire, chaque cible peut être innervée par un nombre important d'axones (Chen and Regehr, 2000; Katz and Shatz, 1996; Lohof et al., 1996; Wyatt and Balice-Gordon, 2003).

Malgré le fait que la synapse soit fonctionnelle dès sa formation, elle doit subir un long processus de maturation. Ceci dépend entre autres de l'activité neuronale qui permet de façonner l'organisation et la fonction synaptique. De plus, la formation et la maturation ne sont pas des mécanismes purement neuronaux, mais ils requièrent la contribution des cellules gliales. Ces mécanismes de formation et de maturation sont plus étudiés et mieux compris à la JNM et seront discutés dans la section suivante.

2.6. Synaptogenèse à la JNM : « Neuromuscular synaptogenesis: coordinating partners with multiple functions »

La JNM est la synapse formée entre un motoneurone et une fibre musculaire qui contrôle le mouvement volontaire. Cette synapse est abondamment utilisée pour l'étude de la synaptogenèse en raison de son accessibilité et son organisation d'apparence simple. Cependant, la simplicité de la JNM nécessite un processus complexe de formation et de maturation. Ces processus requièrent la collaboration de la terminaison présynaptique, la fibre musculaire ainsi que les cellules gliales. De plus, sa maturation est grandement influencée par l'activité pour ainsi former une synapse hautement spécialisée, efficace et fiable.

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« NEUROMUSCULAR SYNAPTOGENESIS: COORDINATING PARTNERS WITH MULTIPLE FUNCTIONS »

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1. Abstract

The formation of highly efficient and reliable synapses at the neuromuscular junction (NMJ) relies on dynamic molecular interactions. Studies of the development and maturation of the NMJ have focused on events that are dependent on synaptic activity and that require the coordinated actions of nerve- and muscle-derived molecules with different targets and effects. More recently, perisynaptic Schwann cells — the glial cells at NMJs — have become an important focus of research. These glia concomitantly contribute to pre- and postsynaptic maturation while undergoing maturation themselves. Thus, an intricate 'danse à trois' regulates the maturation of the NMJ to form a highly efficient communication unit, in which fine glial processes lie in close proximity to a highly concentrated population of postsynaptic receptors and perfectly aligned presynaptic release sites.

2. Introduction

During embryonic development in vertebrates, motor neuron axons grow long distances from the spinal cord to reach their distal targets[1]. They form the link between the CNS and the rest of the body — particularly the striated muscles that effect voluntary movements. The direction of this long-distance axonal travel towards specific muscles to be innervated requires well-orchestrated interactions among axons, axonal Schwann cells and environmental cues[1, 2]. Owing to its large size, accessibility and simple organization, the neuromuscular junction (NMJ), which enables the nervous system to control the muscular system, has been used extensively to study synapse formation[3, 4]. However, this apparent simplicity belies the complex processes that take place during synapse formation and that are necessary for NMJ function. Indeed, the formation of perfectly aligned presynaptic assemblies and postsynaptic compartments requires an intricate interplay between molecular signals. Ultimately, the two elements become tuned functionally to one another, with the level of presynaptic transmitter release adapted to the capacity of postsynaptic activation.

Synapse formation is followed by another major step, synapse maturation, which leads to the stabilization of all of the synaptic elements to form a reliable synapse. It has long been acknowledged that the maturations of pre- and postsynaptic elements at the NMJ are interdependent; however, until recently, the elaborate interactions between these two compartments were poorly understood. Presynaptic maturation was previously assumed to be controlled by molecules that are released by the postsynaptic apparatus, and vice versa[5, 6, 7, 8]. However, recent advances in molecular analysis (for instance, improvements in fusion proteins and cell-specific gene deletion) have revealed a more complex scenario, wherein some molecules regulate both pre- and postsynaptic organization. Thus, it is possible that the formation and maturation of pre- and postsynaptic compartments are in fact tightly coordinated by shared molecular mechanisms. Moreover, although NMJ formation was originally thought to be regulated by only pre- and postsynaptic mechanisms, more recent evidence has implicated a role for glial molecular mechanisms in coordinating the maturation of the two synaptic elements. Indeed, it was reported that perisynaptic Schwann cells (PSCs;

also known as terminal Schwann cells) produce and release synaptogenic factors[9, 10, 11, 12, 13, 14], and their own maturation is modulated by pre- and postsynaptic molecules[15, 16, 17, 18, 19, 20]. This tri-directional interaction is also required for key activity-dependent changes that are involved in NMJ maturation and is particularly important during synaptic competition and elimination. Indeed, PSCs detect activity from competing synapses[18] and are responsible for debris clearance and the elimination of superfluous nerve terminals[21, 22]. Thus, PSCs could represent a link between the activity-dependent and molecularly regulated changes that are essential for the maturation of the NMJ.

In this Review, we discuss the growing evidence of a tri-directional molecular and activity-dependent crosstalk among presynaptic terminals, postsynaptic muscle fibres and PSCs at the developing NMJ. This coordination structures and organizes the presynaptic release machinery and postsynaptic receptors, and thus enables the tight assembly of an efficient and reliable communication unit.

3. Molecular mechanisms of NMJ development

NMJ formation, which is initiated during embryonic development (around embryonic day 12 (E12) to E13.5 in rodents)[5, 7, 23, 24], is preceded by the guidance of the axon towards the muscle fibre that is to be innervated and involves accurate molecular signalling to ensure the formation of precise nerve–muscle contacts. A period of maturation follows that leads to morphological and functional changes to the pre- and postsynaptic elements. Maturation of the NMJ depends on molecular mechanisms that elaborate the complex pre- and postsynaptic organization and that reduce the number of axonal terminals at a single NMJ until only one remains; these mechanisms are necessary for efficient synaptic communication. Presynaptic terminals must express synaptic proteins to form active zones, whereas postsynaptic elements express high densities of aggregated receptors at the crests of postsynaptic folds. Together, it is the 'synaptic elements' — namely, the presynaptic nerve terminal, the postsynaptic muscle fibre and the associated perisynaptic glia — that collectively coordinate the maturation of the NMJ.

Pathfinding, guidance and pre-patterning. During embryonic development, pools of motor neurons in the spinal cord form organized motor columns (already present by E6–7 in chick embryos)[25, 26, 27], which can be classified according to their location (rostrocaudal, mediolateral or dorsoventral) within the spinal cord[27, 28]. This columnar organization seems to be crucial for establishing the proper inputs of each class of motor neuron to their specific effector targets to control complex locomotor behaviour[27, 29, 30]. This well-structured organization suggests that elaborate mechanisms are required to guide the right group of motor axons to their specific final target. It is now recognized that pathfinding is not controlled by the target muscle per se[31] but instead by guidance cues along the path of the motor neuron[27, 32], and that this process is dependent on neuronal activity[33]. These mechanisms, and the wide range of genes and transcription factors involved, have been discussed elsewhere (reviewed in Refs 1,27).

When motor axons reach their final target (the timing of which varies according to the muscle and its location; around E12.5 for the mouse diaphragm)[23], muscle fibres already show a certain level of maturation, as indicated by the presence of clusters of ionotropic acetylcholine receptors (AChRs) on their surface[23, 34, 35]. The presence of AChR clusters on the fibres prior to the arrival of the motor axon indicates that these AChRs are not directly linked to synaptic activity. This has raised the question of whether the pre-patterning (that is, nerve-independent clustering) of AChRs dictates the location of NMJs on muscle fibres[23, 34, 36, 37, 38]. Although initial work in cell culture has shown that NMJs do not form preferentially at pre-existing AChR clusters[39, 40], evidence obtained from mammals and other vertebrates suggests that AChR pre-patterns have a role in defining the location of nerve–muscle contacts[37, 38, 41, 42].

Upon contact with muscles, motor axons innervate muscle fibres extensively to generate polyinnervated NMJs and large motor units[43, 44, 45]. Although NMJs are functional at this stage, the motor neurons are not efficient enough to induce muscle contractions in a refined and precise way; this efficiency requires NMJ maturation. Even though pre- and postsynaptic maturation are discussed separately below, the two processes are interdependent and involve molecules that are derived from, and act on, all three synaptic elements.

Postsynaptic maturation. The postsynaptic compartment undergoes major rearrangement during NMJ maturation, including increases in the number, stability, density and clustering of AChRs; these changes are mediated by key molecular mechanisms.

The proteoglycan agrin is the most studied molecule involved in postsynaptic organization. Mice that lack agrin (*Agrn*$^{-/-}$ mice) show major defects in postsynaptic AChR organization: AChRs in these animals are uniformly distributed on the surface of the muscle fibre and do not cluster after muscle innervation[46]. These mice also show presynaptic defects, such as poor arborization of motor axons[46] and aimless overgrowth of nerves[23]. However, in these animals, the pre-patterned AChR clusters that form prior to innervation are not affected[23, 34, 46]. Interestingly, intramuscular injection of recombinant agrin induces the formation of AChR clusters in denervated muscles and the formation of ectopic AChR clusters in adult muscles[47, 48, 49]. Agrin can be synthesized and released by neurons, muscle fibres and

Schwann cells (hereafter defined as SCs when referring to Schwann cells that are associated with axons or when the type is unclear), and has varying AChR-clustering efficacy depending on alternative splicing of the agrin gene[50, 51]. Generally, neuronal forms of agrin are more potent at clustering AChRs[11, 52, 53]. However, AChR clustering can be enhanced by the active PSC-derived form of agrin — for instance, at the end of tadpole metamorphosis or during nerve regeneration at the amphibian NMJ[11].

Agrin triggers the autophosphorylation of the muscle-specific tyrosine kinase receptor (MUSK)[54]. MUSK is enriched in the postsynaptic endplate, colocalizes with AChR[55] and has a pivotal role in AChR clustering. Mice lacking MUSK (*Musk*$^{-/-}$ mice) show a more severe phenotype than do *Agrn*$^{-/-}$ mice: *Musk*$^{-/-}$ mice show neither AChR pre-patterns nor nerve-induced clustering of AChRs, and their motor axons grow aimlessly over the muscle[54]. Agrin interacts with MUSK via low-density lipoprotein receptor-related protein 4 (LRP4), a transmembrane protein that is enriched at the NMJ[56, 57, 58]. Mice that lack this lipoprotein die at birth and show major defects that are comparable to those seen in *Musk*$^{-/-}$ mice[56]. Indeed, LRP4 links agrin and MUSK activation, and is required for AChR clustering[56, 57, 58]. Furthermore, the association of LRP4 with MUSK is sufficient to trigger MUSK autophosphorylation — even in the absence of agrin — and LRP4 is important for the pre-patterning of AChR clusters prior to the arrival of the nerve terminal[57, 58]. As a tyrosine kinase receptor, activated MUSK influences numerous downstream signalling pathways (reviewed in Ref. 59) and promotes the accumulation of synaptic membrane proteins such as rapsyn and neuregulin receptors. As well as being a self-associating protein, rapsyn can cluster AChRs, dystroglycan and other important NMJ proteins. Mice that are null for rapsyn show diffusely distributed AChRs over the surface of the muscle fibre and have impaired neuromuscular transmission[60] (reviewed in Ref. 8).

WNT ligands are secreted molecules that regulate many aspects of embryonic development, including cell survival, cell death, cell proliferation and axon guidance, AChR aggregation and AChR clustering[61, 62], as well as the differentiation of the pre- and postsynaptic elements of the NMJ (reviewed in Refs 62,63). In zebrafish, WNT ligands interact with MUSK — before the nerve arrives at the NMJ — to induce the pre-patterning of

AChRs on muscle fibres[7, 64]. WNT proteins could subsequently participate in AChR clustering while nerve–muscle contacts form[65, 66]. WNTs seem to be both positive and negative regulators of AChR clustering. In cultured mouse myotubes, WNT3, a WNT protein that is expressed by nerve terminals at the mouse NMJ, acts with agrin to increase AChR clustering[65]. By contrast, WNT3A, another isoform of WNT, suppresses the expression of rapsyn and thus inhibits AChR aggregation at the surface of cultured myotubes[66]. Moreover, WNT can be released by motor neurons and by other cell types, including glial cells[14,67]. In *Drosophila melanogaster*, Wingless proteins (which are WNT orthologues) from glia at the NMJ facilitate the clustering of glutamatergic postsynaptic receptors[14]. However, although the roles of WNT proteins in postsynaptic maturation are gradually being revealed, their *in vivo* contributions at the mammalian NMJ remain poorly understood owing to their diverse actions[62, 63]and cellular sources[14, 67].

The maturation of the NMJ must coincide with an increase in the synthesis of AChR and its associated proteins such as rapsyn and MUSK. Neuregulins — in particular, neuregulin 1 — that are expressed by motor neurons, muscle fibres and SCs[68, 69, 70] may regulate the transcription of AChR-encoding genes and functional receptor expression[70, 71]. Indeed, NMJs do not form properly in mice that lack neuregulin 1 or its receptors, ERBB2 and ERBB3 (Refs 16,17,72). Extracellular neuregulin 1 binds to these tyrosine kinase receptors of the ERBB family to promote the synthesis of AChR and its associated proteins[68, 71, 73]. However, the exact signalling pathway that is involved in promoting AChR synthesis and clustering remains elusive, and studies have questioned whether neuregulins and their receptors are necessary for NMJ formation[15, 74]. For instance, the specific ablation of muscle ERBB2 and ERBB4 receptors in mice did not result in major defects[15], whereas overexpression of a constitutively active form of the ERBB2 receptor in muscle fibres induced the aberrant formation of NMJs[75]. One study showed that motor-neuron-derived neuregulin 1 was not necessary for proper AChR density and clustering, and that muscle neuregulin 1 was not necessary for postsynaptic maturation[74]. The ambiguity associated with the role of neuregulin 1 may be partly due to the multiple cellular targets and functions of neuregulin 1, notably in SC survival and maturation[16, 17, 72, 76]. Indeed, SCs are absent in mice that lack neuregulin 1, ERBB2 or ERBB3, and NMJs in these mice do not form properly and are not

maintained[16, 17, 72]. As PSCs express neuregulins and their receptors[69], it has been proposed that the function of neuregulin 1 — in particular, its effect on postsynaptic maturation — may be mediated indirectly via PSCs[8, 15] (rather than by its direct effects on the muscle fibre). If this is the case, then both neuronal and PSC-derived neuregulins could be important for the maturation and maintenance of the NMJ, as well as for the regulation of the synthesis of AChR and its associated proteins. It remains unclear whether the NMJ defects observed in mice that are null for neuregulin 1, ERBB2 or ERBB3 are due to the absence of SCs or the lack of neuregulin signalling, or both. Studies examining the effect of deleting PSC-derived neuregulin 1 or neuregulin 1 receptors at the surface of PSCs would help to resolve this issue. Nonetheless, it is apparent that a combination of molecules that are derived from various sources must act in a concerted way to regulate and promote AChR clustering, as well as postsynaptic assembly and maturation (Table 1).

Table 1 : Molecular signalling for NMJ maturation.

Molecules	Sources	Targets	Roles	Species	Refs
Postsynaptic maturation					
Agrin	NT, MF and SC*	MUSK–LRP4 complex	MUSK activation, leading to ↑AChR clustering	Mouse, frog and *D. melanogaster*	11,23,50, 54
LRP4	MF and NT‡	MUSK	MUSK activation, leading to ↑AChR clustering	Mouse	56–58,109, 110,112
WNT ligands	MF, NT and SC§	MUSK (WNT9A, WNT11, WNT16)	Agrin-dependent MUSK activation, leading to ↑AChR clustering	Mouse, zebrafish and *D. melanogaster*	61,64–66
		MUSK (WNT3)	Agrin-independent MUSK activation, leading to ↑AChR clustering		
		Target of WNT3A is not clear	Suppression of rapsyn expression, leading to ↓AChR clustering		
ACh	NT	AChRs	↓AChR synthesis and clustering	Mouse	78–80,185
Neuregulin 1	NT and SC‖	ERBB receptors	↑Synthesis and clustering of AChRs and associated proteins	Mouse	15,16,68, 71–74
TGFβ	SC	TGFBR1–TGFBR2 complex	↑Agrin expression	Frog	12
		Punt receptors	↑Agrin expression	*D. melanogaster*	13
Synaptic laminins	MF	Integrins on MF (for laminin β2), dystroglycan on MF (for laminins α4 and α5)	↑AChR clustering	Mouse	81,88,105
Presynaptic maturation					
Synaptic laminins	MF	N-type (early) or P/Q-type (late development) VGCCs on NT (for laminin β2); target for laminins α4 or α5 is not clear	• Regulation of active-zone proteins • Presynaptic organization	Mouse	6,88,89, 186
FGF7, FGF10 and FGF22	MF	FGFR2B on NT	↑Vesicle clustering	Mouse	93
Collagen α2, α3 and α6 chains (IV)	Not clear	Not clear	↑Vesicle clustering	Mouse	93
BDNF	MF	Pro-BDNF: p75NTR	Retraction of NT	Mouse	102,104
		Mature BDNF: TRKB	Nerve survival		
GDNF	MF and SC¶	Not clear	Regulation of number of innervating axons	Mouse	98,101,187
WNT ligands	MF, NT and SC#	Not clear	Presynaptic branching and maturation	*D. melanogaster*	113,114
LRP4	MF and NT	Not clear	• Presynaptic maturation (*in vivo*) • Synaptic vesicles and active-zone protein clustering (shown *in vitro*)	Mouse	110,112
TGFβ	SC	TGFBR1–TGFBR2 complex	Increase in nerve–muscle contacts and maturation	Frog and *D. melanogaster*	12,13
PSC maturation					
Neuregulin 1	NT and SC	ERBBs	SC proliferation and survival	Mouse	15–17
Synaptic laminins	MF	Not clear	Laminins β2 and α4 or α5 control SC proliferation and migration	Mouse	19,20
ATP	NT	P2YRs	SC activation	Mouse	18

ACh, acetylcholine; AChR, ACh receptor; BDNF, brain-derived neurotrophic factor; *D. melanogaster*, *Drosophila melanogaster*; FGF, fibroblast growth factor; FGFR2B, fibroblast growth factor receptor 2B; GDNF, glial-cell-derived neurotrophic factor; LRP4, low-density lipoprotein receptor-related protein 4; MF, muscle fibre; MUSK, muscle-specific tyrosine kinase receptor; NMJ, neuromuscular junction; NT, nerve terminal; P2YR, purinergic type 2Y receptor; p75NTR, p75 neurotrophin receptor; PSC, perisynaptic Schwann cell; SC, Schwann cell; TGFβ, transforming growth factor-β; TGFBR, transforming growth factor-β receptor; TRKB, tropomyosin-related kinase B; VGCC, voltage-gated calcium channel. *Neuronal isoform is more potent, muscle isoform is dispensable and SC isoform enhances AChR aggregation. ‡Expression of neuronal LRP4 has not been determined (see main text for details). §At the *D. melanogaster* NMJ, SC-associated WNT (Wingless) signalling regulates glutamate receptor clustering. ‖Not yet clear. ¶Overexpression of GDNF by SCs did not cause hyperinnervation. #The SC-derived WNT-induced contribution to presynaptic regulation remains unknown.

Although most of the mechanisms described above promote the synthesis and clustering of AChRs, other molecular signals promote the removal of improperly localized AChRs and limit the number of AChRs. Interestingly, this regulation is partly activity-dependent, as neuronal ACh release inhibits the expression of AChRs and promotes their endocytosis[77, 78]. NMJs that lack choline acetyltransferase (ChAT) — which exhibit very low levels of ACh synthesis — are larger, more complex and have a broader distribution of AChR clusters, which suggests that ACh antagonizes AChR clustering[78, 79, 80]. In fact, synaptic activity may trigger positive- or negative-feedback mechanisms depending on the state of postsynaptic AChRs. As the nerve–muscle contact is formed, nerve terminals release positive signals — such as agrin — that promote AChR clustering. However, if AChRs are not stabilized, then cholinergic neural activity promotes their disruption and prevents further AChR synthesis and clustering. Interestingly, the phenotype observed in *Agrn*$^{-/-}$ mice — the uniform, non-clustered distribution of AChRs — is partially rescued in mice that lack both agrin and ChAT[78]. Such feedback mechanisms may also help to explain why AChRs are only found at synapses: other proteins, such as MUSK and rapsyn, ensure AChR stability and thus counteract the effect of cholinergic neural activity.

Similar to AChR clustering, the accumulation of agrin in the extracellular matrix (ECM) must be controlled to maintain proper receptor clustering. This may be achieved by matrix metalloproteinases (MMPs), which regulate the ECM by cleaving matrix proteins[81]. For instance, purified MMP3 can directly cleave agrin, lowering its levels in the ECM[82, 83, 84, 85]. MMP3-null mutant mice show an increased density of AChRs, as well as agrin immunoreactivity[82, 85]. MMP3 is secreted in its pro-form and requires cleavage to become active (reviewed in Ref. 86). This activation might be activity-dependent, as denervated muscles exhibit high levels of agrin and presumably reduced levels of active MMP3 (Ref. 85). Interestingly, MMP3 is found in the ECM around PSCs and is absent from active-zone areas[82, 83, 85]. Given the similar positioning of PSC processes and MMPs at the edge of the synaptic cleft, it is tempting to propose that MMPs that are from, or activated by, PSCs decrease perisynaptic levels of agrin, leaving it concentrated in the endplate area, where maximal AChR clustering is observed.

Presynaptic maturation. Presynaptic nerve terminals also undergo major changes during NMJ formation. Specifically, there is an increase in the number of nerve ramifications and active zones, and in the levels of associated proteins; there are changes in the distribution and types of calcium channels expressed; and there is a decrease in the number of nerve terminals in contact with a single muscle fibre. Unlike postsynaptic maturation, fewer molecular signals that control presynaptic differentiation have been identified, and the molecular mechanisms that underlie presynaptic maturation are less well understood.

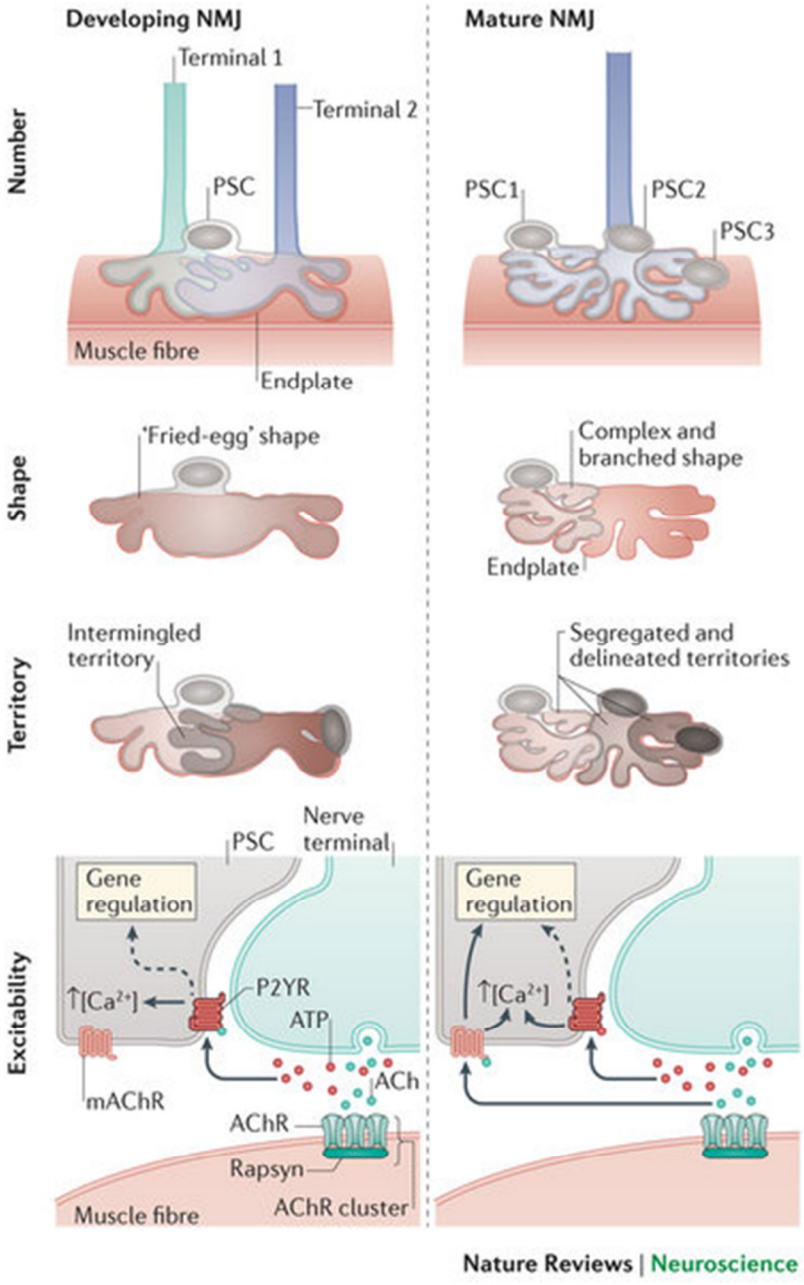
Laminins are glycoproteins that are considered to be important regulators of presynaptic terminal maturation. They are heterotrimeric (comprising α, β and γ subunits) muscle-derived glycoproteins and are included in the basal lamina of the ECM. The absence of laminins results in mild to severe defects in NMJ development[6, 19, 87, 88]. For example, NMJs from mice that lack the laminin β2 chain (which is present in all synaptic laminins) have fewer active zones, synaptic vesicles that do not cluster and reduced synaptic activity. In addition, the processes of SCs in these animals invade the synaptic cleft[87, 88]. Laminin β2 binds to presynaptic N-type voltage-gated calcium channels during early stages of development and to P-type and Q-type channels at later stages[81, 89](different channels are involved in transmitter release at these respective stages of development)[89, 90]. The interaction of laminin β2 with calcium channels regulates active-zone proteins such as bassoon and the distribution of calcium channels[89, 91]. Other synaptic laminins, such as laminin α4 and laminin α5, are required for NMJ maturation and for the proper alignment of pre- and postsynaptic elements (reviewed in Ref. 81).

Certain fibroblast growth factors (FGFs) — namely, FGF7, FGF10 and FGF22 — promote NMJ maturation and vesicle clustering in motor neurons *in vitro*[92, 93] and *in vivo* during embryonic and early postnatal development, as revealed using mice lacking FGF receptor type 2B (FGFR2B;*Fgfr2b*$^{-/-}$ mice)[93]. Muscle fibres are the main sources of FGFs, which signal at the NMJ mainly through presynaptic FGFR2B[93, 94]. Another molecule involved in NMJ maturation is type IV collagen, which is found primarily in the basal lamina[93, 95]. Vesicle clustering is promoted by collagen IV α2 chains during embryonic stages, and by collagen IV α3 and α6 chains during postnatal development[93]. Interestingly, FGFs

(FGF7, FGF10 and FGF22), laminin β2 and collagen IV (α2, α3 and α6 chains) were all found to be important for presynaptic maturation, and the three classes of molecules were shown to act sequentially from embryonic to postnatal stages[93]. At least for FGF7, FGF10, FGF22 and collagen IV (α3 and α6 chains), this idea is consistent with the levels of expression of mRNA encoding these proteins at different developmental stages[93]. Type XIII collagen may also be important for pre- and postsynaptic maturation, as NMJs from mice that lack collagen XIII have fewer active zones, abnormal AChR clustering and improper presynaptic–postsynaptic alignment, all of which correlate with synaptic defects[96]. Finally, the extracellular domain of the signal regulatory protein-α (SIRPα), which is a member of the immunoglobulin family, promotes synaptic-vesicle clustering in cultured chick spinal cord motor neurons[97].

Glial-cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) also regulate presynaptic maturation. GDNF is expressed by muscle fibres and SCs, and promotes motor neuron survival[98, 99]. Injection of GDNF, or the genetic overexpression of its muscle isoform, leads to an increase in the number of nerve terminals innervating the same NMJ and delays synapse elimination[100, 101]. SCs also express GDNF, but the role of SC-derived GDNF in NMJ maturation remains unclear[101]. Like GDNF, the application of BDNF to the muscles of neonatal mice delays synapse elimination[102]. BDNF is initially synthesized as its precursor form (pro-BDNF), which is then cleaved to generate the mature form (mBDNF)[103]. Through different receptor interactions, pro-BDNF favours synapse elimination, whereas mBDNF promotes the maintenance of synapses[102, 104]. Moreover, the conversion from pro-BDNF to mBDNF may be regulated by synaptic MMP3 and MMP9 (Refs 102,104). As MMP3 localizes in the ECM that surrounds PSCs[85], it is again appealing to propose that PSC-derived MMPs might convert pro-BDNF to mBDNF and thus influence presynaptic maturation.

Box 1: Maturation of PSCs.



Perisynaptic Schwann cells (PSCs) are generated from stem cells that originate from the neural crest and follow motor axons into the muscles, where they reach their final location at the neuromuscular junction (NMJ) (reviewed in Refs 5,162). Immature Schwann cells begin to take on a PSC phenotype once they reach their location at the NMJ. As the number of PSCs at an NMJ increases[18, 150, 151], their shapes become increasingly complex, their motility

decreases and their territories become segregated (see the panels in the figure illustrating the changes in the number[18, 149, 151], shape[149] and territory[149] of PSCs). These changes have been attributed to the spatial constraints that dictate PSC territories, suggesting that PSCs actively compete for available space[149]. Interestingly, this period of PSC remodelling coincides with the peak of postnatal maturation of the NMJ; in mice, this occurs during the first postnatal week. During this period, PSCs show a high level of phagocytic activity[146]when pruning connections during synapse maturation[21, 22], and they express receptors to detect and decode neurotransmission[18].

Interestingly, the PSC receptors that detect neurotransmission differ between developing and mature NMJs (see the figure panel labelled 'excitability')[18, 154]. Whereas PSCs at immature NMJs rely mainly on purinergic signalling[18], PSCs at mature NMJs use purinergic receptors (including purinergic type 2Y receptors; P2YRs) and muscarinic metabotropic acetylcholine receptors (mAChRs) to detect released neurotransmitters[154]. This differentiation may be important for regulating the expression of a number of genes, particularly those under the control of mAChR activation[163], such as the gene encoding glial fibrillary acidic protein (GFAP). One intriguing possibility is that the receptor types involved may be adapted to the state of NMJ plasticity, such that low mAChR activity is permissive for growth and remodelling, whereas higher mAChR activation promotes stability and the modulation of synaptic activity and efficacy.

Coordinated tri-directional maturation of the NMJ. In order to build synapses that are adapted and functionally tuned, the maturation of the NMJ must be well coordinated. Many molecules and pathways, from all three synaptic elements at the NMJ, are involved in aspects of this process (Fig. 1; Table 1).

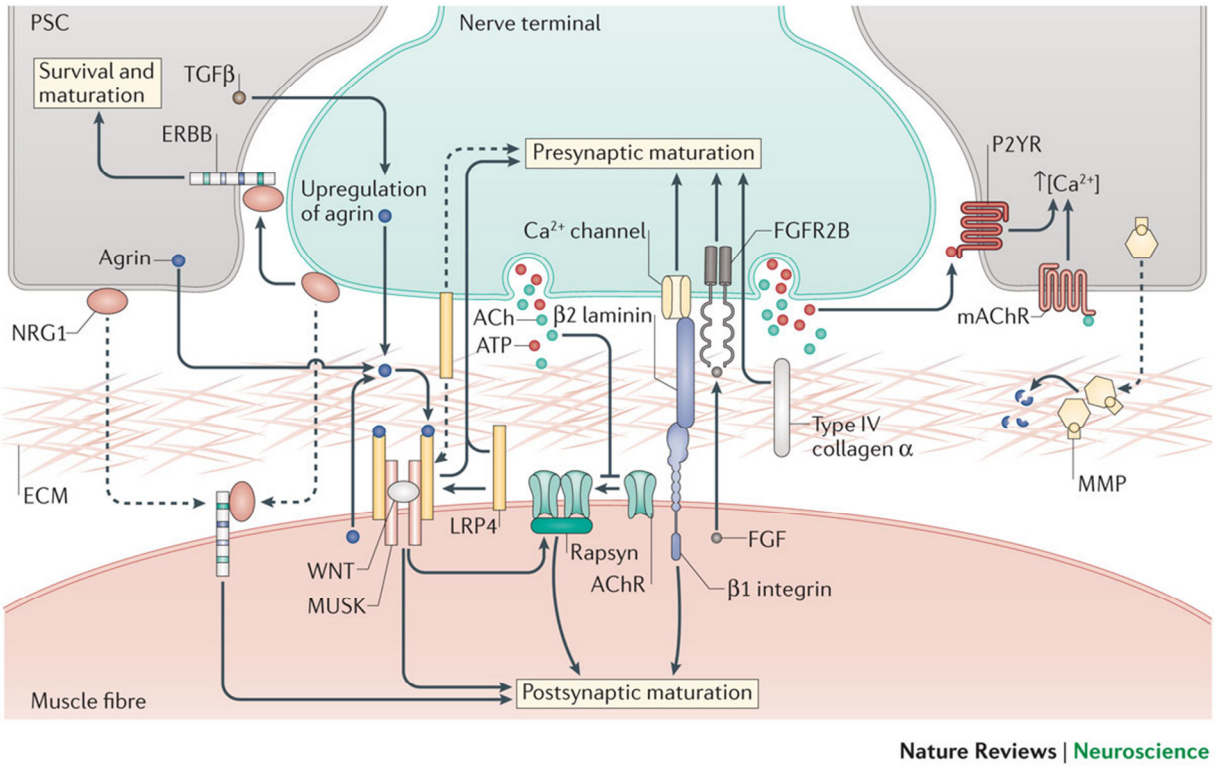


Figure 1. Synapse formation at the NMJ: generating a functional communication unit.
Parallel signalling pathways at the neuromuscular junction (NMJ) are involved in the coordinated maturation of the presynaptic terminal (green), the postsynaptic muscle fibre (red) and the perisynaptic Schwann cells (PSCs; grey); dotted lines indicate pathways that are still being debated. Agrin, which is released by the nerve terminal, muscle fibre and surrounding PSCs, acts on the LRP4–MUSK complex, which comprises low-density lipoprotein receptor-related protein 4 (LRP4) and muscle-specific tyrosine kinase receptor (MUSK). The phosphorylation of MUSK leads to rapsyn-mediated clustering of ionotropic acetylcholine receptors (AChRs) and postsynaptic maturation. AChR clustering can also be enhanced by WNT ligands (associated with MUSK in the figure), whereas release of ACh inhibits AChR clustering. LRP4 is mainly of postsynaptic origin, although neuronal LRP4 may also have a

role (see main text for details). LRP4 acts as a co-receptor for agrin and stimulates AChR clustering as well as presynaptic maturation by clustering synaptic vesicles and active-zone proteins (not shown). Neuregulin 1 (NRG1) can be released by the nerve terminal and/or surrounding PSCs, and binds to PSC-expressed or postsynaptic ERBB receptors (ERBB2 or ERBB3). The binding of NRG1 to ERBB receptors on PSCs promotes PSC survival and maturation. Although the exact signalling pathway is controversial, the activation of postsynaptic ERBB receptors by NRG1 may increase levels of postsynaptic proteins such as rapsyn, MUSK and AChR, and could have a role in AChR clustering, which overall leads to postsynaptic maturation. Synaptic laminins, such as laminins β2, α4 and α5, are released by the muscle fibre. They form heterotrimeric glycoproteins that are included in the basal lamina and are important for proper pre- and postsynaptic alignment and maturation, as well as PSC maturation (not shown). Laminin β2 binds to presynaptic calcium channels and regulates active-zone proteins (not shown). Postsynaptically, laminins interact with integrin β1, which increases AChR clustering. Fibroblast growth factors (specifically FGF7, FGF10 and FGF22) are released by the muscle fibre and activate mainly presynaptic type 2B FGF receptors (FGFR2B); thus, they are important for vesicle clustering and presynaptic maturation, as are type IV collagen α chains (α2, α3 and α6). PSC-derived transforming growth factor-β (TGFβ) induces presynaptic maturation and postsynaptic differentiation by upregulating the expression of agrin. Synaptically released ATP is detected by PSC-expressed purinergic type 2Y receptors (P2YRs) and triggers increases in intra-PSC Ca^{2+} concentrations. PSCs also express muscarinic AChRs (mAChRs), and their activation by the local application of ACh triggers increases in intra-PSC Ca^{2+} concentrations. However, mAChRs are not activated by endogenous ACh release. Matrix metalloproteinases (MMPs) in the extracellular matrix (ECM) that surrounds PSCs regulate the composition of the ECM and cleave matrix proteins such as agrin, triggering its removal from the ECM.

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Muscle-derived laminins that contain α4, α5 or β2 chains have an autocrine role in postsynaptic maturation[105]. Laminins containing the β2 chain bind to integrin receptors containing the β1 subunit in the ECM to increase AChR clustering, and laminins with an α4

or α5 chain bind to dystroglycan, which stabilizes AChRs in the postsynaptic membrane and participates in the recruitment of acetylcholinesterase to the NMJ[106, 107, 108] (reviewed in Ref. 81). Thus, the anterograde and retrograde effects of synaptic laminins may be responsible for proper pre- and postsynaptic alignment. Laminins also control glial maturation: synaptic laminin 521 (which comprises α5, β2 and γ1 subunits) actively inhibits PSCs from extending their processes into the synaptic cleft[19]. In mice that lack the laminin β2 chain, the invasion of the NMJ synaptic cleft by PSC processes could explain many of the abnormalities in NMJ function, as PSCs could block synaptic transmission and neuron– muscle interactions[88]. Therefore, laminins coordinate the maturation of the NMJ and ensure the correct relative positioning of each synaptic element[19].

Like laminins, LRP4 acts bidirectionally to coordinate pre- and postsynaptic development. LRP4 acts as an agrin co-receptor to stimulate AChR clustering[57, 58, 109], but it is also necessary for presynaptic maturation (which is reflected by the clustering of synaptic vesicles and active-zone proteins)[110]. Muscle-derived LRP4 may act as a retrograde signal to regulate presynaptic maturation in a MUSK-independent manner[110]. By contrast, MUSK may indirectly participate in presynaptic differentiation by clustering LRP4 (Refs 59,110,111), thus concentrating the bidirectional signalling of LRP4. Although the expression of LRP4 by muscle fibres has been established[56, 57], a recent study suggests that neuronal LRP4 may also contribute to NMJ maturation[112]. More-severe NMJ deficits were observed in mutant mice that lacked both muscle- and motor-neuron-derived LRP4 than in mice that only lacked muscle-derived LRP4 (Ref. 112). However, direct evidence of LRP4 protein or RNA expression specifically in motor neurons remains undetermined[112].

WNT ligands may also have effects beyond AChR clustering. For instance, disrupting Wingless protein activity in *D. melanogaster* results in both pre- and postsynaptic NMJ defects, which suggests that, at least in flies, Wingless proteins serve as both anterograde and retrograde signals to promote NMJ formation[113, 114]. BDNF and GDNF could also have multiple roles at developing NMJs, as muscle fibres and SCs express receptors for both neurotrophic factors[115, 116, 117]. In neuron–muscle cultures, GDNF increases the membrane levels of AChRs in the muscle fibre without altering AChR synthesis[118], whereas in cultured

myotubes BDNF can inhibit agrin-induced AChR clustering[115] and maintain AChR clusters[117]. Moreover, the regulation of PSC activity by neurotrophic factors at the adult mouse NMJ[119] indicates that such molecules may have broader effects on NMJ synaptogenesis.

As highlighted above, PSCs participate in many important steps of synapse maturation. SCs rely on pre- and postsynaptic elements for survival and maturation (Box 1; Table 1), but they also contribute to pre- and postsynaptic maturation and NMJ maintenance. For instance, SCs are absent in mice that lack neuregulin 1, ERBB2 or ERBB3. Although contact formation seems to occur normally in these mice, nerve terminals subsequently retract and motor neurons degenerate shortly afterwards[16, 72]. This strongly suggests that SCs are not important for the initial process of synapse formation but are essential for the survival of motor neurons and for the maintenance of NMJs during synapse maturation. SCs in general are important for neuronal survival[120, 121], and PSCs may have similar roles at the NMJ. One study examined the contribution of PSCs at the NMJ using complement-mediated cell lysis to specifically ablate PSCs at the amphibian NMJ *in vivo*[10]. Without PSCs, the NMJs were smaller, had fewer branches and underwent a long-term loss of synapses[10]. Similar observations were made at the mouse NMJ following selective PSC ablation with a ganglioside-specific autoantibody[122, 123].

4. Activity-dependent NMJ maturation

Although the NMJ is functional upon its formation, it undergoes maturation for a prolonged period. Like all synapses in the nervous system, it depends on synaptic activity to develop an optimal organization and proper synaptic efficacy to accomplish its function. Activity shapes both the postsynaptic apparatus[8, 78, 79] and presynaptic organization, as well as synaptic function. One of the most fascinating activity-induced changes at the NMJ is the dramatic decrease in the number of synaptic connections. In mature NMJs, a single nerve terminal contacts the postsynaptic cell; however, during early stages of development, each muscle fibre is innervated by several nerve terminals that branch from different motor neurons[44, 45]. As a result, competition ensues between nerve terminals for the same postsynaptic endplate area. This competition is activity-dependent and leads to the elimination of supernumerary inputs; it is regulated by pre- and postsynaptic elements and by PSCs associated with the synapse. A similar activity-dependent competition occurs between regenerated axons during the reinnervation of muscle fibres following nerve injury (Box 2).

Unlike the pathways that regulate pre- and postsynaptic maturation, little is known about the molecular mechanisms that regulate activity-dependent synaptic competition. Moreover, the relationship between the activity-dependent processes and the molecular pathways that drive synapse formation and maturation remains ill defined. We discuss below synaptic competition and elimination, with a particular focus on the possible underlying molecular mechanisms and the role of glial cells during these processes.

Synaptic competition and elimination. Between the late embryonic period and the early postnatal period, nerve terminals at the same NMJ compete with one another for the sole innervation of the muscle fibre[4, 45, 124, 125]. This process is important for the development of proper synaptic connections and synaptic function[43, 44, 126, 127]. *In vivo* time-lapse imaging of synaptic elimination at dually innervated NMJs revealed that one axon progressively vacates a synaptic area, which is then gradually taken over by a competing nerve input[125, 128]. Typically, a 'losing' terminal ultimately retracts in the form of a 'retraction bulb', which is eliminated by

PSCs[21,125]. This competition process is highly dynamic, and competing terminals continuously vie for territories. Indeed, the outcome of the competition is not preordained, and the territories of nerve terminals can even shift back and forth[125], such that a terminal that initially occupies a smaller area could take over the territory and ultimately 'win' the competition[125]. Interestingly, a recent study showed that terminals that were considered to be losing the competition (that is, smaller terminals) — and even retraction bulbs at a distance from the endplate — were able to reverse their fate and grow back to occupy the NMJ if their competitor was ablated[129].

The difficulty in predicting a winning nerve terminal solely on the basis of morphological and structural observations suggests the mechanisms involved in synaptic competition and elimination are complex. It has been suggested that the outcome of synaptic competition and elimination depends on the efficacy and structure of nerve terminals — properties that are dependent on neuronal activity[130, 131, 132]. The general idea is that, at early stages of synaptic competition (for example, during the first postnatal days in mice), terminals that compete at the same NMJ could have comparable synaptic efficacies and presynaptic areas that occupy equally sized postsynaptic territories. Then, one input is gradually strengthened and occupies a larger area at the expense of the other nerve terminals[44, 133]. At dually innervated NMJs, a disparity in the synaptic efficacies of competing inputs gradually builds up, resulting in a clear distinction between a strong input (with a high release of neurotransmitter) and a weak input (with a low release of neurotransmitter)[18, 130,132] (Fig. 2).

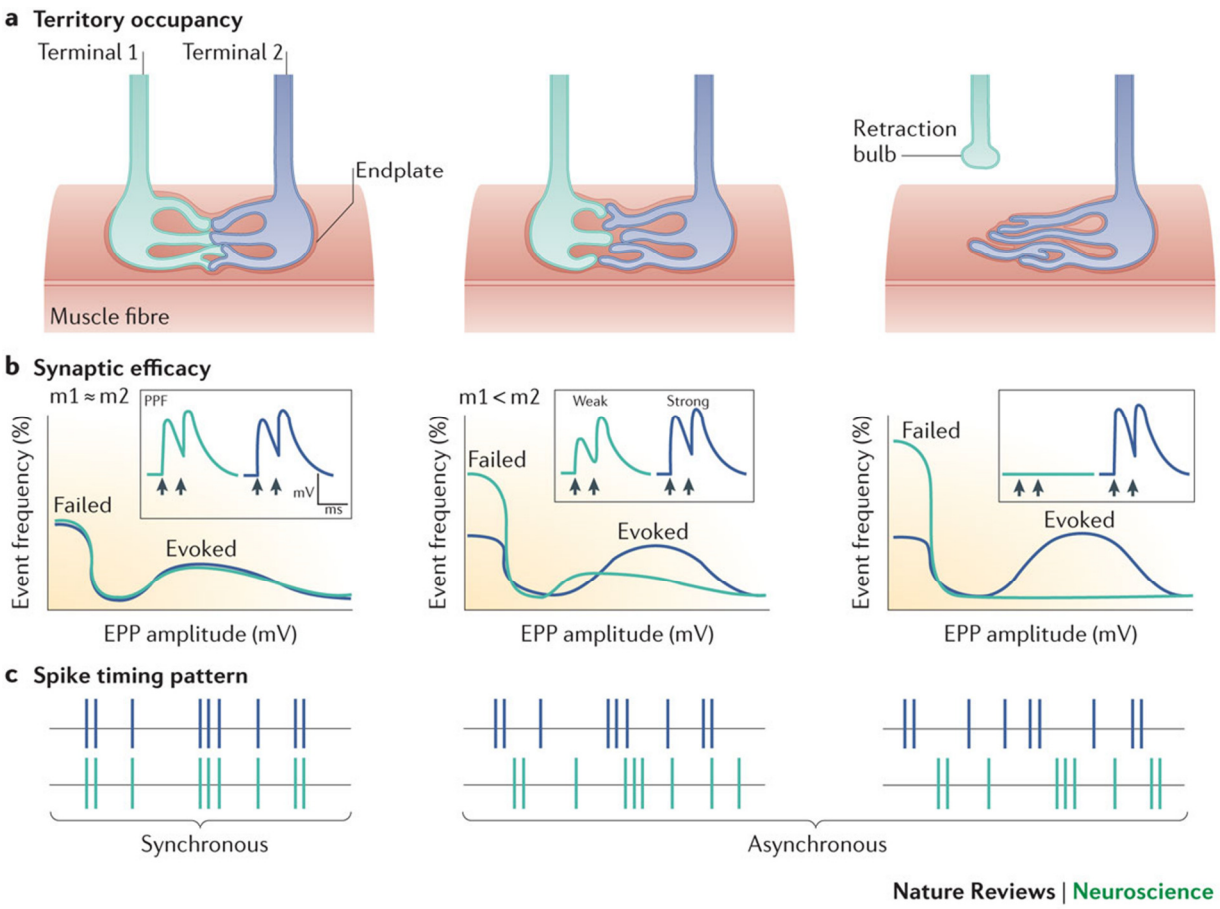

Figure 2. Synaptic competition and elimination at the NMJ.

Morphological and functional changes during postnatal maturation are associated with synaptic competition and elimination. **a** | A dually innervated neuromuscular junction (NMJ) with two nerve terminals (green and blue) competing for the same endplate (represented by the darker red on the pale red muscle fibre) is represented schematically. Competing terminals start with similar territories (left panel; territories could be intermingled, but this is not shown here). Gradually, territories segregate and one terminal (blue) occupies a larger area (middle panel), leading to the elimination of the losing axon (right panel). **b** | Competing terminals could have a similar synaptic efficacy (left panel), as defined by the small differences in quantal content 'm', the similar rate of failed and evoked endplate potentials (EPPs), and the similar paired pulse facilitation (PPF) profiles of each of the terminals (left graph). One input (blue) gradually becomes stronger (middle graph), with a larger quantal content, lower failure rate and lower PPF (which is defined as the increase in the amplitude of the second EPP compared to the first one) and a larger number of evoked EPPs with higher amplitudes.

(Experiments supporting these observations were performed in a condition of low probability of release, with low Ca^{2+} and high Mg^{2+} concentrations)[18, 130,132]. Once the losing terminal (green) retracts (right graph), it no longer induces synaptic activity at this given NMJ (100% failure). **c** | Patterns of neuronal firing change from synchronous motor-unit activity (left panel) to asynchronous activity (middle and right panels), which leads to synapse elimination. The functional changes depicted in parts **b** and **c** are indirectly associated with the morphological changes presented in part **a**.

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Although initially a controversial idea[134, 135, 136], there is evidence to suggest that synaptic inputs that are more active are favoured during synaptic competition[131, 137, 138, 139, 140, 141]. For instance, Buffelli *et al.*[131] deleted ChAT in a subset of motor axons in mice to cause a robust neuronal-subset-specific reduction in neurotransmission and hence a disparity in synaptic efficacy between affected and unaffected subsets. This manipulation resulted in maintenance of the more-active inputs and elimination of the others[131]. In addition to relative synaptic efficacy, the pattern of activity of competing terminals (spike timing) is an important determinant of synapse elimination. During the early phase of synaptic competition (postnatal day 0 (P0) to P4), motor-unit activity from competing terminals is synchronous, but these inputs become gradually asynchronous during the period when synapse elimination is prominent (P4–P10). Motor units reach complete asynchrony at the end of the synaptic competition and elimination process (at ~P14 in mice)[139, 142]. Interestingly, inducing asynchrony between spikes from competing axons promotes synapse elimination, whereas imposed synchrony extends the period of synaptic competition[137].

Asynchrony — that is, distinct firing patterns from different presynaptic terminals — could allow the muscle fibre and/or PSCs to detect and distinguish between independent inputs and then actively participate in the strengthening or weakening of individual terminals. For example, focal blockade of AChRs in a portion of an endplate area caused presynaptic retraction from this specific area, whereas blockade of an entire endplate area did not induce presynaptic loss[138]. This suggests that the postsynaptic element must distinguish between

inputs to promote survival or elimination. More-active nerve terminals are also more likely to fire out of synchrony with their 'quieter' competitors; therefore, a number of spikes will occur in the most active input when its competitors are silent[137]. Thus, both the level and the pattern of activity shape synaptic competition and elimination.

Although a direct link between synaptic strength, structure and elimination has yet to be elucidated, there is evidence to support the notion that these three factors are interdependent[44, 129, 131]. For example, a slight decrease in synaptic activity could weaken the interaction between a terminal and a muscle fibre, resulting in a vacated territory as the less active terminal is remodelled. This territory could then be occupied by the competing terminal, and this new occupation may increase the feedback from the muscle fibre and PSCs to reinforce this terminal and result in improved efficacy. An experiment in which both synaptic activity and the territory occupancy of competing terminals could be monitored and modulated would provide valuable information on the relationship between synaptic organization and activity during synapse competition. In parallel with the effects of synaptic activity and territory occupancy, competing terminals may constantly battle against each other for survival factors such as GDNF and BDNF[101, 102, 104]. Indeed, pro-BDNF and mBDNF serve as punishment and reward signals, respectively, to either eliminate or maintain competing nerve terminals, leading to the maintenance of more-active inputs[102, 104]. Thus, this would ultimately favour a nerve terminal that has a larger synaptic strength and a better pattern of synaptic activity, and that is in a more advantageous position to benefit from survival factors.

The process of synaptic competition requires a constant remodelling of the NMJ such that the molecular mechanisms that are involved in synapse maturation may also be dynamically regulated during synaptic competition. The elimination of nerve terminals must involve activity-dependent molecular mechanisms to weaken some nerve–muscle interactions while stabilizing others. Hence, one could hypothesize that the mechanisms involved in AChR clustering and stabilization (including MUSK signalling and receptor anchoring by rapsyn) are regulated in an activity-dependent manner. As some terminals are eliminated, the postsynaptic territory can be either re-occupied by neighbouring inputs or left vacated[22, 125]. If left unoccupied, the postsynaptic apparatus will be remodelled: the improperly localized AChRs

will be dispersed and removed[125, 143]. Consistent with this possibility, during postnatal development the endplate area changes from an 'en plaque' oval structure to a branched 'pretzel-like' shape owing to the loss of AChRs from some regions[143]. As ACh regulates the expression and clustering of AChRs, this transition could be activity-dependent[77, 78, 79, 80]. Indeed, this physical transformation temporally corresponds with synaptic competition and elimination events, which themselves are activity-dependent[8, 143]. Moreover, activity-dependent changes may be regulated by many molecules, such as laminins, as the transition into a pretzel-like shape is delayed at NMJs in mice that lack laminin α5 (Ref. 105).

Glial roles in synaptic competition and elimination. As axons gradually retract, they leave behind debris-containing synaptic organelles known as axosomes[21, 144] (Fig. 3). These are formed by the engulfment of axonal endings by PSCs and are subsequently entirely contained within these cells[21]. Axosomes have been observed following the elimination of terminal branches — leading to segregation of the territories of competing terminals[21] — and during the retraction of nerves as retraction bulbs, which leads to synapse elimination[21] (Fig. 3a). Consistent with the well-known role of glial cells in debris clearance[145], the retraction of terminals is associated with high lysosomal activity, which suggests that axosomes are processed through the lysosomal pathway of SCs[22,146]. Interestingly, axonal debris was cleared more slowly in a mouse model of a lysosomal storage disease[146]. At the fly NMJ, the phagocytosis of presynaptic debris results from coordinated efforts of glial and muscle cells[147, 148]. These cells phagocytose presynaptic debris, including debris from weak and unstable synaptic boutons. This is an activity-dependent phenomenon, as the accumulation of debris increases following high-frequency stimulation of motor neurons[148].

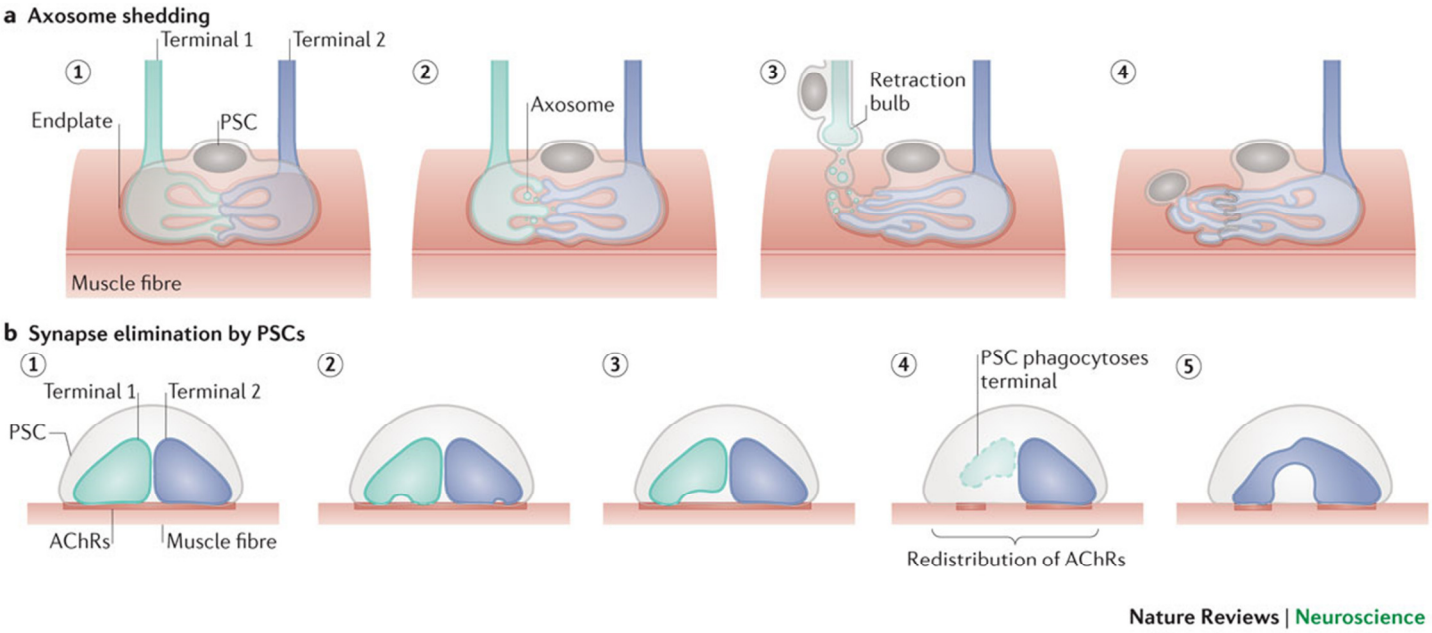

Figure 3. PSCs eliminate supernumerary connections.

a | A schematic representation of the losing nerve terminal shedding axosomes[21, 144] is shown. Step 1: during synaptic competition, competing nerve terminals (in green and blue) may occupy similar territories of the muscle fibre endplate (red) at the neuromuscular junction and are covered by a perisynaptic Schwann cell (PSC; translucent grey). Step 2: PSCs are thought to engulf the endings of nerve terminals, leaving organelles containing synaptic debris (axosomes) that are processed by the PSC lysosomal pathway (not shown). This decreases the size of the territory occupied by the losing terminal (terminal 1; green). This territory can be gradually occupied by its competitor (terminal 2; blue). Step 3: PSCs continue to phagocytose the losing terminal until the nerve retracts into a retraction bulb, which is ensheathed by Schwann cell processes. Step 4: following the elimination of the losing terminal, a single terminal (terminal 2; blue) innervates the muscle fibre. **b** | Mechanism of synapse elimination by PSCs[22] is shown. Step 1: a schematic representation shows a PSC (translucent grey) covering two nerve terminals (terminal 1, green; terminal 2, blue), which are competing to innervate the same muscle fibre (red). Steps 2 and 3: the PSC seems to compete with nerve terminals for acetylcholine receptors (AChRs; red band) by extending its processes between the nerve terminals and the muscle fibre, partially blocking synaptic neurotransmission. Step 4: the distribution of AChRs can be remodelled in places that no longer receive neurotransmitters. In parallel, the PSC phagocytoses nerve endings (in this case, the green

terminal) that are in contact with the muscle fibre, on a stochastic basis[22]. Step 5: the vacated area can be occupied by a nearby terminal (blue).

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With regard to synapse reorganization and elimination, a key question is how the nerve terminals to be maintained — or pruned and eliminated — are identified. Two recent studies implicate PSCs in synapse elimination. First, Smith *et al.*[22] showed that during development, PSCs arrive at the NMJ and cover all terminals with no apparent preference. Early in the process of synapse elimination (that is, during embryonic and early postnatal stages), PSCs extend their processes between the nerve terminals and muscle fibres, obstructing AChRs and synaptic communication. It has been proposed that PSCs compete with nerve terminals to be in close proximity to AChR-covered surfaces in order to make even more contact with AChRs than the terminal boutons. PSCs then phagocytose and remove synaptic branches from different terminals, resulting in the random elimination of terminals. The eliminated terminals leave behind vacant territory that can be occupied by non-eliminated neighbouring inputs[22] (Fig. 3b). Interestingly, given the tight relationship between AChRs and glial coverage, it has been proposed that PSCs may also sculpt the postsynaptic endplate[22], perhaps via an increase in MMP3 activity, which would cause the cleavage of agrin and thus the elimination of unstable AChRs[83, 85]. Such mechanisms could contribute to the transition of the endplate into a pretzel-like shape, and would be consistent with the change of PSCs from a 'fried-egg' shape to a branched morphology during postnatal development[149] (Box 1). However, these hypotheses remain to be tested.

Smith *et al.*[22] propose that PSCs do not select a winning nerve terminal per se but instead randomly eliminate branches, eventually leading to a sole survivor. This study provides extensive and important information on the ultrastructural organization of polyinnervated NMJs. It also highlights the complex structural interaction between competing nerve terminals, the postsynaptic apparatus and the surrounding PSCs. However, it does not fully explain the role of neuronal activity in this scenario. Our group proposes another mechanism by which PSCs might be involved in synapse competition and elimination[18]. As

weaker terminals are more likely to be eliminated, PSCs may be able to differentiate between weaker and stronger terminals, and could therefore actively participate in the targeted elimination of 'losing' synapses. PSCs must possess certain qualities to actively decide which synapses to eliminate. As exemplified by the fact that most NMJs on the soleus muscles are covered by a single PSC during this period[18, 150, 151], a single PSC must be able to discriminate between competing inputs and accurately compare their relative synaptic strengths (as relative synaptic strength is a major determinant of synaptic competition). This condition was tested at dually innervated NMJs using a nerve–muscle preparation in which the activity of individual terminals could be independently evoked and recorded while observing the increases in intra-PSC Ca^{2+} levels (which reflect PSC activity) that were evoked by synaptic activity[152, 153, 154]. We showed that a single PSC at a dually innervated NMJ detects neurotransmitter that is released from each nerve terminal[18] (Fig. 4). Moreover, Ca^{2+} responses that were induced within the same PSC by neurotransmission from different competing terminals were proportional to the relative synaptic efficacy of each terminal (Fig. 4).

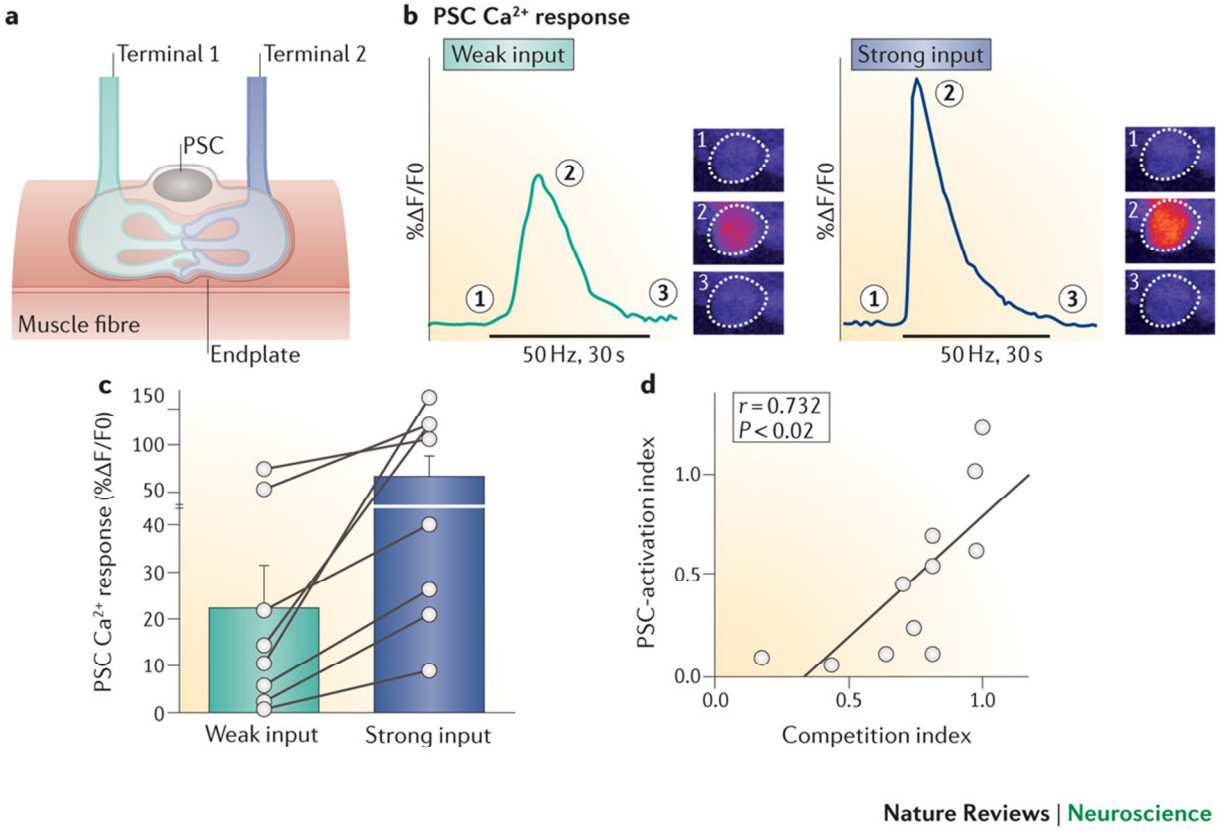

Figure 4. PSCs can differentiate between competing synaptic terminals.

a | A single perisynaptic Schwann cell (PSC; translucent grey) in close association with two terminals (green and blue) during synaptic competition is shown. **b,c** | PSCs can distinguish between strong (blue) and weak (green) inputs. Neurotransmitter release evoked by selective stimulation of the motor neuron associated with the stronger input induces intra-PSC Ca^{2+} responses of a greater amplitude (as indicated by the larger percentage change in the calcium-indicator fluorescence over basal fluorescence; %ΔF/F0) than those induced by neurotransmitter release from the weaker input. Insets in part **b** are false-colour confocal images of changes in intra-PSC Ca^{2+} level before stimulation (inset 1), at the peak of the response (inset 2) and after stimulation (inset 3). Part **c** shows intra-PSC Ca^{2+} responses (mean ± SEM) that were induced by strong and weak competing terminals. Each point represents a single experiment, and the black bars join the intra-PSC Ca^{2+} responses from the weak and strong nerve terminals that are competing at the same neuromuscular junction (NMJ). **d** | The amplitudes of intra-PSC Ca^{2+} responses (the PSC-activation index, which is defined as the amplitude of the intra-PSC Ca^{2+} response induced by the weak input divided by the amplitude

of the intra-PSC Ca^{2+} response induced by the strong input) are tightly correlated with the synaptic strength (the competition index, which is defined as the quantal content of the weak input divided by the quantal content of the strong input and is a major determinant of the outcome of synaptic competition) of competing terminals. Each point is a single experiment, and the black line represents the linear regression relationship (*r*) between the PSC-activation and synaptic competition indices. Terminals with higher competition and PSC-activation indices are less likely to be eliminated. Parts **b–d** have been modified and republished with permission of the Society for Neuroscience, from: Glial cells decipher synaptic competition at the mammalian neuromuscular junction. Darabid, H., Arbour, D. & Robitaille, R. *J. Neurosci.* **33**, 1297–1313 (2014); permission conveyed through Copyright Clearance Center, Inc.[18].

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This tight relationship between terminal synaptic efficacy and intra-PSC Ca^{2+} responses does not only correspond to the amounts of neurotransmitter released, but also depends on intrinsic PSC properties, including PSC-receptor segregation[18]. Indeed, a single PSC seems to organize its receptors in functional groups to face each competing terminal18, an arrangement that could enable a more efficient detection of released neurotransmitter and a more accurate differentiation of competing inputs. When more than one PSC is present at a dually innervated NMJ, each PSC differentiates the weak input from the strong input using similar mechanisms[18]. Thus, PSCs integrate synaptic properties by constantly monitoring the strength of competing terminals. This also implies that PSCs must provide specific feedback that either promotes or represses synapse elimination. Although such a feedback mechanism has not yet been elucidated, PSCs could target elimination machinery specifically towards weaker inputs, or — as PSCs can regulate synaptic activity at mature NMJs (see below)[153, 155] — they could differentially modulate the efficacy of competing terminals, preferentially strengthening one terminal over the other.

The mechanisms proposed by Smith et al.[22] and by our group[18] are not mutually exclusive and could be complementary during synaptic competition. Indeed, at late embryonic and early postnatal stages, many motor neurons innervate the same NMJ (muscle fibres could typically receive more than ten different inputs)[43], and this polyinnervated stage coincides with the arrival of the first PSCs. Regardless of their number, PSCs are in contact with all terminals and show no apparent preference[22, 43]. In this situation, PSCs may randomly eliminate branches from different terminals, as suggested by Smith et al.[22]. Gradually, the number of competing inputs would decrease and the segregation of territories would increase, such that each competing terminal would occupy a precise and delineated territory. When only a few competing terminals remain at an NMJ, PSCs — which can decode the relative synaptic efficacy of different inputs — could trigger the elimination of the weaker inputs. In doing so, PSCs may ensure that the most efficient and suitable nerve terminal is maintained.

Once mono-innervation is achieved, presynaptic and postsynaptic elements continue to rely on PSCs for proper functioning. Indeed, PSCs modulate the fine-tuning of neurotransmission at mature frog and mouse NMJs[153, 155] in a Ca^{2+}-dependent manner[153, 155], whereby the outcome of synaptic regulation is governed by the amplitude and kinetics of intra-PSC Ca^{2+} signalling. The ablation of PSCs at the mature amphibian NMJ results in structural and functional deficiencies[10], including an increase in nerve-terminal retractions, and decreases in both nerve-evoked muscle-twitch tension and the presynaptic release of neurotransmitter[10]. Thus, PSCs are important for synapse maintenance and refinement, as well as for synapse maturation and activity-dependent changes.

Box 2: NMJ formation following injury and in disease

The peripheral nervous system has a remarkable ability to regenerate[164, 165]. Following nerve damage, the distal sections of injured axons remain functional for a given period before Wallerian degeneration occurs. This time period is known to directly depend on the length of the degenerating nerve stump[166, 167]. Wallerian degeneration involves many events: changes to the blood–nerve barrier, such as increased permeability; the degeneration of the distal axon; the dedifferentiation and proliferation of Schwann cells, which start invading the synaptic cleft[168, 169]; the recruitment of macrophages; and the reorganization of the extracellular matrix[170]. The combined goal of all of these events is to generate an innervation-permissive environment so that regenerated axons can reinnervate the muscle endplate once Wallerian degeneration is complete.

During reinnervation, axons continue to grow beyond the boundary of a denervated neuromuscular junction (NMJ; which is delineated by the endplate area and the clustered acetylcholine receptors (AChRs)), where they travel along peripheral Schwann cell bridges that have been formed during denervation[165]. These 'escaped fibres' can reach another synaptic site, leading — along with the axonal reinnervation — to the polyinnervation of NMJs[171, 172].

Remarkably, reinnervation shares several features with synapse formation during development. First, in both cases, NMJs that are polyinnervated undergo a period of synaptic competition, which leads to monoinnervation[171, 172, 173]. Second, as with elimination in the developing NMJ, elimination during reinnervation depends on and is shaped by synaptic activity[137, 174]. Therefore, there is evidence to suggest that the processes that occur during NMJ formation are recapitulated following nerve injury. This would imply that during reinnervation perisynaptic Schwann cells (PSCs) might adapt their sensibility in response to neurotransmission until it resembles that observed in immature PSCs. This suggests that the relative contributions of muscarinic and purinergic signalling in PSCs can also be adapted.

Although these NMJ-forming processes can be repeated, severe diseases of the NMJ can arise if a single step of NMJ formation is compromised. For example, the production of

autoantibodies targeting specific proteins that are present in the endplate leads to neuromuscular diseases such as myasthenia gravis, which is characterized by muscle weakness and fatigue. Autoantibodies that are involved in the more prevalent neuromuscular diseases include those that target AChRs[175], muscle-specific tyrosine kinase receptor (MUSK)[111, 176, 177, 178] and low-density lipoprotein receptor-related protein 4 (LRP4)[179, 180](reviewed in Ref. 181). Furthermore, mutations in the genes encoding proteins that are essential for NMJ formation, such as MUSK[59] or proteins associated with the basal lamina, lead to congenital neuromuscular diseases[182]. Recently, motor-endplate disease, a severe genetic disorder in mice that is characterized by progressive muscular weakness and that leads to paralysis and premature death, has been attributed to a mutation in the gene encoding the voltage-gated sodium channel Nav1.6. Interestingly, this mutation leads to the apoptosis of PSCs during synapse formation[183, 184].

Thus, a better understanding of NMJ formation and maturation will help to elucidate both the regenerative processes that occur after nerve injury and how altered maturation leads to major defects. Moreover, considering the NMJ as a functional unit composed of a presynaptic terminal, a muscle fibre and associated PSCs may lead to the identification of additional therapeutic targets to treat neuromuscular diseases.

5. Conclusions and perspectives

Owing to its simplicity and accessibility, the NMJ has contributed substantially to our knowledge of synapse formation and maturation. These processes rely on complex molecular interactions that involve synaptogenic factors from muscle fibres, nerve terminals and PSCs. These factors ensure a coordinated maturation of the NMJ such that presynaptic, postsynaptic and glial partners are morphologically matched and functionally tuned. This coordination is essential to accurately position the hundreds of presynaptic active zones to face the complex postsynaptic organization at the motor endplate. This tight presynaptic–postsynaptic pairing is further compounded by PSC processes, which express receptors that allow them to regulate NMJ efficacy[153, 155].

The maturation of synapses at the NMJ is influenced by changes in synaptic activity and can therefore be tuned as a function of the environment. Moreover, such activity shapes NMJ connectivity by promoting the maintenance of certain inputs and the elimination of redundant connections after competition.

Recent studies have revealed that PSCs regulate many aspects of NMJ formation — from receptor clustering and presynaptic differentiation to activity-dependent synapse pruning and the modulation of synaptic activity. Interestingly, although synapse formation is best studied at the NMJ, the contribution of PSCs to synaptogenesis is less well understood than that of their CNS counterparts[156, 157]. Indeed, many CNS glial-dependent pathways have been described, including those involving glypicans, thrombospondins, hevin and SPARC (secreted protein acidic and rich in cysteine)[158, 159, 160, 161]. Similar mechanisms are probably involved at the NMJ; however, an assessment of these mechanisms is complicated by the lack of genetic and molecular tools for specifically targeting PSCs. Therefore, it will be important to develop such tools to unveil the contributions of PSCs and to better understand NMJ formation and maturation.

Furthermore, a functional link between the activity-dependent processes discussed here and the molecular pathways that drive synapse formation and maturation is yet to be elucidated. Considering the high number of intricate interactions among the three elements, it is possible that the molecular mechanisms involved are under some form of activity-dependent regulation. Furthermore, if this is the case, then the activity-dependent regulation of the molecular pathways of each compartment must be accurately controlled to shape a tightly organized communication unit. In summary, determining how the different molecular and activity-dependent mechanisms influence one another is an important issue that needs to be addressed.

Understanding NMJ formation and the contribution of glia to this process would have a marked impact on our knowledge of the changes that occur at the NMJ with ageing, following nerve injury or in the contexts of various motor neuron diseases. In particular, understanding the mechanisms that regulate the sprouting and extension of PSC processes would prove to be invaluable, as these are some of the most important events that regulate NMJ reformation following injury. Studying NMJ formation as a tripartite, integrated ensemble process would therefore give us a better understanding of numerous diseases, thus potentially expanding the spectrum of therapeutic targets.

6. Glossary

Neuromuscular junction

(NMJ). A unitary functional structure composed of a single axon terminal innervating a muscle fibre. The presynaptic terminal is covered by specialized glial cells called perisynaptic Schwann cells.

Perisynaptic Schwann cells

(PSCs). Non-myelinating glial cells at the neuromuscular junction. They originate from the neural crest but differ structurally and phenotypically from axonal myelinating or axonal non-myelinating Schwann cells.

Active zones

Areas on the surface of the presynaptic terminal that are characterized by their electron-dense appearance owing to the high concentration of proteins involved in Ca^{2+}-dependent synaptic-vesicle exocytosis and recycling.

Motor columns

Groups of motor neurons that innervate selective sets of muscles.

Pre-patterning

A nerve-independent phenomenon that occurs prior to the arrival of motor axons whereby acetylcholine receptors cluster in the central region of the muscle fibres (along the longitudinal axis), purportedly defining the location of nerve–muscle contact.

AChR clustering

The gathering of acetylcholine receptors (AChRs), which is regulated by molecular mechanisms. It is one of the initial steps of synapse maturation.

Synapse elimination

A reduction in the number of synaptic contacts that results from activity-dependent synaptic competition.

Ganglioside

Member of a family of oligoglycosylceramide plasma-membrane lipids that was originally discovered after its isolation from ganglion cells and which is predominantly found in the nervous system. Antibodies against some disialosyl epitopes of gangliosides can be used to specifically ablate perisynaptic Schwann cells at the mammalian neuromuscular junction.

Retraction bulb

Enlarged distal part of the axon undergoing retraction (that is, axosomal shedding); it is commonly observed during synapse elimination.

Spike timing

Pattern of temporal correlation between presynaptic and postsynaptic activities. Synchronous and asynchronous patterns of activity are observed during early and late phases of synapse elimination, respectively.

Asynchronous activity

Uncorrelated timing of synaptic inputs onto the muscle fibre, leading to an out-of-phase activation.

PSC-receptor segregation

Spatial grouping and separation of receptors on the surface of perisynaptic Schwann cell (PSC) processes. Presumably, this grouping enables the PSC to detect neurotransmitter release from each competing terminal at dually innervated neuromuscular junctions. This segregation was described for purinergic type 2Y receptors, which mediate intra-PSC calcium activity during synaptic competition.

Muscle-twitch tension

Tension elicited by a muscle contraction that is evoked by a suprathreshold stimulation of the muscle or the nerve input.

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2.7. La compétition et l'élimination synaptique durant le développement

La compétition et l'élimination synaptique sont des processus généralisés dans le système nerveux. Similairement à la JNM, plusieurs données décrivent l'importance du raffinement des connexions synaptiques dans le SNC ainsi que l'implication des cellules gliales dans ce phénomène. Pour cette raison, je vais brièvement décrire la compétition et l'élimination ainsi que la contribution des cellules gliales dans la maturation du SNC.

Dans le SNC, des connexions synaptiques excédentaires sont formées de façon à ce que beaucoup plus de synapses soient observées durant le développement que ce qui est maintenu dans le système nerveux mature. Il s'ensuit alors un processus de compétition et d'élimination durant lequel plusieurs terminaisons luttent pour l'innervation de la même cellule cible. La présence de connexions surnuméraires et la nécessité de l'élimination synaptique sont répandues dans le SNC et ont été observé dans de nombreuses structures incluant le noyau géniculé latéral du thalamus (relai visuel des connexions de la rétine; Campbell and Shatz, 1992; Chen and Regehr, 2000; Del Rio and Feller, 2006; Hooks and Chen, 2006), le cervelet (Hashimoto and Kano, 2013; Lohof et al., 1996; Mariani and Changeux, 1981), le Calyx de Held (Holcomb et al., 2013) et le cortex (LaMantia and Rakic, 1990; LeVay et al., 1980). L'élimination sélective de synapses durant cette période permettrait d'ajuster les territoires synaptiques ainsi que de réguler la localisation, le nombre et le type de synapses maintenues, ce qui définit l'organisation des circuits neuronaux (Clarke and Barres, 2013; Eroglu and Barres, 2010).

2.7.1. L'activité synaptique relative définit l'issue de la compétition synaptique

L'un des premiers exemples marquants de l'importance de la compétition et l'élimination synaptique est l'apparition des colonnes de dominance oculaire dans le cortex visuel (Hubel et al., 1977; LeVay et al., 1980). À la naissance, la couche IV du cortex visuelle de mammifères reçoit des connexions diffuses en provenance des deux yeux. Cependant, lors de la maturation, il y a une réorganisation des connexions et l'apparition de bandes dont les neurones reçoivent majoritairement des connexions d'un seul œil. De cette façon, des bandes innervées par l'œil gauche sont en alternance avec des bandes innervées par l'œil droit (Hubel et al., 1977). L'apparition des colonnes de dominance oculaire est grandement dépendante de l'activité de chaque œil. Maintenir un œil fermer de façon permanente induit la réorganisation à long terme des bandes qui y sont associées et qui seront éventuellement innervées par des connexions de l'œil ouvert (Hubel et al., 1977).

Il est de plus en plus évident que les processus de compétition et d'élimination synaptiques sont grandement dépendants de l'activité et de l'expérience environnementale qui façonnent la connectivité nerveuse. Bien que certaines études à la JNM montrent que des terminaisons moins actives peuvent tout de même être maintenues (Barber and Lichtman, 1999; Callaway et al., 1987; Costanzo et al., 1999, 2000), il semble que les terminaisons les plus actives sont favorisées pour gagner la compétition (Balice-Gordon and Lichtman, 1994; Buffelli et al., 2003; Busetto et al., 2000; Colman et al., 1997; Favero et al., 2012; Ribchester and Taxt, 1983; Ridge and Betz, 1984; Schafer et al., 2012; Stevens et al., 2007).

Plus précisément, le niveau d'activité relative entre les terminaisons en compétition dicte le raffinement des connexions. À la naissance, les projections de chaque œil dans le noyau géniculé latéral du thalamus sont diffuses et sont en compétition les unes avec les autres. Cependant, ces projections deviennent très ordonnées et ségrégées durant le développement (Sretavan and Shatz, 1986). Ce processus de ségrégation est dépendant de l'activité neuronale sous forme de vagues rétiniennes spontanées (Wong et al., 1993). Les

projections dont l'activité est augmentée sont favorisées lors de la compétition synaptique (Stellwagen and Shatz, 2002). De façon surprenante, aucune modification des territoires synaptiques occupés par les projections de chaque œil n'est observée lorsque l'activité de toutes les projections est augmentée. Ceci montre que la compétition synaptique est dépendante de l'activité relative, et non absolue, entre les terminaisons en compétition. De manière similaire à la JNM, un blocage global de l'activité entraine un retard d'élimination (Balice-Gordon and Lichtman, 1994). Cependant, diminuer la neurotransmission d'un groupe de terminaisons axonales entraîne le maintien des terminaisons les plus actives (Buffelli et al., 2003).

Il semble donc que le réarrangement des connexions synaptiques dépend de l'activité et de l'expérience sensorielle et que l'efficacité synaptique relative dicte l'établissent des circuits neuronaux. Tel que montré dans la section 2.6, l'élimination synaptique à la JNM dépend d'une importante contribution des cellules gliales. De plus, il semble que cette propriété soit conservée dans l'ensemble du système nerveux puisque les cellules gliales participent aussi à l'élimination synaptique dans le SNC. En effet, la synaptogenèse et le remodelage de la connectivité nécessitent une grande contribution des cellules gliales qui participent à la formation et à la maturation synaptiques, ce qui est discuté dans la section suivante.

2.8. Régulation de la synaptogenèse par les cellules gliales dans le SNC

2.8.1. Formation et maturation synaptique

Un des rôles des cellules gliales récemment identifié est la formation et la maturation des connexions synaptiques (Clarke and Barres, 2013; Eroglu and Barres, 2010). Il est maintenant connu que les astrocytes contrôlent la formation et la maturation synaptique par l'action de signaux synaptogéniques.

Par exemple, les thrombospondines sont parmi les molécules clés libérées par les astrocytes qui favorisent la formation et l'intégrité structurale des synapses excitatrices (Christopherson et al., 2005). L'absence de différentes isoformes de cette protéine entraîne une diminution du nombre de synapses (Christopherson et al., 2005). Il semble que les thrombospondines recrutent différentes molécules d'adhésion et d'échafaudage à la synapse (Sigrist and Plested, 2009) et peuvent interagir avec les intégrines et neuroligines qui sont essentiels pour la formation synaptique (DeFreitas et al., 1995; Xu et al., 2010).

Les astrocytes régulent aussi la maturation des synapses par la relâche de protéines de la matrice extracellulaire, Hevin et SPARC. Hevin favorise la formation et la maturation des synapses alors que SPARC antagonise la fonction de Hevin. Ceci permettrait de réguler le nombre et la taille des synapses (Kucukdereli et al., 2011).

Non seulement les facteurs relâchés par les astrocytes régulent la formation de la synapse, mais ils participent aussi à sa maturation. Les glypicanes sont des molécules ancrées sur la membrane extracellulaire des astrocytes et peuvent être clivées et relâchées. Ces dernières participent à la maturation en favorisant le recrutement de récepteurs postsynaptiques et ainsi régulent l'efficacité et la fonction synaptique (Allen et al., 2012). De la même manière, des particules de cholestérol contenant l'apolipoprotéine E, d'origine

astrocytaire, ont étés impliquées dans la maturation et l'amélioration de l'efficacité synaptique (Goritz et al., 2005).

Bref, il semble que les cellules gliales participent à la formation et à la maturation de synapses. Dès lors, cette propriété serait conservée dans l'ensemble du système nerveux puisque, les CSPs à la JNM et les astrocytes dans le SNC participent aux évènements de la synaptogenèse.

2.8.2. Élimination et remodelage synaptique

Les cellules gliales participent aussi à l'élimination et au remodelage des connexions synaptiques (Chen and Regehr, 2000; Katz and Shatz, 1996; Lohof et al., 1996; Wyatt and Balice-Gordon, 2003).

Jusqu'à récemment, les mécanismes responsables de l'élimination synaptique et les types cellulaires impliqués étaient inconnus. Les études des dernières années montrent que les microglies jouent un rôle clé dans l'élimination de synapses (Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007). Par exemple, dans le relai visuel du thalamus, l'élimination par les microglies des connexions surnuméraires, en provenance des projections des cellules ganglionnaires de la rétine, est possible par l'action de la voie classique de la cascade du complément (Stevens et al., 2007). En effet, les synapses expriment C1q, protéine d'initiation de la cascade du complément. Les microglies expriment le récepteur CR3. Ce récepteur, par l'intermédiaire du segment C3b qui est accroché au C1q, permet la phagocytose des synapses exprimant le C1q. De plus, la phagocytose dépendante de C1q est régulée par l'activité de façon à ce qu'une augmentation ou réduction de l'activité favorise la phagocytose et l'élimination des connexions les moins actives (Schafer et al., 2012; Stevens et al., 2007).

Il est intéressant de noter que les astrocytes régulent l'expression des protéines du complément pour promouvoir l'élimination synaptique. En effet, les astrocytes secrètent le

Facteur de croissance transformant β (*Transforming Growth Factor β*) qui peut augmenter l'expression neuronale de C1q de manière dépendante de l'activité (Bialas and Stevens, 2013). Plus important encore, une étude récente montre clairement que les astrocytes participent activement au raffinement des connexions synaptiques en éliminant directement certains contacts synaptiques. En effet, les astrocytes favorisent le désassemblage et l'élimination des synapses indésirables du SNC (Chung et al., 2013). Ce processus requiert les voies phagocytaires dépendantes de MEGF10 et MERTK et il est grandement régulé par l'activité (Chung et al., 2013). La délétion génétique de MEGF10 ou MERTK entraine une augmentation du nombre de synapses qui ont une force synaptique anormalement faible (Chung et al., 2013). De manière intéressante, MEGF10 est l'orthologue de Draper chez la drosophile (Fuentes-Medel et al., 2009; Ziegenfuss et al., 2008). À la JNM de la drosophile, Draper serait impliqué dans l'élimination synaptique par les cellules gliales qui participent à phagocyter les débris présynaptiques, spécialement ceux associés à des boutons synaptiques faibles ou instables (Fuentes-Medel et al., 2009).

L'élimination synaptique dépendrait aussi de l'organisation des prolongements gliaux à proximité des synapses. Dans le cervelet, une délétion génétique des récepteurs-canaux impliqués dans la détection de neurotransmetteurs par les cellules de Bergmann (analogue des astrocytes dans le cervelet), provoque une rétraction des prolongements gliaux qui enveloppent les synapses, une augmentation du nombre de contacts synaptiques et un retard de l'élimination synaptique (Iino et al., 2001). Ceci pourrait s'expliquer par l'absence d'une interaction entre les prolongements gliaux et les synapses. Il pourrait donc y avoir un lien entre la détection de l'activité synaptique par les cellules gliales et la régulation de la connectivité. En effet, une hypothèse intéressante qui n'a pas été testée est que cette altération de l'élimination synaptique pourrait être due à l'absence des récepteurs-canaux gliaux ce qui pourrait perturber la capacité des cellules gliales à détecter les subtilités de l'activité synaptique comme que décrit dans la section 2.4.

En conclusion, les cellules gliales jouent un rôle important dans l'élimination synaptique. Qu'il s'agisse des microglies et des astrocytes dans le SNC ou des CSPs à la JNM, les cellules gliales participent activement au raffinement de la connectivité. Ceci pourrait avoir

des conséquences cruciales pour le fonctionnement du système et l'étude de maladies neuro-développementales. Par exemple, altérer les mécanismes de phagocytose par les microglies ou les astrocytes provoque un retard d'élimination synaptique et la présence d'un plus grand nombre de synapses (Bialas and Stevens, 2013; Chung et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007). Dans certains contextes, une élimination aberrante de synapses par la microglie a été récemment associée à des déficits de comportements sociaux liés à des troubles semblables à ceux observés dans l'autisme (Zhan et al., 2014). Cependant, des interrogations majeures subsistent et restent sans réponse : comment l'élimination est-elle dirigée vers des synapses spécifiques alors que d'autres sont épargnées ? Comment l'activité synaptique influence l'élimination de synapses par les cellules gliales ?

3. Buts de la thèse

Les cellules gliales à la JNM, jouent un rôle important dans l'élimination synaptique puisqu'elles sont connues pour enlever des branches synaptiques de terminaisons en compétition et sont responsables de l'élimination complète de terminaisons nerveuses de la JNM (Bishop et al., 2004; Smith et al., 2013; Song et al., 2008). Cependant, la manière dont cette élimination est dirigée est dirigée spécifiquement vers des synapses particulières considérées comme faibles ou mal adaptées demeure inconnue. De plus, la contribution des cellules gliales à l'activité synaptique menant à l'élimination synaptique demeure inexplorée.

Puisque les CSPs à la JNM mature sont connues pour détecter le niveau d'activité synaptique et de réguler la neurotransmission (Robitaille, 1998; Rousse et al., 2010; Todd et al., 2010), nous proposons l'hypothèse suivante :

Les CSPs détectent le niveau d'activité des terminaisons en compétition, régulent leur activité et influencent l'issue de l'élimination synaptique

La figure 2.6 illustre l'hypothèse testée dans cette thèse. Pour tester cette hypothèse, nous avons posé les questions suivantes qui sont traitées dans deux études :

Section 4. Premier article de recherche : « *Glial Cells Decipher Synaptic Competition at the Mammalian Neuromuscular Junction* »

- Est-ce que les CSPs détectent l'activité des terminaisons en compétition ?
- Est-ce que les CSPs décodent l'efficacité des terminaisons en compétition ?
- Quels sont les récepteurs et mécanismes impliqués ?

Section 5. Deuxième article de recherche: « *Glial Cells Regulate Synaptic Plasticity to Influence Synaptic Competition at the Mammalian Neuromuscular Junction* »

- Est-ce que les terminaisons en compétition montrent différents niveaux de plasticité synaptique ?

- Est-ce que les CSPs régulent l'activité et la plasticité des terminaisons en compétition ?
- Est-ce que l'activité des CSPs est nécessaire pour une élimination et connectivité synaptique adéquate ?

Pour ce faire, nous avons étudié l'excitabilité des CSPs à l'aide de l'imagerie calcique en simultané avec l'activité synaptique électrophysiologique des terminaisons en compétition à une même JNM. De plus, nous avons spécifiquement bloqué l'activité des CSPs, *in situ* et *in vivo*, pour voir l'impact sur la plasticité synaptique des terminaisons en compétition ainsi que sur l'élimination synaptique.

Nous montrons que les CSPs détectent et décodent l'activité des terminaisons en compétition lors du développement de la JNM. De plus, nous montrons que les CSPs potentialisent préférentiellement l'activité des terminaisons fortes et influencent l'issue de l'élimination synaptique. Cette étude démontre, pour la première fois, le lien entre l'activité synaptique des terminaisons en compétition, sa modulation par les CSPs ainsi que l'impact sur l'élimination et la connectivité nerveuse.

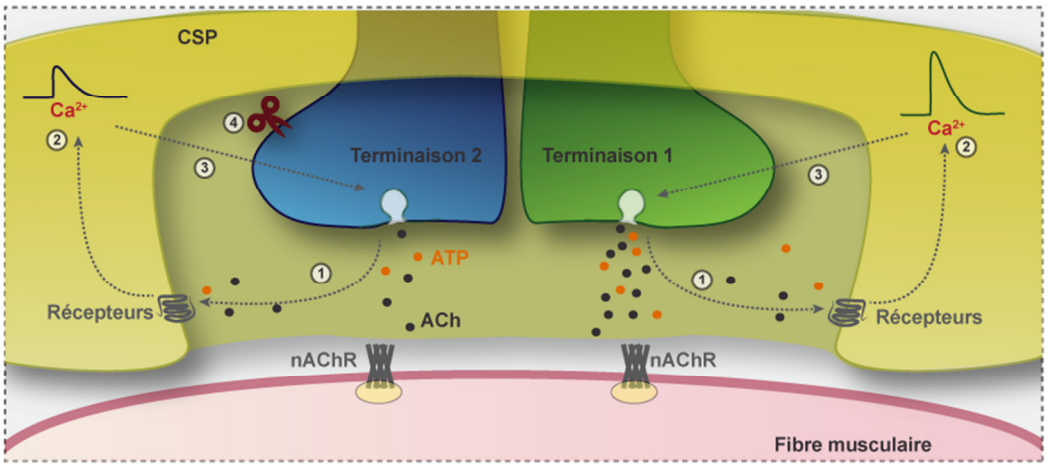

Figure 2.6. Hypothèses de la détection et la régulation de l'activité des terminaisons en compétition par les CSPs à la JNM.

1. Une CSP à une JNM polyinnervée détecte l'activité des terminaisons en compétition.
2. Puisque les terminaisons en compétition peuvent avoir différentes efficacités synaptiques, une CSP décode l'efficacité des terminaisons en compétition.
3. Les CSPs régulent l'activité synaptique des terminaisons en compétition en favorisant une terminaison par rapport à l'autre.
4. Le renforcement préférentiel d'une terminaison par les CSPs influence l'issue de la compétition et l'élimination synaptique.

4. Premier article de recherche

4.1. Introduction au premier article de recherche

Comme décrit dans l'introduction, le développement du système nerveux inclut une période durant laquelle plusieurs connexions surnuméraires sont en compétition pour l'innervation d'une même cellule cible. Cette compétition est basée sur l'activité neuronale et mène au renforcement de certaines terminaisons nerveuses et à l'élimination de d'autres. De manière générale, les terminaisons qui montrent la meilleure activité sont favorisées alors que les synapses mal adaptées sont éliminées.

Jusqu'à récemment, les mécanismes et les types cellulaires responsables de l'élimination synaptique étaient inconnus. Les études de la dernière décennie montrent que les cellules gliales jouent un rôle clé dans l'élimination de synapses. Cependant, il demeure inconnu si les cellules gliales peuvent décoder les niveaux d'activité des terminaisons en compétition ce qui est un déterminant majeur de l'issue de la compétition synaptique. Ce décodage glial de l'activité des terminaisons est un préalable pour que ces cellules gliales puissent fournir un feedback approprié aux terminaisons. Ceci est d'un intérêt particulier puisqu'il est connu que les cellules gliales dans le système nerveux mature régulent l'activité des neurones et que cette régulation dépend de leur capacité à décoder les propriétés synaptiques.

Par conséquent, nous avons analysé la capacité des cellules gliales à décoder l'activité des terminaisons nerveuses au cours de la compétition synaptique et nous avons étudié les mécanismes impliqués. Pour cette fin, nous avons profité de la jonction neuromusculaire, un modèle simple et le bien caractérisé, et nous avons combiné l'imagerie Ca^{2+} des cellules gliales, un rapporteur fiable de leur activité avec des enregistrements synaptiques de jonctions neuromusculaires poly-innervées de souriceaux.

Les pages qui suivent présentent les résultats de cette partie publiés dans l'article :

Darabid H, Arbour D, Robitaille R. (2013). *Glial cells decipher synaptic competition at the mammalian neuromuscular junction*. Journal of Neuroscience. 33:1297-313.

4.2. « GLIAL CELLS DECIPHER SYNAPTIC COMPETITION AT THE MAMMALIAN NEUROMUSCULAR JUNCTION »

The Journal of Neuroscience 33:1297-313.

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H.D. performed all research except some immunohistochemistry experiments. D.A. performed immunohistochemistry experiments.

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4.2.1. Abstract

It is now accepted that glial cells actively interact with neurons and modulate their activity in many regions of the nervous system. Importantly, modulation of synaptic activity by glial cells depends on the proper detection and decoding of synaptic activity. However, it remains unknown whether glial cells are capable of decoding synaptic activity and properties during early postdevelopmental stages, in particular when different presynaptic nerve terminals compete for the control of the same synaptic site. This may be particularly relevant because a major determinant of the outcome of synaptic competition process is the relative synaptic strength of competing terminals whereby stronger terminals are more likely to occupy postsynaptic territory and become stabilized while weaker terminals are often eliminated. Hence, because of their ability to decode synaptic activity, glial cells should be able to integrate neuronal information of competing terminals. Using simultaneous glial Ca^{2+}imaging and synaptic recordings of dually innervated mouse neuromuscular junctions, we report that single glial cells decipher the strength of competing nerve terminals. Activity of single glial cells, revealed by Ca^{2+} responses, reflects the synaptic strength of each competing nerve terminal and the state of synaptic competition. This deciphering is mediated by functionally segregated purinergic receptors and intrinsic properties of glial cells. Our results indicate that glial cells decode ongoing synaptic competition and, hence, are poised to influence its outcome.

4.2.2. Introduction

Glial cells were long considered as a support element for neurons providing essential energy metabolites and maintaining the ionic balance of the extracellular environment. However, there is now a large body of evidence revealing that glial cells actively interact with neurons and are important for proper information processing in the nervous system. Indeed, glial cells detect synaptic activity at the synapse and, in turn, release neuroactive molecules that modulate neurotransmission (Robitaille, 1998; Fellin et al., 2004; Panatier et al., 2006; Henneberger et al., 2010; Todd et al., 2010; Panatier et al., 2011).

Involvement of glial cells in the regulation of synaptic activity and plasticity heavily depends on their ability to detect and decode synaptic activity (Auld and Robitaille, 2003; Todd et al., 2010; Panatier et al., 2011). However, these neuron–glia interactions were addressed solely in the mature nervous system, whereas their activity at immature synapses remains ill-defined despite their essential role in synapse formation and elimination (Ullian et al., 2004; Christopherson et al., 2005; Eroglu and Barres, 2010). An event where the ability of glial cells to detect synaptic activity could come to play is the postnatal synaptic competition that occurs between supernumerary nerve terminals competing for the same synaptic territory (Lohof et al., 1996; Chen and Regehr, 2000; Wyatt and Balice-Gordon, 2003). This competition results in the strengthening of certain synaptic inputs and weakening and elimination of others (Colman et al., 1997; Kopp et al., 2000; Keller-Peck et al., 2001; Walsh and Lichtman, 2003; Bishop et al., 2004). Synaptic competition and elimination are in part dependent on the relative activity of the competing nerve terminals (Balice-Gordon and Lichtman, 1994; Stellwagen and Shatz, 2002; Buffelli et al., 2003) where the most active inputs (i.e., releasing more neurotransmitters) are more likely to be strengthened and maintained (Busetto et al., 2000; Buffelli et al., 2003). Hence, glial cells could alter the outcome of synaptic competition because of their ability to detect and modulate transmitter release. However, a fundamental prerequisite remains undetermined: glial cells must be able to detect ongoing synaptic competition and decipher the strength of each competing nerve terminal. To address this question, we used the mammalian neuromuscular junction (NMJ), a

well-characterized model for the study of synaptic competition (Kopp et al., 2000; Walsh and Lichtman, 2003; Chung and Barres, 2009) and neuron–glia interactions (Robitaille, 1998; Todd et al., 2010).

Using simultaneous Ca^{2+} imaging of perisynaptic Schwann cells (PSCs), glial cells at the NMJ, and synaptic recordings of dually innervated NMJs, we show that a single PSC deciphers ongoing synaptic competition. Based on their intrinsic properties and segregated functional $P2_Y$ receptors, PSCs decode the properties of each competing input, identifying the strong from the weak input. Hence, PSCs are in tune with the synaptic competition process, selectively decoding the state of each competing nerve terminals.

4.2.3. Materials and Methods

Animals and nerve-muscle preparation.

All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Comité de déontologie animale of Université de Montréal. P7–P8 male CD-1 mice (Charles River) were anesthetized by a lethal intraperitoneal injection of a ketamine (15 mg/ml) and xylazine (1 mg/ml) mixture. The soleus muscle (SOL) and its innervation to the ventral roots were dissected according to Kopp et al. (2000) under oxygenated (95% O_2, 5% CO_2) Rees' Ringer's solution (in mM): 110 NaCl, 5 KCl, 1 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 11 glucose, 0.3 glutamate, 0.4 glutamine, 5 BES, 4.34×10^{-7} cocarboxylase and 0.036 choline chloride. The pH of oxygenated solution was at 7.3. The SOL and its innervation were pinned in a Sylgard-coated recording chamber, and the ventral roots were carefully divided in three segments that were independently stimulated by three suction-stimulating electrodes (square pulses; 0.1 mV to 2.0 V, 0.1 ms duration) using a Master-8 stimulator (AMPI). This preparation allowed us to stimulate two distinct inputs competing at single NMJ, as described in Figure 1*A*.

Immunohistochemistry of NMJ components.

Immunohistochemical labeling was performed according to Todd et al. (2010). In brief, dissected SOL muscles were pinned in a Sylgard-coated dish and fixed for 10 min in 4% formaldehyde at room temperature and then permeabilized in 100% cold methanol for 6 min at −20°C. Nonspecific labeling was minimized by incubating the muscles in a solution of 10% normal donkey serum (NDS) and 0.01% Triton X-100 for 20 min. Nerve-muscle preparations were incubated overnight at 4°C with a rabbit anti-S100β (1:250, Dako, with 0.01% Triton X-100 and 2% NDS). After rinsing, muscles were incubated in a goat anti-neurofilament (1:250; SC16143, Santa Cruz Biotechnology) and chicken anti-synaptotagmin (1:250; AB9356, MilliporeBioscience Research Reagents) for 90 min. Secondary antibodies (Alexa 488 α-goat, FITC α-chicken and 647 α-rabbit, 1:500) were incubated together for 60 min at

room temperature. Muscles were incubated with α-bungarotoxin (Alexa 594, 0.75 μg/ml) for 45 min. After each step, muscles were rinsed in PBS containing 0.01% Triton X-100 for 5 min. The preparations were then mounted in the Prolong Gold antifade reagent containing DAPI (Invitrogen) to visualize nuclear material. All labels were observed simultaneously using the spectral detection feature of an Olympus FV1000 confocal microscope. Pinholes were set to obtain an airy disk value of 1. No further manipulations of the images were performed after the acquisition.

The identification and count of PSCs at NMJs were performed on muscles that were either used for electrophysiological recordings or freshly dissected and processed directly for immunostaining. When the same muscles were used for electrophysiological recordings and immunostaining, the recorded NMJs were identified *post hoc* using their specific location on the muscle using the distance of the fiber from the nerve entry as reference and the pattern of nerve branching since only one NMJ was present on each muscle fiber. The following parameters were used to identify and count PSCs at NMJs: cells needed to be S100β immunopositive, labeled by DAPI and located within the endplate area. PSCs somata were well separated and easily identified even though their processes are known to intermingle around the nerve terminals.

Immunohistochemistry of $P2_Y1$ receptors.

We could not label PSCs using the rabbit anti-S100β as indicated above because type 1 $P2_Y$ receptors ($P2_Y1R$) were labeled using a polyclonal antibody also generated in rabbit. Instead, PSCs were selectively loaded using a single cell electroporation method. Dissected SOL muscles were pinned in a Sylgard-coated recording chamber and constantly perfused with oxygenated Rees' Ringer's solution. A glass pipette (5–8 MΩ) was filled with 5 mg/ml fixable anionic Dextran, AlexaFluor-594 (10,000 MW; Invitrogen). The pipette was mounted on a pipette holder with a platinum stimulating wire connected to a Master-8 stimulator (AMPI), and the reference electrode was placed in the bath. The pipette was positioned close to the soma of a PSC using a micromanipulator under visual guidance. One to 3 single square pulses (10 ms duration; 10 V amplitude) were applied to open the cell membrane (Nevian and

Helmchen, 2007) and allow the loading of AlexaFluor-594 into the cell. The procedure was repeated to load other visible PSCs at the same NMJ. PSCs from 3 to 4 NMJs were loaded per muscle. Muscles were allowed to rest for 30 min before fixation and immunohistochemistry staining. Loaded PSCs/NMJs were identified *post hoc* using the specific shape of the PSCs and the fiber distance from the nerve entry as a reference as indicated above. Three muscles were used as control to confirm that electroporated PSCs were also immunopositive for S100β (data not shown).

Muscles were then incubated overnight with a rabbit antibody against $P2_Y1$ receptors (1:1500; 34–7200, Invitrogen). After rinsing, muscles were incubated in a goat antineurofilament (1:250; SC16143, Santa Cruz Biotechnology) and mouse antisynaptic vesicular protein SV2 IgG1 (1:1500; Developmental Studies Hybridoma Bank) for 120 min at room temperature. Preparations were incubated with Alexa Fluor 488 α-goat and Alexa Fluor 647 α-rabbit (1:500) secondary antibodies for 60 min and then incubated with DL488 α-mouse IgG1 (1:500) for 60 min at room temperature. Finally, muscles were incubated with α-bungarotoxin (Alexa Fluor 594, 0.75 μg/ml) for 45 min. After each step, muscles were washed in PBS containing 0.01% Triton X-100 (3 times, 5 min each). The preparations were then mounted in the Prolong Gold antifade reagent and all labels observed simultaneously using the spectral detection feature of an Olympus FV1000 confocal microscope.

Immunohistochemistry of muscarinic receptors M5 and M3.

For labeling muscarinic receptors (mAChRs), muscles were incubated overnight at 4°C with a rabbit anti-S100β (1:250, Dako) to label PSCs. After rinsing, muscles were incubated with a chicken antineurofilament to label axons and nerve terminals (1:1000; 212–901-D84, Rockland), mouse anti-SV2 IgG1 to label active zones (1:1500; Developmental Studies Hybridoma Bank), and goat anti-M3 (1:500, SC31486, Santa Cruz Biotechnology) or M5 (1:500, SC7478, Santa Cruz Biotechnology) to label mAChRs, all at room temperature for 120 min. Nerve-muscle preparations were then sequentially incubated in the following secondary antibody solutions for 60 min at room temperature: Alexa Fluor 488 α-chicken (1:500, antineurofilament primary antibody) with Alexa Fluor 647 α-goat (1:500 for M5 or 1:1000 for

M3), DL488 α-mouse IgG1 (1:500; anti SV2 primary antibody), and finally Alexa Fluor 405 α-rabbit (1:500). Preparations were rinsed with PBS containing 0.01% Triton X-100 (3 times, 5 min each) in between each antibody incubation. Finally, muscles were incubated with α-bungarotoxin (Alexa Fluor 594, 0.75 μg/ml) for 45 min and, after washout, preparations were mounted in the Prolong Gold antifade reagent. All labels were observed simultaneously using an Olympus FV1000 confocal microscope.

Electrophysiological recordings of synaptic transmission.

Intracellular recordings of endplate potentials (EPPs) were performed at 28°C-30°C using glass microelectrodes (40–70 MΩ, filled with KCl, 3M). Only NMJs that showed EPPs evoked by the independent stimulation of two of the three ventral roots were studied. Each suction electrode contained only a single axon that innervated the NMJ because only a single EPP with given amplitude and rise time was induced by the stimulation. Increasing threshold stimulation intensity never evoked any sudden increase in EPP amplitude (EPP steps), as would be expected if multiple axons innervating a single poly-innervated NMJs were present within the stimulated stimulating electrode (Redfern and Thesleff, 1971; Busetto et al., 2000; Buffelli et al., 2002). Synaptic responses were recorded using an Axoclamp 2B amplifier (Axon Instruments) and further amplified (100×) and filtered at 2 kHz by a Warner Instruments DC amplifier. EPPs were recorded with WinWCP software (John Dempster, University of Strathclyde, Strathclyde, United Kingdom).

Synaptic strength of each nerve terminal in competition at a given NMJ was determined by measuring their respective quantal content and paired-pulse facilitation. These were determined using a modified Ringer's solution with low Ca^{2+} (1 mM)/high Mg^{2+} (6–7 mM) solution. The ventral roots were stimulated at a frequency of 0.2 Hz with an intensity that was twice the threshold for eliciting EPPs. Quantal content (m) for each input was determined using the method of failures (Del Castillo and Katz, 1954): $m = \log_e$ (no. of nerve impulses/no. of failures).

To determine the paired-pulse facilitation (F), two stimuli (0.1 ms duration) were given with an interval of 10 ms and facilitation was calculated as the mean amplitude of the second responses (B) divided by the mean amplitude of the first responses (A) (including failures).

Ca^{2+} imaging of PSCs.

PSCs were loaded with the Ca^{2+} indicator fluo-4AM (10 μM, Invitrogen) by incubating nerve-muscle preparations for two times 35 min at room temperature. The loading solution also contained 0.02% pluronic acid (Invitrogen) and 0.5% dimethylsulfoxide (Sigma) added to the preoxygenated normal Ringer solution. This method is known to load preferentially PSCs at NMJs, and cells were easily identifiable in fluorescent illumination for the Ca^{2+} indicator (fluo-4) and transmitted light (Rochon et al., 2001; Rousse et al., 2010; Todd et al., 2010). Ca^{2+} responses were elicited in PSCs by independently stimulating each ventral root at 50 Hz for 30 s. Preparations were allowed to rest for 20 min between stimulation of the two competing inputs.

Changes in fluorescence intensity were monitored using a Bio-Rad MRC600 laser-scanning confocal microscope equipped with an argon ion laser. The imaging area was focused on a single NMJ, and all PSCs present at that NMJ were imaged at once. The preparation was excited with a 488 nm laser line and the emitted fluorescence was detected through a long-pass filter at 590 nm. A 60× water-immersion lens was used (0.90 NA; Olympus). Relative changes in fluorescence intensity were analyzed over PSCs soma and were expressed as $\%\Delta F/F = F - F_{rest}/F_{rest} \times 100$.

In some experiments, Ca^{2+} responses in PSCs were elicited by agonists applied locally using a Picospritzer II (Parker Instruments, positive pressure pulses of 10 PSI, 150 ms) from a glass pipette (5 MΩ, ~2-μm-tip diameter) positioned at proximity of the cells. Agonists, ATP (3 μM, Sigma), muscarine (5 μM, Sigma), or acetylcholine (5 μM) were diluted in the extracellular Ringer solution used for the experiment.

All Ca^{2+} imaging experiments were performed in normal Ca^{2+}/Mg^{2+} concentration Ringer's solution at 28°C to 30°C. Muscle contractions were prevented by blocking postsynaptic cholinergic receptors with D-tubocurarine chloride (2.8–3.9 μM, Sigma) or 20 μM α-bungarotoxin (Invitrogen). For experiments where synaptic strength was first established in low Ca^{2+}/high Mg^{2+}Rees' solution, preparations were perfused with normal Ca^{2+}/Mg^{2+} Ringer for 30 min before imaging Ca^{2+}.

Antagonist applications.

In some experiments, antagonists of $P2_Y$ (Reactive Blue 2 [RB2], 20 μM, Alexis) or muscarinic receptors (atropine, 20–80 μM or scopolamine, 20 μM, Sigma) were bath applied for 20–45 min before the start of the experiment. Stock solutions were prepared in the same extracellular Rees' solutions.

TEA application.

Neurotransmitter released by either weak or strong nerve terminal was increased using a K^+ channel blocker, tetraethyl ammonium (TEA, 0.5 mM). First, a dually innervated NMJ was identified and synaptic strength of competing terminals determined. The nonpotentiated input was stimulated first at high frequency (50 Hz for 30 s), and evoked PSC Ca^{2+} responses were recorded under normal Ringer solution. Then, TEA was bath applied for 30 min and synaptic activity was monitored. Then, the second input (now potentiated by TEA) was stimulated at high frequency and the corresponding PSC Ca^{2+} responses were recorded. In some control experiments, agonists (ATP, 10 μM) were locally applied in the presence of TEA by micropressure as indicated above.

Statistical analysis.

Results are presented as mean ± SEM, and *N* represents the number of PSCs. Unless otherwise stated, only one PSC/NMJ was kept for further analyses. Paired *t* tests were performed when comparing responses induced by the two competing terminals during the

same experiment. When data were found not to conform to normality, Mann–Whitney U tests were used. Unpaired *t* tests were performed to compare two different conditions from different experiments. One-way Kruskal–Wallis ANOVA test with Dunn's Multiple-Comparison post-test were used to compare three groups or more. Analyses were deemed significant at $p < 0.05$.

4.2.4. Results

The NMJ has proven to be one of the most useful models to study synaptic competition and elimination. However, although the presynaptic and postsynaptic changes have been studied, very little is known about PSCs during synaptic competition and elimination at the NMJ.

In this work, we investigated PSC properties and activity at P7–P8 dually innervated NMJs and their ability to decipher ongoing synaptic competition.

PSCs at dually innervated NMJs

More than 60% of NMJs are poly-innervated and undergoing synaptic competition at P7–P8 mice (Kopp et al., 2000). Lichtman and colleagues showed that each competing nerve terminal could occupy a segregated territory of endplate area (Gan and Lichtman, 1998; Walsh and Lichtman, 2003; Bishop et al., 2004), whereas Hirata et al. (1997) showed that one or two PSCs are present at poly-innervated rat NMJs.

Here, the state of synaptic innervation was determined using the presence of synaptic responses evoked by independent stimulation of ventral roots (Fig. 1*A*) and by immunohistological labeling of PSCs and presynaptic and postsynaptic elements (Fig. 1*B*). The number of PSCs was based on the criteria described in the experimental procedures using S100β labeling of PSCs combined with nuclear staining with DAPI (Fig. 1*C1–2*). Consistent with Hirata et al. (1997), we found an average of 1.6 ± 0.1 (8 muscles, 37 NMJs) PSCs at poly-innervated NMJs and 2.7 ± 0.2 at mono-innervated NMJs (Fig. 1*D*, *E*; 8 muscles, 14 NMJs; unpaired *t* test, $p < 0.0001$).We observed that 43.3% of poly-innervated NMJs were covered by one PSC, whereas 51.3% were covered by two PSCs. Only two NMJs had three PSCs (5.4%). In contrast, mono-innervated NMJs were generally associated with two or more PSCs (14.3% with one PSC, 21.4% with two PSCs, 42.9% with three PSCs, and 21.4% with four PSCs; Fig. 1*E*). Importantly, it appears that the number of PSCs is directly related to the

state of synaptic competition (i.e., mono-innervated vs poly-innervated) where fewer cells appear to be present during the course of synaptic competition. Furthermore, these results show that both competing nerve terminals at a poly-innervated NMJ were often covered by a single PSC. In this work, only dually innervated NMJs were studied while the number of PSCs present was determined.

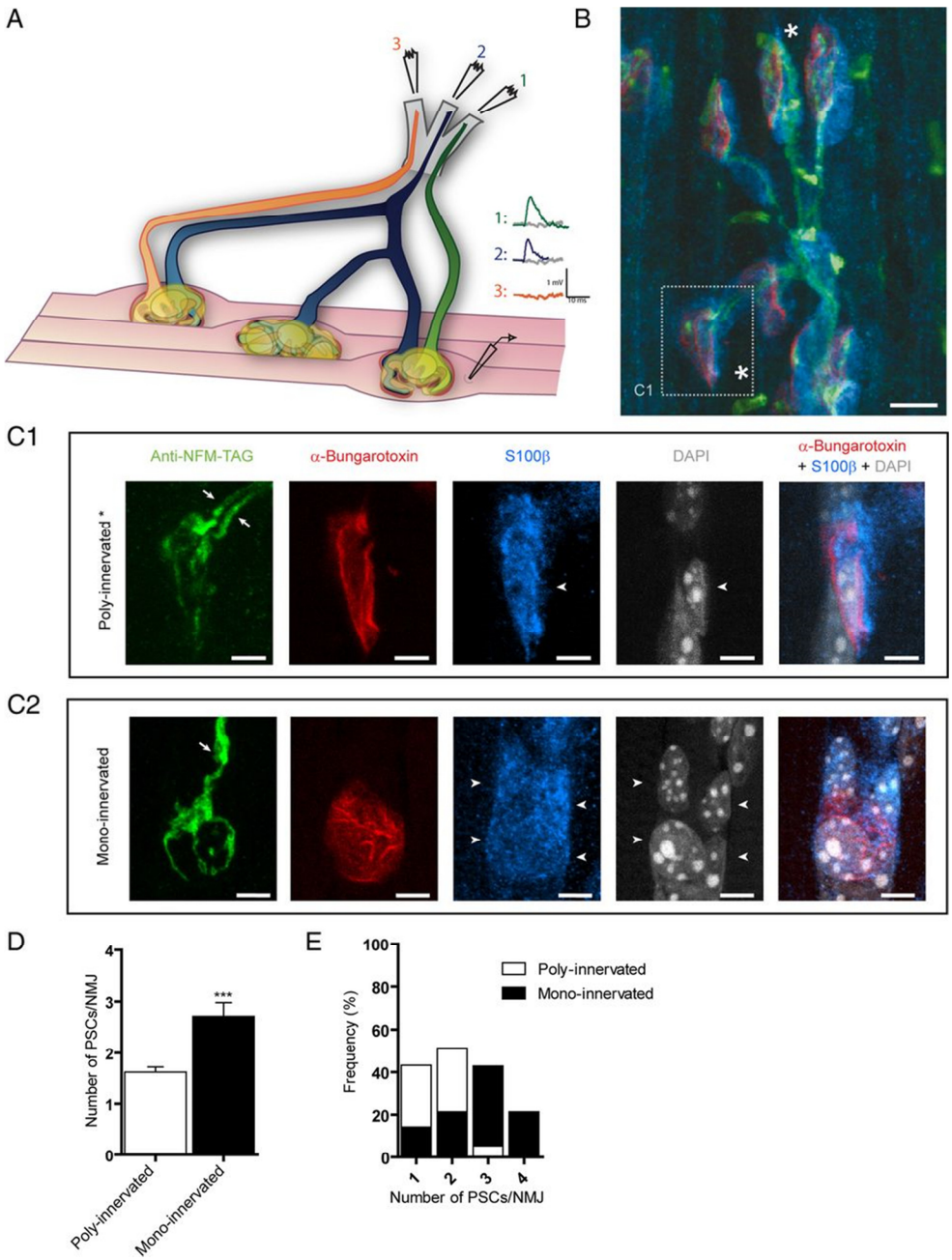

Figure 1. PSCs during synaptic competition at the mammalian NMJ.

A, Diagram illustrating the experimental situation where transmitter release from a dually innervated NMJ was recorded with a sharp microelectrode and synaptic responses evoked by independent stimulation of two different ventral roots. B, Labeling of presynaptic terminals (green), PSCs (blue), and postsynaptic nAChR (red) of P7 Soleus NMJs. At this age, most

NMJs were poly-innervated (as shown in C1 and marked with "*"), but some NMJs were already mono-innervated. C1, At dually innervated NMJs, two independent axonal branches (in green, arrows) converged at the same endplate area (red). Also note that a single PSC (blue) covered the whole endplate area and both nerve terminals. PSCs counted (arrowheads) were positive for S100β (blue) and DAPI (gray) staining and were located at the endplate (red). C2, At mono-innervated NMJs, one axon (in green, arrow) was observed at the endplate area (red). This NMJ was from another muscle preparation than in B and C1. D, Histogram of the number of PSCs at poly-innervated and mono-innervated NMJs. Note that there were more PSCs at mono-innervated than poly-innervated NMJs. Unpaired t test, $p < 0.0001$. E, Distribution of the number of PSCs at mono-innervated and poly-innervated NMJs. Note that most poly-innervated NMJs had 1 or 2 PSCs. Scale bars: B, 10 μm; C1, C2, 5 μm.

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A single PSC detects neurotransmitter release from competing nerve terminals at dually innervated NMJs

A fundamental prerequisite for PSCs to decode ongoing synaptic competition is that they are synaptically responsive, detecting synaptic activity generated by both competing nerve terminals. To test this possibility, we examined the ability of PSCs to respond to synaptic activity of each individual competing nerve terminal evoked by independent ventral root stimulation. Dually innervated NMJs with one or two PSCs were chosen, and each competing nerve terminal was independently stimulated at 50 Hz for 30 s, a pattern and frequency reported *in vivo* for tonic firing of motor neurons innervating the SOL at P7–P8 (Gorassini et al., 2000; Eken et al., 2008). PSC activity was assessed by monitoring changes in intracellular Ca^{2+} levels, known to be a reliable reporter of their level of excitation and responsiveness (Jahromi et al., 1992; Robitaille, 1998; Rochon et al., 2001).

As shown in Figure 2, independent stimulation of each competing nerve terminal induced a Ca^{2+} response at the soma of the same PSC. From all tested NMJs, 85.7% of PSCs

responded to independent neurotransmitter release from each competing input (36 PSCs of 42). The average amplitude of PSC Ca^{2+} response to the activity of the first input was 76.2 ± 14.9% ΔF/F0, whereas it was 62.4 ± 20.3% ΔF/F0 in response to the stimulation of the second input (Fig. 2*D*; $N = 11$, paired *t* test, $p > 0.05$).

These results indicate that PSCs at dually innervated NMJs detect transmitter release from the two competing nerve terminals. As such, these results indicate that PSCs are synaptically responsive.

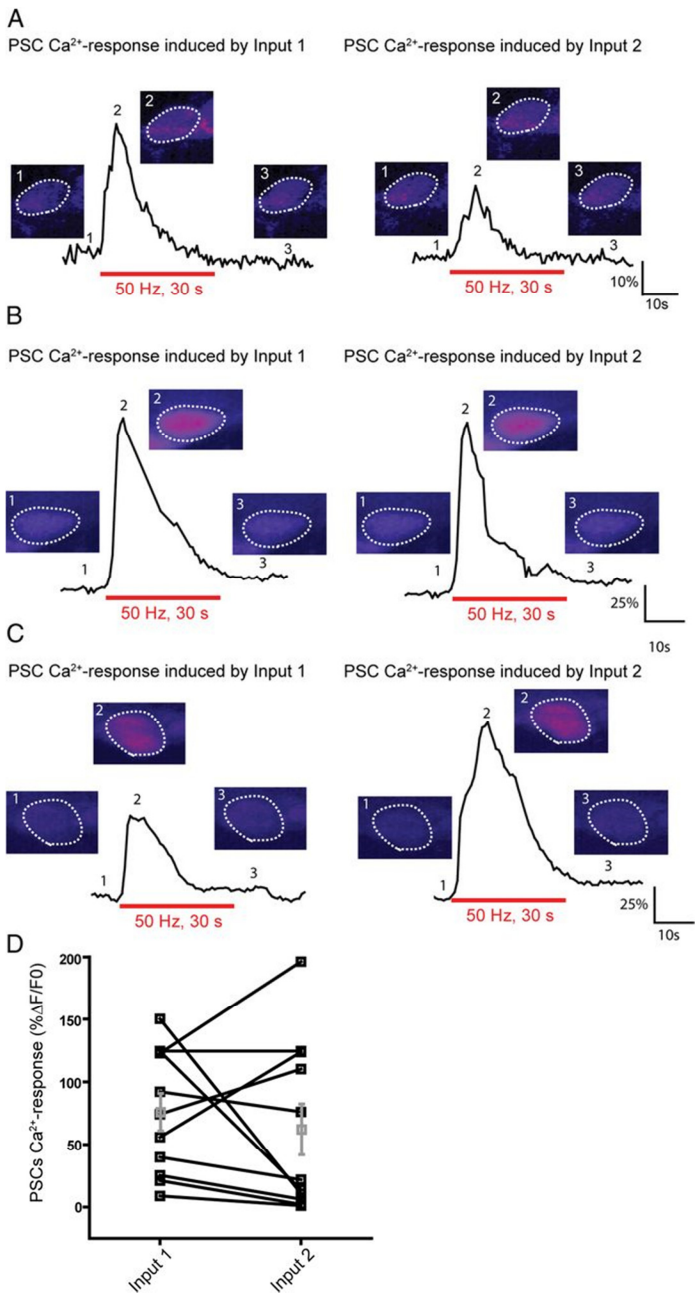

Figure 2. A single PSC detects synaptic activity of competing nerve terminals at dually innervated NMJs.

A, Example of Ca^{2+}responses elicited in a PSC by independent stimulation of the two competing nerve terminals (Input 1 and Input 2). False color images of the changes in fluorescence of Fluo-4 illustrating Ca^{2+}levels before, at the peak of the response, and after the stimulation. Note that the first input (Input 1) induced a bigger Ca^{2+} response than the second

one (Input 2). ***B***, An example of PSC Ca^{2+}responses where both inputs induced similar PSC Ca^{2+}responses. ***C***, A third example where the first input induced a smaller PSC Ca^{2+} response than the second one. ***D***, Plot illustrating the diversity of PSC Ca^{2+} responses induced by two inputs at poly-innervated NMJs.

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A single PSC decodes the synaptic strength of competing nerve terminals

Although a single PSC at a dually innervated endplates responded to transmitters released by each individual nerve terminal, our data revealed a large variability in the amplitude of the responses evoked by the two terminals (Fig. 2*D*). Indeed, we observed that the amplitude of PSC Ca^{2+} responses induced by the stimulation of the first competing input was sometimes higher than those induced by the second input in 51% of the tested NMJs (Fig. 2*A*, *D*), similar in 25% (Fig. 2*B*, *D*) or lower in 24% (Fig. 2*C*, *D*) of the remaining responding PSCs.

One possibility might be that Ca^{2+} responses in PSCs reflected the level of synaptic strength of each competing terminal. This would be consistent with the properties of PSCs at mature NMJs that decode different levels of synaptic activity by producing different Ca^{2+} responses (Rousse et al., 2010; Todd et al., 2010). Based on the disparity in synaptic strength between competing terminals (Colman et al., 1997; Kopp et al., 2000) and the relationship between transmitter release and the responsiveness of PSCs, we hypothesized that the weaker of the competing nerve terminals would induce smaller PSC Ca^{2+}responses than the stronger one.

To test this hypothesis, we performed simultaneous intracellular recordings from dually innervated NMJs combined with PSCs Ca^{2+} imaging. Synaptic strength of each nerve terminal was determined using two approaches; the quantal content (m) using a binomial

analysis of the failure rate and paired pulse facilitation (Fig. 3*A*, *B*). At dually innervated NMJs, it is known that strong inputs show bigger quantal content and smaller paired pulse facilitation than weak ones (Kopp et al., 2000). We only considered an input to be stronger than the other when its quantal content was higher and its facilitation significantly smaller than the other one (Fig. 3*A*, *B*).

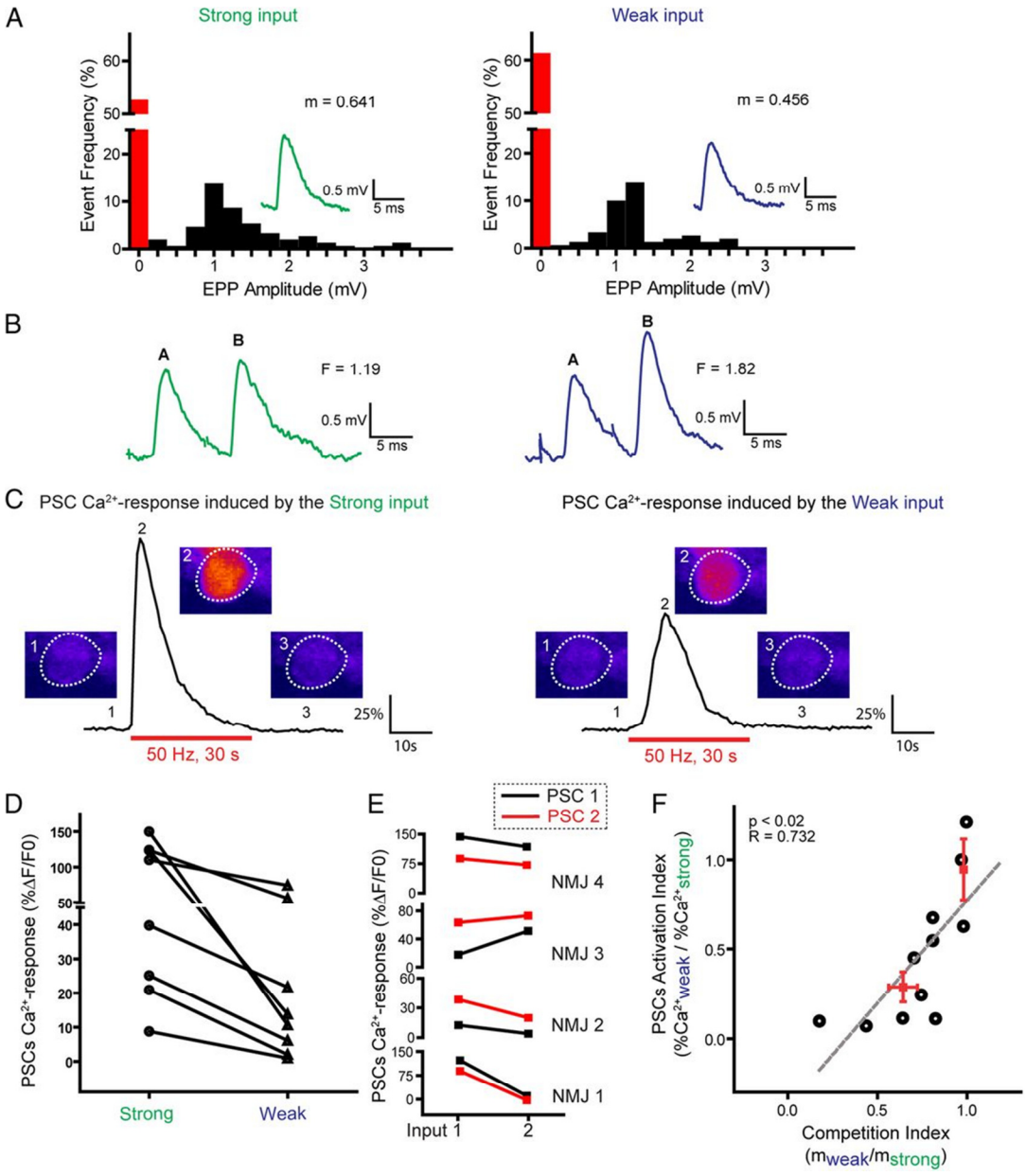

Figure 3. A single PSC decodes synaptic strength of competing nerve terminals at dually innervated NMJs.

A, Distribution of EPP amplitude induced by stimulation of the strong and weak inputs, including failures. Inset, Examples of EPPs and calculated quantal content (m). ***B***, Examples of EPPs and facilitation values (F) obtained from the paired-pulse (10 ms interval) independent stimulation of strong and weak inputs shown in ***A***. ***C***, Ca^{2+}responses elicited in a

PSC by independent stimulation of the strong and weak competing inputs (same as in ***A*** and ***B***). False color confocal images of changes in fluorescence of Fluo-4 illustrating Ca^{2+} levels before, at the peak of the response, and after stimulation. ***D***, Plot of the amplitude of PSC Ca^{2+} responses induced by strong (circle) and weak (triangle) terminals. PSC Ca^{2+} responses induced by terminals in competition at the same NMJ are connected with a black line. Note that strong inputs always induced larger Ca^{2+} responses than weak inputs. ***E***, Ca^{2+}responses induced in two PSCs at the same dually innervated NMJs. Note that the relationship between Ca^{2+} responses elicited by the two inputs was the same for both PSCs (black and red) at the same NMJ. ***F***, Relationship between the relative synaptic strength of the competing nerve terminals (CI, *x*-axis) and the relative responsiveness of the PSCs upon their activation by the independent activity of the competing nerve terminals (PSC activation index, *y*-axis). Each point (●) represents an individual experiment, and the red points are the average for the experiments where synaptic strength ratio was close to 1 and close to 0.5. The strong linear relationship ($r = 0.732$; $p < 0.02$) indicates that PSC responsiveness is tightly influenced by the disparity of the strength of the competing nerve terminals.

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Interestingly, sustained and repeated activity of strong inputs (50 Hz, 30 s) always elicited larger Ca^{2+} responses in PSCs than the weak ones (75.3 ± 20.1% ΔF/F0 vs 23.2 ± 9.6% ΔF/F0, $N = 8$; paired *t* test, $p = 0.02$) (Fig. 3*C*, *D*). Importantly, stimulating the weak input before the strong one or vice-versa, did not influence the ability of PSCs to respond to the synaptic activity and did not influence the size of the Ca^{2+} response elicited (data not shown). As a direct corollary of this observation, one would predict that competing inputs with similar synaptic strength at the same NMJ should trigger similar Ca^{2+} responses in PSCs. As expected, competing nerve terminals with similar synaptic strength (quantal content 0.32 ± 0.06 vs 0.33 ± 0.06, $N = 4$; paired *t* test, $p > 0.05$) showed similar PSC Ca^{2+} responses (119.2 ± 27.9% ΔF/F0 vs 107.3 ± 9.4% ΔF/F0, $N = 4$; paired *t* test,$p > 0.05$). Interestingly, similar observations were made when two PSCs were present at dually innervated NMJs (Fig. 3*E*),

indicating that both PSCs performed the same decoding of synaptic strength and underwent similar interactions with both competing nerve terminals.

Decoding by PSCs may be limited simply to the determination of the weakest and strongest inputs, leading to a bimodal distribution. Alternatively, it is also possible that PSCs decode the relative strength of each input, whereby a linear relationship between PSC Ca^{2+} responses and synaptic strength should be observed. To examine this possibility, we represented synaptic competition and the state of relative synaptic efficacy of competing nerve terminals using a competition index (CI). The CI was calculated as follows: CI = quantal content of weak input/quantal content of strong input. It represented the disparity in the synaptic strength of the competing nerve terminals, a key parameter of synaptic competition. A CI of 1 indicated that the two competing terminals had similar synaptic strength, whereas a CI < 1 meant that an input was stronger than the other. We then plotted the relative PSC responsiveness to the competing nerve terminals using a PSC activation index (PAI). PAI was calculated as follows: PAI = PSC Ca^{2+} response induced by weak input/PSC Ca^{2+} response induced by strong input.

It represented the disparity in PSC Ca^{2+} responses induced by each competing nerve terminals. A PAI of 1 indicated that the two terminals induced similar PSC Ca^{2+} responses, and a PAI < 1 meant that one input induced a bigger PSC Ca^{2+} response than the other.

As shown in Figure 3*F*, the PAI is tightly related to the CI (linear regression, $r = 0.732$; slope significantly non-0, $p < 0.02$). Competing terminals with similar synaptic strength (CI ≈ 1) induced similar PSC Ca^{2+} responses (PAI ≈ 1), whereas those with different synaptic strength (CI < 1) induced different PSC Ca^{2+} responses (PAI < 1). Importantly, the state of the competition was directly reflected in PSC responsiveness as indicated by the tight relationship between PAI and CI. Hence, these results suggest that PSC responsiveness was tightly related to the relative synaptic strength of competing terminals. Furthermore, a single PSC at a dually innervated NMJ differentiated weak and strong inputs and decoded their relative synaptic strength.

Decoding by PSCs does not solely depend on neurotransmitter release

Because of PSC sensitivity to neurotransmitter release, the difference in their responsiveness to strong and weak nerve terminals may simply be the result of the different level of transmitter release produced by each terminal. However, it is also known that the responsiveness of PSCs at adult NMJs is also in part determined by their intrinsic properties (Rousse et al., 2010). Hence, we examined whether differential PSC responses to the activity of competing nerve terminals were solely the result of the level of transmitter release or whether their intrinsic properties were adapted to each competing nerve terminal. To test this hypothesis, we increased the level of neurotransmitter released by the weak nerve terminal using a K^+ channel blocker, TEA (0.5 mM). TEA did not directly alter the responsiveness of PSCs to local application of agonists (data not shown). This is consistent with the observation that PSC Ca^{2+} responses are not dependent on voltage-gated channels and membrane excitability but rather on G-protein coupled receptors (Robitaille, 1998; Wang et al., 2006; Rousse et al., 2010). If PSC responsiveness were solely determined by the level of transmitter release, one would predict that Ca^{2+} responses elicited by an increased level of transmitter release from the weak nerve terminal should evoke larger Ca^{2+} responses in PSCs.

We first determined in control situation the amount of transmitter released during the high-frequency stimulation because this was the synaptic activity that triggered PSC activation. We calculated the cumulative quantal release of neurotransmitters by strong and weak competing inputs during the high-frequency stimulation (HFS; 50 Hz, 30 s): EPP amplitude (% Ctrl) × quantal content, at each time point during the HFS. As expected, the strongest input had a higher cumulative quantal release than the weakest one, suggesting that more neurotransmitter was released (Fig. 4*A–C*). At the end of the HFS (time 30 s), the strongest input had a cumulative quantal release of 289.9 ± 40.8, which was significantly higher than the cumulative quantal release of the weakest input (161.9 ± 18.1, $N = 4$; paired t test, $p < 0.05$). Importantly, this difference was noticeable early during the HFS, a period during which PSC activation took place. Indeed, the strongest input had a cumulative

quantal release of 35. ± 5.1 after the first 3 s, which was significantly higher than the one of the weakest input (21.5 ± 2.3, $N = 4$; paired t test, $p < 0.05$, Fig. 4*C*). Also, as shown before, the strong input that released more neurotransmitter induced larger PSC Ca^{2+} responses than the weak input (strong: 127.0 ± 8.3% ΔF/F0 vs weak: 38.6 ± 15.7% ΔF/F0; $N = 4$; paired t test $p < 0.05$).

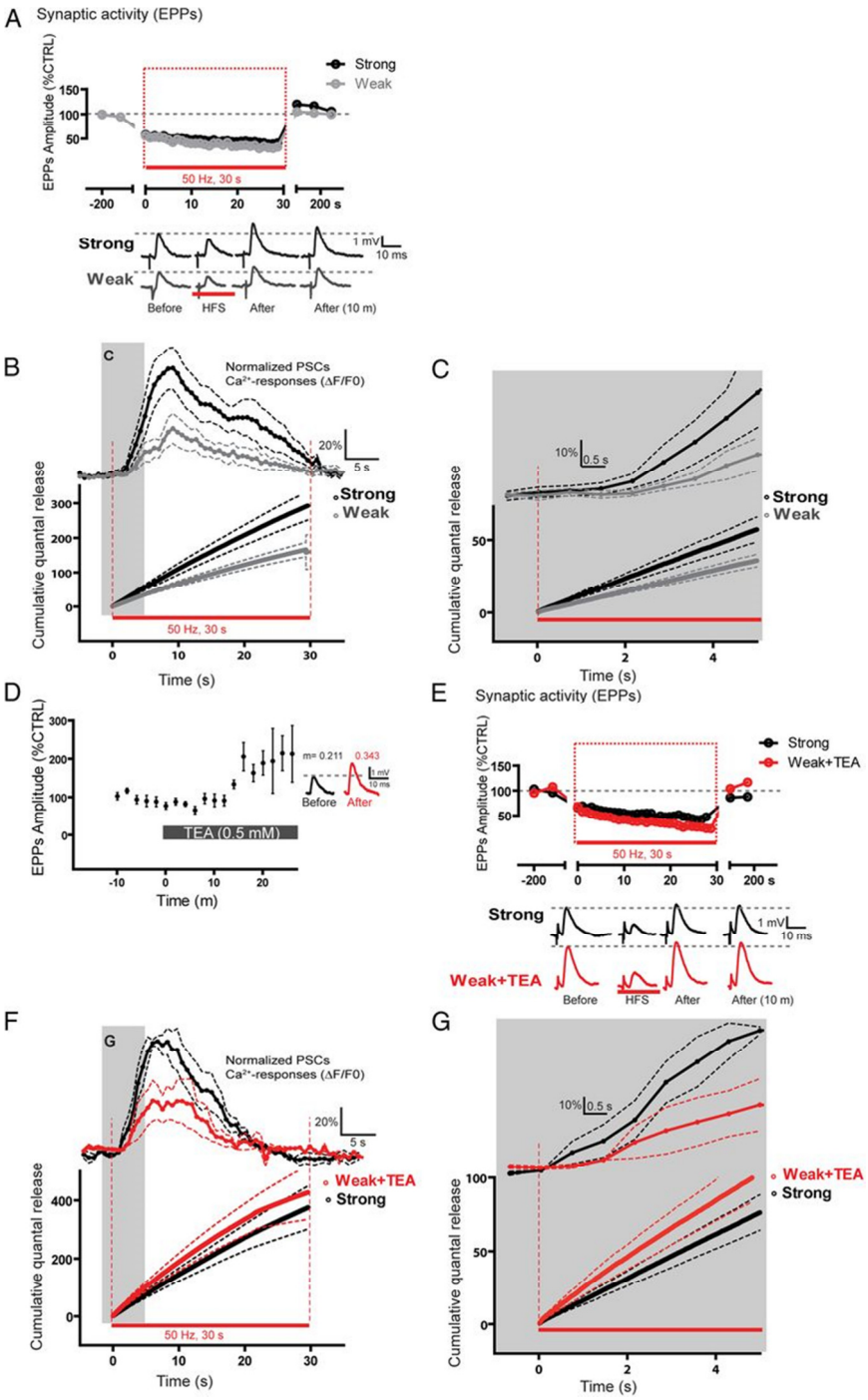

Figure 4. PSC Ca^{2+} responses do not solely depend on the amount of neurotransmitters released.

A, Top, Changes in EPP amplitude before, during, and after motor nerve stimulation (red bar) of the strong (black) and weak (gray) competing inputs at the same NMJ. Period of high-

frequency stimulation used to elicit PSCs Ca^{2+}responses is highlighted (red rectangle). Bottom, Examples of EPPs induced by stimulation of strong and weak inputs before, during, and after high-frequency stimulation. ***B***, Cumulative quantal release ± SEM (dotted lines) obtained during the high-frequency stimulation (red box in A) of strong (black) and weak inputs (gray) at dually innervated NMJs. Top, Corresponding average PSC Ca^{2+} responses ± SEM (dotted lines) induced by synaptic activity of weak (gray) and strong (black) inputs. Ca^{2+} responses are temporally aligned with the timing of the synaptic activity. Note that the strong input had a higher cumulative quantal release and evoked larger PSC Ca^{2+} responses. The gray zone frames the part illustrated in ***C***. ***C***, Similar representation as in ***B***, but illustrating the first 5 s of the stimulation period to emphasize the different kinetics of transmitter release and PSC responses at the onset of activity. ***D***, Effect of TEA (0.5 mM) bath application on EPP amplitude of weak inputs at dually innervated NMJs. Inset, Examples of EPPs and the calculated quantal content (m) before and during TEA application. Note that TEA increased both EPP amplitude and quantal content of weak terminals. ***E***, Top, Changes in EPP amplitude before, during, and after motor nerve stimulation (red bar) for the strong (black) and weak TEA-potentiated (red) competing inputs of the same NMJ. The period of high-frequency stimulation eliciting PSC Ca^{2+} responses is highlighted (red rectangle). Bottom, Examples of EPPs induced by the stimulation of strong and weak inputs before, during, and after high-frequency stimulation. ***F***, Cumulative quantal release ± SEM (dotted lines) obtained during high-frequency stimulation (red rectangle in E) of weak TEA-potentiated (red) and strong inputs (black) at dually innervated NMJs. Transmitter release of the weak nerve terminal was potentiated by TEA (0.5 mM) so that it was similar or even slightly larger than the levels observed at nonpotentiated strong inputs. Top, Average of the corresponding PSC Ca^{2+} responses ± SEM (dotted lines) induced by synaptic activity of the unchanged strong (black) and the weak potentiated input by TEA (red). Note that PSC Ca^{2+} responses triggered by potentiated weak input were still smaller than those triggered by the nonpotentiated strong input. The gray zone frames the part of the figure illustrated in ***G***. ***G***, Similar representation as in ***F***, but illustrating the first 5 s of the stimulation period.

In another set of experiments, we determined the basal level of quantal release and synaptic strength of competing nerve terminals; and then, the strong input was stimulated (HFS; 50 Hz, 30 s), monitored PSC Ca^{2+} responses, and calculated the cumulative quantal release of neurotransmitters (Fig. 4*F*). Then, TEA (0.5 mM) was bath applied, and changes in transmitter release of the weak terminal were monitored. As shown in Figure 4*D*, bath application of TEA potentiated transmitter release of the weak nerve terminal to 189.1 ± 31.6% of control after 20 min ($N = 4$; paired *t* test, $p < 0.05$). TEA raised the quantal content of the weak nerve terminal from 0.11 ± 0.03 to 0.59 ± 0.17 ($N = 4$; paired *t* test, $p < 0.05$), a level that was no longer significantly smaller than the one of the nonpotentiated strong nerve terminal. The cumulative quantal release from the weak TEA-potentiated input was not different from the strong input in absence of TEA (strong: 375.3 ± 73.76 vs weak + TEA: 427.1 ± 92.19;$N = 4$; paired *t* test, $p > 0.05$, Fig. 4*E–G*).

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If decoding by PSCs solely depended on the amount of neurotransmitters released, the potentiated weak input should have induced larger PSC Ca^{2+} responses. However, as shown in Figure 4*F*, *G*, average amplitude of PSCs Ca^{2+} responses induced by neurotransmitters released from the potentiated weak terminals was 25.7 ± 11.0% ΔF/F0, which remained significantly lower than those induced by the strong terminals in the absence of TEA (45.1 ± 13.4% ΔF/F0, $N = 4$; paired *t* test, $p < 0.05$). These data indicate that increasing neurotransmission from the weak input did not alter the amplitude of PSC Ca^{2+} responses. Similar results were obtained when potentiating the strong input (data not shown). Thus, this strongly suggests that decoding of synaptic competition by PSCs did not solely depend on the amount of neurotransmitter released but relied also on their intrinsic properties.

A single PSC decodes the synaptic strength of competing nerve terminals via functionally segregated sets of receptors

We next examined the mechanisms by which a single PSC selectively detected and decoded ongoing synaptic competition, which could be part of PSC intrinsic properties. It has been shown that competing presynaptic terminals are spatially separated and facing their own pool of postsynaptic nicotinic receptors (Gan and Lichtman, 1998; Keller-Peck et al., 2001; Walsh and Lichtman, 2003; Wyatt and Balice-Gordon, 2003; Bishop et al., 2004). We tested whether PSCs used also dedicated pools of receptors to differentiate competing inputs. We used the properties of PSC receptor desensitization known to occur at mature NMJs. PSC receptor desensitization caused a reduction of the amplitude of Ca^{2+} responses at the same PSC when elicited by two series of sustained synaptic activity at interval of 20 min (Rochon et al., 2001). Hence, one would predict that a rundown of the Ca^{2+} response would be observed if PSCs used the same sets of receptors to detect the activity of competing nerve terminals.

First, we examined whether a rundown of Ca^{2+} responses was observed at PSCs of dually innervated NMJs by stimulating the same nerve terminal twice at 20 min interval and monitoring Ca^{2+} responses in the PSC. If PSCs at immature NMJs expressed the same property as in the adult, one would expect to see a rundown of the Ca^{2+} response, with the amplitude of the response evoked by the second stimulation being smaller than the first one. As shown in Figure 5*A*, *D*, a rundown was indeed observed in PSC Ca^{2+} responses where the amplitude to a second motor nerve stimulation was significantly smaller than the first one (first stimulation, 37.5 ± 16.4% ΔF/F0 vs second stimulation, 14.1 ± 11.1% ΔF/F0, $N = 5$; paired *t* test, $p = 0.02$). Importantly, the rundown of the PSC Ca^{2+} response cannot be attributed to a decrease in neurotransmission. Indeed, Figure 5*B* shows that EPP amplitude evoked during the first and second HFS was not significantly different (normalized EPP amplitude: first stimulation, 58.60 ± 7.53% vs second stimulation, 66.09 ± 8.37%, $N = 5$; paired *t* test, $p > 0.05$).

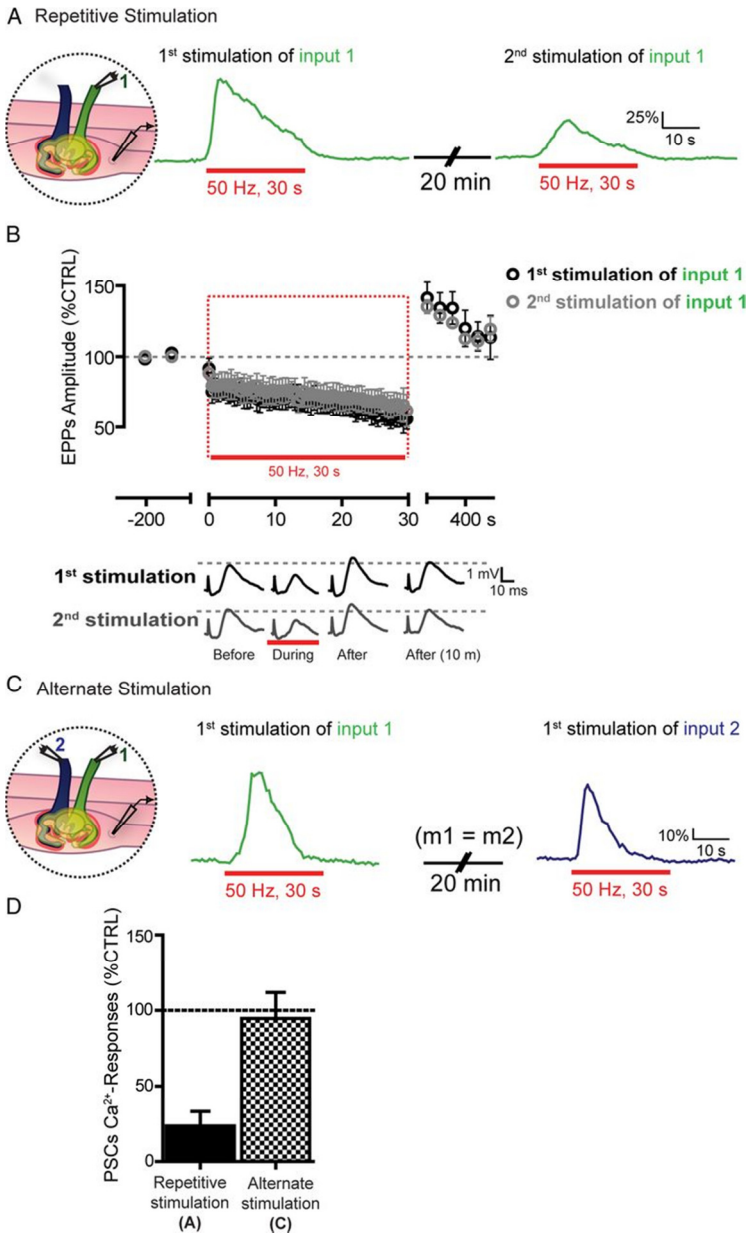

Figure 5. Rundown in PSC Ca^{2+} responses after repetitive stimulations.

A, PSC Ca^{2+} responses induced by the sustained stimulation of the same input (Input 1). Note that the second PSC Ca^{2+} response induced by the second stimulation of the same input was smaller than the first one. ***B***, Top, Changes in EPP amplitude ± SEM before, during, and after the first (black) and second (gray) nerve stimulation of the same input (Input 1). The period of high-frequency stimulation used to elicit PSCs Ca^{2+} responses is highlighted (red rectangle).

Bottom, Examples of EPPs induced by the first and second stimulation of Input 1 before, during, and after the high-frequency stimulation. Note that EPP amplitudes induced by the first and second stimulation of the same input are similar. ***C***, PSC Ca^{2+} responses induced by the alternate stimulation of Input 1 followed, 20 min later, by the stimulation of Input 2 at NMJs where both inputs had similar quantal content (m1 indicates the quantal content of Input 1; and m2, the quantal content of Input 2). Note that Ca^{2+} responses from both PSCs were similar, with no evident rundown. ***D***, Histogram showing the mean of the normalized amplitude of the second PSC Ca^{2+} responses in the repetitive (same input, black, ***A***) and alternate paradigms (alternate inputs, gray, ***C***).

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The rundown of PSC Ca^{2+} responses should not be observed with alternate sequential stimulation of each input whether a PSC decoded synaptic strength of the two competing terminals via different sets of receptors. In this specific set of experiments, we only studied dually innervated NMJs where competing terminals had similar synaptic strength to avoid any variability in PSC responses because of differences in synaptic strength of the terminals. As shown in Figure 5*C*, *D*, no rundown in PSC Ca^{2+}response was observed when alternate stimulation of the two competing nerve terminals was performed. The amplitude of the first Ca^{2+} response was 119.2 ± 27.9% ΔF/F0, whereas the amplitude of the response elicited 20 min later by stimulating the second nerve terminal was 107.3 ± 9.4% ΔF/F0 (Fig. 5*C*, $N = 4$; paired *t* test, $p > 0.05$). These results suggest that a single PSC detected neurotransmitter release and decoded synaptic strength of the competing nerve terminals via functionally segregated sets of receptors.

PSCs activation is not mediated by muscarinic receptors

One possible mechanism that might explain the difference in the responsiveness of PSCs to weak and strong nerve terminals was that PSCs used different types of receptors for each competing input. At adult NMJs, it is known that PSC Ca^{2+} responses are mediated by cholinergic and purinergic receptors activated by the presynaptic release of ACh and ATP during synaptic activity (Robitaille, 1995; Rochon et al., 2001). We used selective antagonist for each receptor type to test their relative contribution to the activation of PSCs by the competing terminals. Because of the rundown of Ca^{2+} responses, amplitude of responses elicited by the strong and weak terminals presented in Figure 3*C*, *D* was used as control.

We first tested the contribution of muscarinic receptors (mAChR) because they are the main receptor system through which PSCs are activated in adult NMJs (Rochon et al., 2001). To test the contribution of these receptors in the decoding of synaptic strength by PSCs, we stimulated independently each of the competing terminals in the presence of the general muscarinic receptor antagonist atropine (60 μM) applied in the bath. Atropine is known to block synaptic-dependent activation of PSCs of adult mammalian NMJs (Rochon et al., 2001). Surprisingly, atropine had no effect on PSC Ca^{2+} responses induced by the stimulation of the strong or weak input (Fig. 6*A*, *C*). Indeed, the amplitude of PSC Ca^{2+} responses induced by neurotransmission from the strong input in the presence of atropine was not different from control (70.5 ± 22.1% ΔF/F0 in atropine, $N = 4$ vs 75.3 ± 20.1% ΔF/F0 in control, $N = 8$; one-way Kruskal–Wallis ANOVA test, Dunn's Multiple-Comparison post-test, $p > 0.05$). This was also the case for PSC Ca^{2+} responses induced by neurotransmission from the weak input where no significant difference was noticed (37.6 ± 18.8% ΔF/F0 in atropine, $N = 4$ vs 26.3 ± 10.4% ΔF/F0 in control, $N = 7$; one-way Kruskal–Wallis ANOVA test, Dunn's Multiple-Comparison post-test, $p > 0.05$). Similar results were obtained with scopolamine, another large spectrum muscarinic antagonist (data not shown).

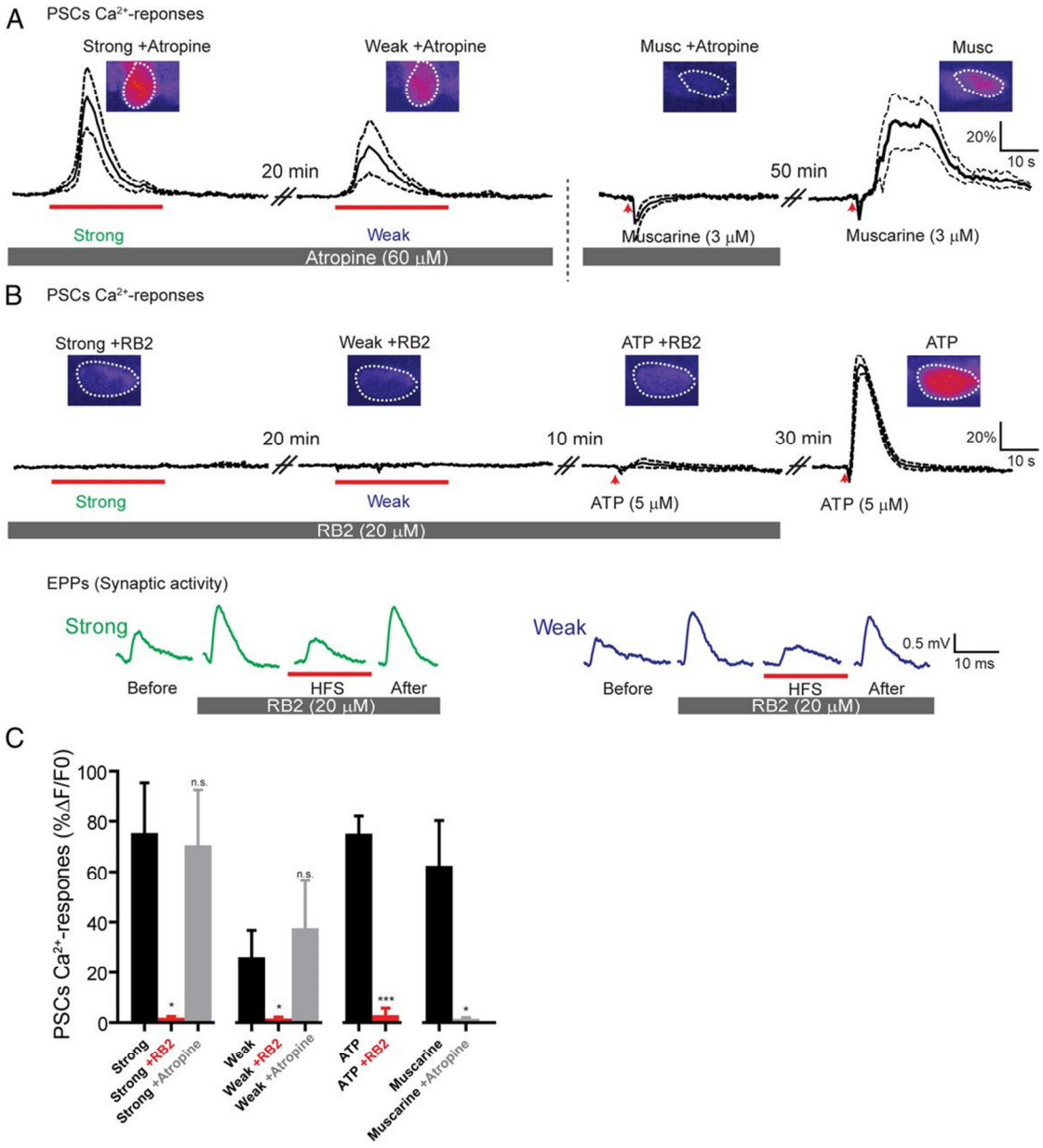

Figure 6. PSCs Ca^{2+} responses are mediated by $P2_Y$ receptors, not muscarinic receptors.

A, PSC responsiveness to ventral root stimulation during bath application of the mAChR antagonist, atropine (60 μM). Note that atropine did not prevent PSC Ca^{2+}responses induced by the strong and the weak input stimulations but antagonized responses induced by local application of the mAChR agonist, muscarine (3 μM). The dark trace represents the average of PSC Ca^{2+} responses; and the dotted lines represent SEM. Inset, False color confocal images showing changes in fluorescence of Fluo-4 at the peak of the response. ***B***, Top, PSC Ca^{2+} activity evoked in the presence of the $P2_Y$ receptor antagonist, RB2 (20 μM). Note that

no Ca^{2+} responses were evoked in the presence of RB2 either by stimulation of the strong or the weak input. Also, responses induced by local application of the $P2_Y$ receptor agonist, ATP (3 μM), were antagonized. Bottom, Examples of EPPs induced by the strong and weak inputs before and during RB2 application. Note that the lack of PSC Ca^{2+}responses cannot be attributed to the absence of synaptic activity because EPPs were still elicited by motor nerve stimulation. ***C***, Histogram showing PSC Ca^{2+} responses induced by strong and weak inputs and local application of agonists in the different conditions studied.

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We next examined whether the lack of contribution of mAChR to PSC activation was the result of the absence of functional mAChR. We tested this possibility by locally applying the mAChR agonist muscarine (3 μM) on PSCs. As shown in Figure 6*A*,*C*, local application of muscarine elicited PSC Ca^{2+} responses after atropine washout (62.45 ± 17.84% ΔF/F0, $N = 4$). No Ca^{2+}responses were induced by local muscarine application in the presence of atropine (1.524 ± 0.5374% ΔF/F0, $N = 4$, paired *t*test, $p < 0.05$, Fig. 6*C*), further confirming the efficiency of the muscarinic antagonist.

These results indicates that PSC mAChRs were not activated by neurotransmitter release from the strong or the weak input with the stimulation parameters used and were not required for the proper decoding of the synaptic strength by PSCs in the experimental conditions tested.

PSC activation is mediated by purinergic receptors

We next tested the contribution of purinergic receptors because synaptic ATP is known to activate PSCs via these receptors (Rochon et al., 2001; Todd and Robitaille, 2006). We stimulated independently each of the competing terminals in the presence of the general

$P2_Y$ receptor antagonist, RB2 (Claes et al., 2004; De Lorenzo et al., 2006), applied in the bath perfusion. As shown in Figure 6*B*, *C*, neither the stimulation of the strong nor the weak input elicited a detectable PSC Ca^{2+} response in the presence of RB2 (strong with RB2, 2.0 ± 0.5% ΔF/F0, $N = 5$ vs control, 75.3 ± 20.1% ΔF/F0 $N = 8$; weak with RB2, 2.0 ± 0.6% ΔF/F0, $N = 5$ vs control, 26.3 ± 10.4% ΔF/F0 $N = 7$, one-way Kruskal–Wallis ANOVA test, Dunn's Multiple-Comparison post-test, $p < 0.05$, Fig. 6*C*). Differences in PSC Ca^{2+} responses induced by the stimulation of the strong or weak input were no longer noticeable because no responses were induced. Furthermore, the absence of synaptic-induced Ca^{2+} responses in PSCs cannot be explained by a reduction in transmitter release. Indeed, intracellular recordings showed that EPPs were still evoked by the stimulation of the strong or the weak input in the presence of RB2 (Fig. 6*B*). Hence, the lack of PSC Ca^{2+}responses was not the result of the absence of synaptic activity but the blockade of $P2_Y$ receptors on PSCs.

The responsiveness of PSCs to ATP was further tested by local application of ATP (5 μM) as the agonist. ATP did not elicit Ca^{2+} responses in presence of RB2 (2.9 ± 2.8% ΔF/F0, $N = 4$) but fully recovered after 30 min of washout (75.1 ± 6.9% ΔF/F0,$N = 4$; paired *t* test $p = 0.0007$; washout vs RB2). Therefore, this strongly suggests that decoding of synaptic strength of competing nerve terminals by PSCs was mediated by $P2_Y$ receptors. Together, these results show that PSCs used the same type of receptors, $P2_Y$ receptors, to detect the activity of competing nerve terminals. Although they were present and functional, muscarinic receptors were not used for synaptic communication with PSCs at immature NMJs in the paradigm studied.

Localization and distribution of P2Y receptors on PSCs

Our data indicate that PSCs decoded synaptic competition via functionally segregated $P2_Y$ receptors, whereas muscarinic receptors did not participate in this decoding even though they were present and functional. This differential involvement of the two receptor systems may have a morphological basis such that the purinergic receptors were preferentially

associated with release sites whereas muscarinic receptors were not. Hence, we performed a series of immunohistochemical labeling to determine the relative distribution of purinergic and muscarinic receptors and assessed their respective distribution in relation with presynaptic and glial markers.

We first examined the spatial distribution of $P2_Y$ receptors at poly-innervated NMJs. Poly-innervation was confirmed by the presence of two axons innervating the same endplate, revealed by the neurofilament labeling. We focused on $P2_Y1R$ based on the pharmacological signature of the Ca^{2+} responses (Robitaille, 1995; Rochon et al., 2001). As shown in Figure 7*A*, *C*, $P2_Y1R$ were present at poly-innervated NMJs (13 NMJs, 6 muscles). $P2_Y1R$ labeling was localized at the endplate area along with the presynaptic (SV2 and neurofilament) and postsynaptic (α-bungarotoxin) markers. Interestingly, the $P2_Y1R$ labeling was punctuated with puncta of different sizes and intensity distributed over the NMJ area.

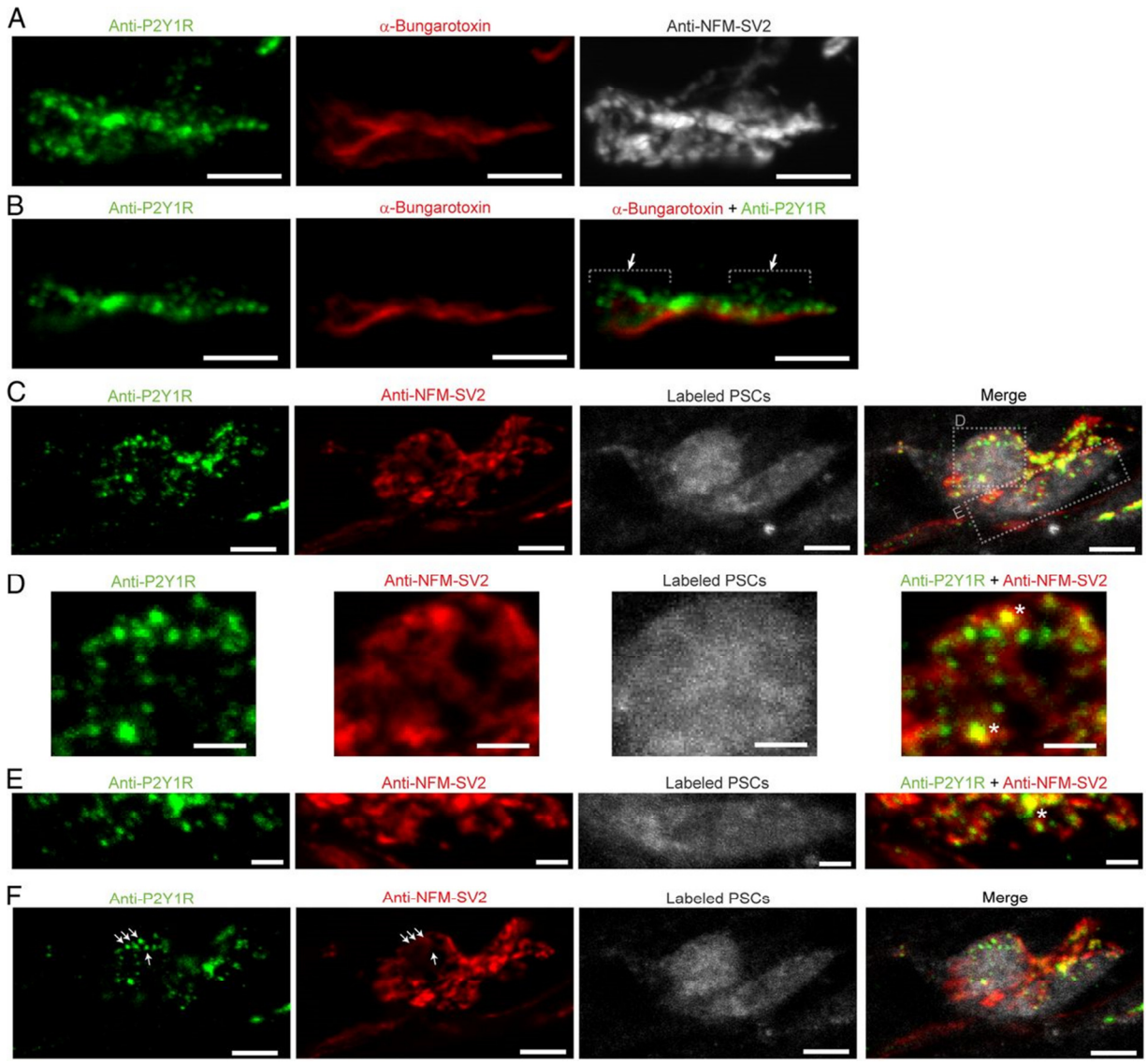

Figure 7. $P2_Y1Rs$ at poly-innervated NMJs.

A, *z*-stack of false color of 9 confocal images of immunohistochemical labeling of type 1 $P2_Y$receptors ($P2_Y1R$; green), postsynaptic nAChRs (α-bungarotoxin; red), and presynaptic terminals (NF-SV2; gray) at a P7 poly-innervated NMJ. Note that $P2_Y1Rs$ were arranged in "hotspots," with a punctuated distribution throughout the endplate area (in red).***B***, A single focal plane of the NMJ presented in ***A*** showing $P2_Y1Rs$ (green) and postsynaptic nAChRs (red). Note that $P2_Y1Rs$ and nAChRs are located at a different level and do not colocalize (dashed lines and arrows represent 2 regions where P2Y1R and nAChR did not overlap). ***C***, Confocal *z*-stack of 5 images of *en face* view of a P7 poly-innervated NMJ showing $P2_Y1R$

(green), presynaptic terminals (NF-SV2; red), and dextran-loaded PSCs (gray). Note that $P2_Y1R$ labeling was present in the area of the dextran-loaded PSCs. Dotted boxes in the right panel highlight the regions enlarged in ***D*** and ***E***. ***D***, Higher magnification of a part of the NMJ presented in ***C***. Note the puncta of $P2_Y1Rs$ (green) over the area of the PSCs, interspersed in between presynaptic labeling and closely associated with presynaptic active zones (NF-SV2; red). Note also that very few $P2_Y1$ hotspots colocalized with presynaptic elements (yellow spots, asterisks in right panel). ***E***, Same as in ***D*** but illustrating the ***E*** dotted box highlighted in ***C***. ***F***, An image of a single plane focusing on the top of the NMJ showing hotspots of $P2_Y1R$ (green) localized within the dextran-loaded PSC (gray) where the presynaptic staining (NF -SV2; red) was out of focus (arrows). Scale bars: ***A***–***C***, ***F***, 5 μm; ***D***, ***E***, 2 μm.

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We next determined with which synaptic compartment the P2Y1R labeling was associated. First, we determined whether the labeling was on the postsynaptic side of the endplate area or whether it was preferentially located in the presynaptic/PSC area. To determine this, we observed NMJs located on the side of muscle fibers. If located in the postsynaptic side, the P2Y1R labeling should be intermingled with the α-bungarotoxin staining. However, as shown in Figure 7*B*, the $P2_Y1R$ labeling was located above the postsynaptic receptor staining, indicating that it was located in the presynaptic and/or PSC compartments.

Next, we used two strategies to determine whether the $P2_Y1R$ were preferentially located in the presynaptic elements or in the PSCs. PSCs were identified using the single-cell electroporation technique (Fig. 7*C*) as the S100β antibody cross-reacted with the one used for labeling the $P2_Y1R$. First, we analyzed the distribution of $P2_Y1R$ puncta (green) in relation with the SV2 labeling, indicative of active zone labeling (red). If the two labels overlapped, indicative of a presynaptic localization, one should see a significant level of yellow pixels (green and red gives yellow) in the overlay image. However, whether the two labels did not

overlap, the initial colors of the labels should be unaltered when overlaid. As shown in Figure 7*D*, *E*, only a few yellow hotspots were observed within the boundary of the PSCs. Interestingly, the hotspots of $P2_Y1R$ were often juxtaposed to active zones revealed by the SV2 labeling. Second, we observed a number of $P2_Y1R$ hotspots located at focal planes above the presynaptic labeling (Fig. 7*F*), probably in PSC compartments that sit on top of the presynaptic terminals. Hence, the presence of $P2_YRs$ in PSCs above the presynaptic terminals and the very poor overlap between the $P2_Y1R$ labeling and the presynaptic markers strongly argue that these receptors were preferentially located in PSCs, juxtaposed to the presynaptic release sites. This is consistent with their role in the detection of transmitter released by the competing nerve terminals.

Localization and distribution of muscarinic receptors on PSCs

We next examined the presence and distribution of muscarinic receptors to determine their distribution in relation with presynaptic markers. Because our data revealed that PSC functional muscarinic receptors were not required for decoding synaptic strength of competing terminals, we hypothesized that muscarinic receptors, unlike $P2_Y$ receptors, were not in close juxtaposition with SV2 positive sites, thus limiting their activation. To test this possibility, we labeled type 3 (mAChR3) and 5 (mAChR5) muscarinic receptors to study their distribution at the NMJ.

As shown in Figure 8, these receptors were present at the NMJ, as revealed by their immunolabeling associated with presynaptic and postsynaptic markers. Similarly to $P2_Y1$ receptors, muscarinic receptors were not found in the postsynaptic compartment as indicated by the distribution of the labeling located above the α-bungarotoxin staining observed on NMJs located on the side of the muscle fiber (Fig. 8*B*).

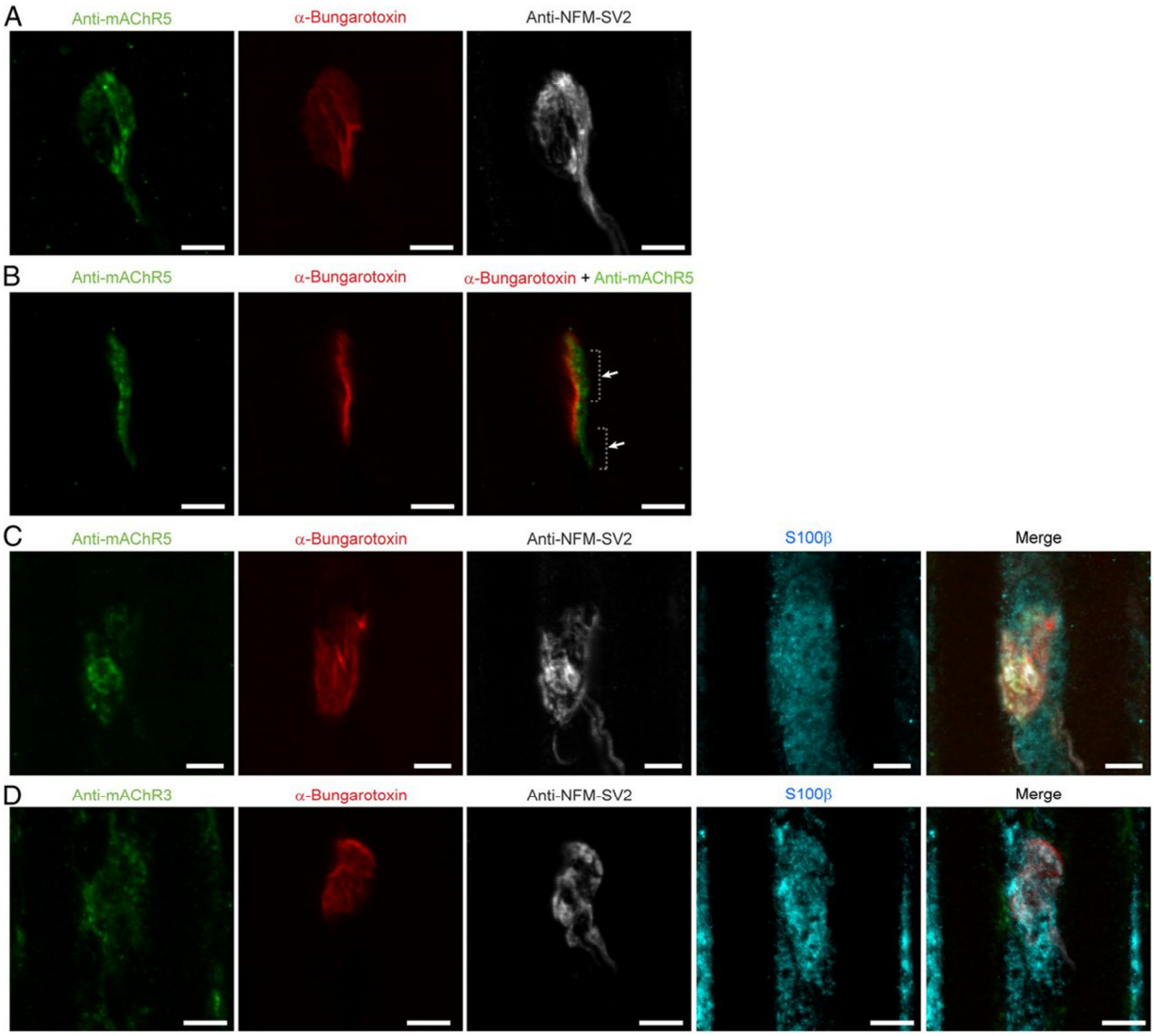

Figure 8. Type 5 and Type 3 mAChRs at poly-innervated NMJs.

A, False color confocal *z*-stack of 10 images of immunohistochemical labeling of Type 5 mAChRs (mAChR5; green), postsynaptic nAChRs (α-bungarotoxin; red), and presynaptic terminals (NF-SV2; gray) at a P7 poly-innervated NMJ. Note the diffuse organization of mAChR5 that covers most of the endplate area in red. ***B***, A single focal plane of the NMJ presented in ***A*** showing mAChR5 (green) and postsynaptic nAChRs (red). Note that mAChR5 and nAChR were located at a different level and did not colocalize (hyphenated lines and arrows show 2 regions where mAChR5 and nAChR did not overlap). ***C***, False color confocal *z*-stack of 5 images of quadruple immunohistochemical labeling of another P7 poly-

innervated NMJ showing the diffuse distribution of mAChR5 (green), postsynaptic nAChRs (α-bungarotoxin; red), presynaptic terminals (NF -SV2; gray), and PSCs (S100β; cyan).***D***, False color confocal *z*-stack of 5 images of quadruple immunohistochemical labeling of another P7 poly-innervated NMJ showing the distribution mAChR3. As for mAChR5, mAChR3 labeling was diffuse and uniform throughout the endplate area (red). Scale bars, 5 μm.

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We next examined the muscarinic receptor distribution to determine whether they were clustered in hotspots. As shown inFigure 8*C*, *D*, even though hotspots and clustering of receptors were sometimes observed, the labeling of mAChR5 and mAChR3 was mostly unstructured (14 NMJs, 5 muscles for mAChR5; 11 NMJs, 4 muscles for mAChR3), presenting a more uniform and even distribution throughout the area of the NMJ. This is similar to what has been reported for muscarinic receptors distribution at NMJs of newborn rats (Garcia et al., 2005). This uniformity in the receptor labeling prevented further characterization of the localization of the synaptic compartments in which they were present. Hence, these data suggest that the muscarinic receptors, unlike $P2_Y1$Rs, were not specifically associated with active zones.

Is there a territorial distribution of $P2_Y$ receptors?

We finally examined the overall distribution of $P2_Y$R to determine whether the decoding of the strong and weak inputs by PSCs could be explained by a preferential association of the $P2_Y$R with the strong nerve terminal. Knowing that competing nerve terminals can occupy defined territories, we postulated that the overall distribution of $P2_Y$R should reflect this territorial organization, resulting in a preferential accumulation of $P2_Y$R hotspots on a portion of the PSC, presumably reflecting the nerve terminal territories.

As shown in Figure 9*A*, it was indeed possible to observe such uneven distribution of $P2_YRs$ whereby some areas showed more numerous and/or brighter hotspots than another area of the poly-innervated NMJ. However, we also observed examples where the overall distribution of $P2_YRs$ hotspots was more evenly spread (Fig. 9*B*). These results may reflect the variability in the strength of the competing nerve terminals.

To ascertain this possibility, we examined the distribution of $P2_YRs$ at mono-innervated NMJs as it could not be influenced by the state of innervation and territorial organization. As shown in Figure 9, we also observed both uneven (Figure 9*C*) and more uniform (Figure 9*D*) distributions of $P2_YR$ hotspots on mono-innervated NMJs. This indicates that the state of innervation and the presence of the overall synaptic strength of each nerve terminal did not account for the distribution of $P2_YRs$.

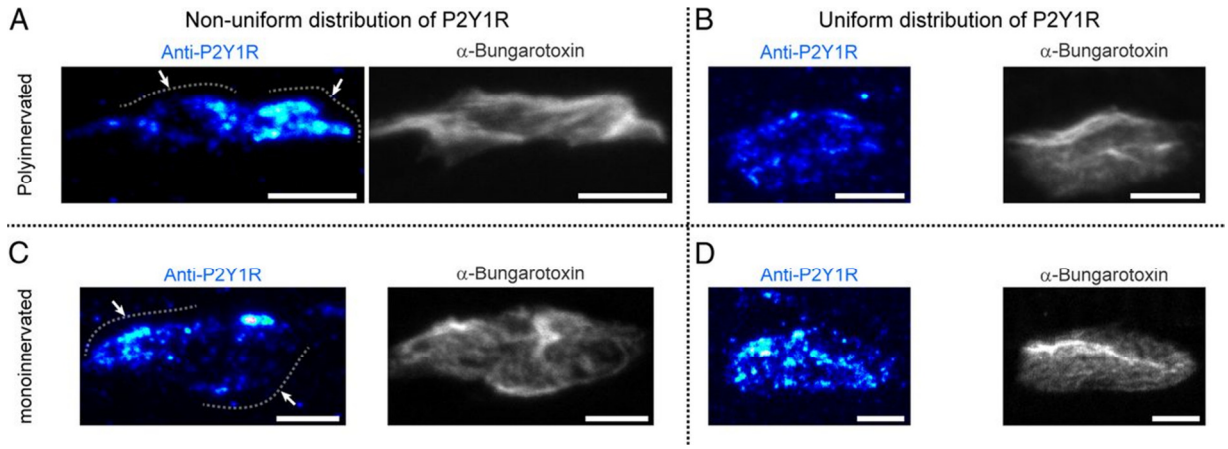

Figure 9. Territorial distribution of P2Y1R receptors at poly-innervated and mono-innervated NMJs.

A, False color confocal z-stack of 16 images of P2Y1R labeling (left; blue) showing two regions (dotted lines and arrows) of different fluorescence intensity at the NMJ. Black/dark blue represents low pixel intensity; and light blue/white, higher intensities. The corresponding AChR labeling delineates the endplate area (right; α-bungarotoxin in gray). Note that the region on the right shows higher fluorescence intensity than the left one, suggesting that more P2Y1Rs were present. B, z-stack of 10 images of another poly-innervated NMJ showing a more uniform distribution of P2Y1Rs (left, blue) throughout the endplate area (right, α-bungarotoxin in gray). C, z-stack of 8 images of a P7 mono-innervated NMJ showing a nonuniform distribution of P2Y1Rs where the left region had higher fluorescence intensity than the right one, suggesting that more P2Y1Rs were present (dotted lines and arrows) over the endplate area (right; α-bungarotoxin in gray). D, z-stack of 10 images of another P7 mono-innervated NMJ showing a more uniform distribution of P2Y1Rs (left; blue) throughout the NMJ (right; α-bungarotoxin in gray). Scale bars, 5 μm.

4.2.5. Discussion

Here we show that glial cells at immature NMJs undergo similar interactions with their neuronal counterparts, as was shown for mature synapses. However, we reveal an additional level of sophistication whereby a single PSC interacts with two independent competing inputs at the same NMJ by decoding their synaptic efficacy based on selective activation of different receptor pools, level of transmitter release, and intrinsic properties of PSCs. These properties should allow PSCs to intervene actively in activity-dependent modulation of synaptic competition.

Responsiveness of PSCs

For PSCs to influence synaptic competition in an activity-dependent manner, an important requirement was that they detect synaptic activity of competing nerve terminals. Similar to adult NMJs (Rochon et al., 2001; Todd et al., 2010), we showed that PSCs were as efficient in detecting transmitter release at this age and developmental stage as indicated by a reliable activation of PSCs (high percentage of responsive cells) that elicited robust and consistent Ca^{2+} elevation.

Interestingly, PSCs at mature and developing NMJs differ by the type of activated receptors. Indeed, PSCs at mature NMJs detect neurotransmitters using a combination of purinergic and muscarinic receptors (Rochon et al., 2001), whereas PSC activation solely depends on $P2_Y$ receptors during synaptic competition at the age studied. Hence, it appears that the purinergic receptor system is predominant during early developmental stages, even though the muscarinic receptor system was present and functional. The lack of muscarinic contribution to synaptic-induced PSC activation, even though the receptors are functional, may be the result of the absence of structured distribution of muscarinic receptors at proximity of active zones.

Therefore, a switch from a purinergic to a mixed muscarinic/purinergic signaling during synapse maturation surely underlies a change in the properties and functions of the PSCs themselves. This is supported by changes in PSC properties after denervation and the expression of glial fibrillary acidic protein known to be regulated by muscarinic receptors (Georgiou et al., 1999). An interesting possibility is that different receptors play different roles so that the muscarinic regulation would mainly stabilize PSCs while the purinergic system would be less restrictive, thus more prominent during synapse formation and maturation where large functional and morphological changes occur. This is also supported by a study showing that PSCs at immature NMJs are highly dynamic, becoming more quiescent with age (Brill et al., 2011).

One PSC, two synaptic inputs

One of the challenges during synaptic competition and elimination is that a single PSC must integrate synaptic information from both competing inputs. A main constraint for a single PSC in such context is to elaborate a strategy to detect synaptic activity from both nerve terminals while maintaining its ability to segregate and identify each activation to a given nerve terminal.

As a whole, our data strongly argue that the selective detection of competing nerve terminals is achieved by maintaining a functional segregation of receptors, with $P2_YR$ clustered in hotspots at proximity of active zones. However, there was no clear territorial distribution of these receptors on a portion of a PSC, associated with a given nerve terminal, that could have explained their differential responsiveness to each input. Instead, our data suggest that this segregation is based on functional properties rather than on a nonuniform distribution of the receptors. Interestingly, the functional segregation of P2YRs and their preferential clustering at proximity of active zones are in accordance with the general organization of NMJs in the midsts of synaptic competition because each presynaptic terminal uses exclusive sets of postsynaptic receptors (Gan and Lichtman, 1998; Keller-Peck et al., 2001; Walsh and Lichtman, 2003; Wyatt and Balice-Gordon, 2003; Bishop et al., 2004). Knowing that the occupancy of the synaptic territory is quite dynamic and changes during the

course of the competition (Turney and Lichtman, 2012), it would be of interest to determine whether PSC receptor properties and distribution also follow similar dynamic alteration in the course of the process.

Receptor distribution and density could also be a determining factor establishing PSC intrinsic properties. Indeed, the inability of TEA to further increase the size of Ca^{2+} responses elicited by synaptic activity could be explained by the presence of different number of receptors and/or receptors at some distance from the release sites. In these conditions, releasing more neurotransmitter would have little effect on PSC Ca^{2+} response because all receptors would be already optimally activated or that there would be sufficient time for the ecto-enzymes to degrade ATP before it reaches the PSC receptors (Robitaille, 1995). Furthermore, PSC receptor distribution and segregation may be regulated by the level of synaptic activity where PSCs could organize the distribution of its fine processes and receptors to optimize the detection of transmitter release occurring at single active zones. The distribution of purinergic receptors, but not the muscarinic ones, in the vicinity of active zones (identified by the SV2 labeling) strongly argues in favor of this interpretation.

In this study, specific Ca^{2+} responses to weak and strong terminals occur throughout the PSC soma. This has two main implications for the decoding of the two inputs. First, the different size of the Ca^{2+} transients does not carry the same information, possibly by activating different Ca^{2+}-dependent mechanisms with different affinity for Ca^{2+}. Second, because of the lack of evidence of morphological segregated distribution of receptors, we propose that the segregated functional distribution of receptors is complemented by the segregation of downstream intracellular machinery. Mechanisms, such as differential cellular machinery distribution or different calcium sources targeted to each nerve terminal, could be present. Such important intrinsic characteristics of PSCs properties will need to be further explored.

Finally, the differential coverage of competing terminals by PSCs could have been another mechanism explaining their differential activation. However, our data are not consistent with this observation. Indeed, according to this scenario at dually innervated NMJs where two PSCs are present, one could predict that each PSC would cover preferentially a

synaptic territory associated with a given nerve terminal whereas the other one would preferentially interact with the other. Hence, if the differential coverage dictates the sensitivity of PSCs, a given PSC would systematically respond with a large Ca^{2+} response from the activity of a given terminal and smaller ones by the activity of the other. This situation would be reversed for the other PSC. However, as shown in Figure 3*F*, we never observed such a phenomenon, suggesting that the level of PSC coverage is not a determinant parameter and that Ca^{2+} responses of both PSCs reflected the state of synaptic competition. This is also in accordance with recent evidence showing that PSCs highly intermingle and overlap during early postdevelopmental stages while they occupy segregated, well-delineated territories in adulthood (Brill et al., 2011).

Impact of PSCs decoding in synaptic competition and elimination

The possible involvement of PSCs in the regulation of synaptic competition has been questioned in previous works where it was proposed that they served more a role of support for axon retraction (Hirata et al., 1997). However, their ability to dynamically decode synaptic strength using their Ca^{2+}-dependent excitability provides strong evidence that PSCs are poised to play a critical role in influencing synaptic competition.

The PSC decoding ability could have different consequences for the regulation of synaptic competition and elimination. First, because of their ability to decipher between strong and weak inputs, as indicated by the different Ca^{2+} responses, it is possible that, in turn, PSCs differentially modulate synaptic efficacy of the competing terminals. For instance, consistent with their ability to decipher levels of synaptic activity at mature NMJs (Rousse et al., 2010; Todd et al., 2010), PSCs could further potentiate synaptic activity of the strong input, at the expense of the weakest one. Such modulation would bias the competition toward the strong input and, ultimately, the outcome of synaptic competition and elimination.

A second consequence could be related to the elimination of the redundant inputs. PSCs are known to actively clear cellular debris at developing NMJs, a critical feature of the developing nervous system (Song et al., 2008). Perhaps their ability to sort out the weak from

the strong input would allow them to selectively target this metabolism to the weak terminal, thus participating actively in the shedding of axon terminals (Chung and Barres, 2009). This could be a central feature of synapse elimination because the mechanism of selecting the branches destined for elimination remains unknown (Song et al., 2008).

Glial cells on synaptic elimination in the CNS

The involvement of glial cells in the refinement of synaptic connectivity has also been addressed in the CNS. For instance, immature astrocytes participate in the axon pruning of redundant inputs by initiating the activation of the complement cascade (Cullheim and Thams, 2007; Stevens et al., 2007; Alexander et al., 2008). Also, genetic manipulation of Ca^{2+}-permeable channels in Bergmann glia caused the retraction of glial processes ensheathing synapses, increased multiple innervations of Purkinje cells by climbing fibers and delayed synapse elimination (Iino et al., 2001). Hence, it is appealing to propose that astrocytes pruning of synapses could be related to their ability to interact with competing inputs and decoding their activity similarly to PSCs at the NMJ.

In conclusion, we report that PSCs decode ongoing synaptic competition and the state of competency and strength of each competing inputs. Because of their ability to modulate and control synaptic activity, their ability to decipher the state of synaptic competition would endow PSCs with a powerful mechanism to alter the outcome of synaptic competition, hence, the quality and reliability of synaptic wiring.

4.2.6. References

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5. Deuxième article de recherche

5.1. Introduction au deuxième article de recherche

Dans la première étude, nous avons montré que les cellules gliales décodent l'efficacité et le niveau d'activité synaptique des terminaisons nerveuses en compétition. Les CSPs sont donc favorablement positionnées pour influencer l'issue de la compétition synaptique puisque le niveau d'activité des terminaisons en compétition est un déterminant majeur.

Ce décodage des CSPs pourrait leur permettre d'interagir activement avec les terminaisons en compétition. Nous avons donc analysé la capacité des cellules gliales à réguler l'activité synaptique des terminaisons nerveuses et à influencer le processus de l'élimination synaptique au cours du développement. Ceci est d'un intérêt particulier puisqu'il est connu que les cellules gliales dans le système nerveux mature régulent l'activité des neurones et que cette régulation dépend de leur capacité à décoder les propriétés synaptiques.

Pour cette fin, nous avons utilisé la jonction neuromusculaire comme modèle et nous avons combiné l'imagerie Ca^{2+} des cellules gliales, avec des enregistrements synaptiques de jonctions neuromusculaires poly-innervées de souriceaux. De plus, nous avons perturbé, *in situ* et *in vivo*, l'activité des CSPs pour analyser leur impact sur l'activité des terminaisons en compétition et sur l'élimination synaptique.

Les pages qui suivent présentent les résultats de cette partie tirés de l'article suivant qui est soumis au journal Neuron :

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Neuron

GLIAL CELLS DIFFERENTIALLY REGULATE SYNAPTIC PLASTICITY TO INFLUENCE SYNAPSE COMPETITION AND ELIMINATION

--Manuscript Draft--

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Abstract:	The precise wiring of synaptic connections requires the elimination of supernumerary inputs competing for innervation of the same target cell. This competition is activity-dependent and results in the strengthening of some inputs while others are eliminated. Although glial cells have been identified as the cell type responsible for the elimination and clearance of terminals, their involvement in activity-dependent synaptic competition remains ill-defined. Here we took advantage of developing neuromuscular junctions of mice to show that perisynaptic glial cells decode the synaptic efficacy of competing terminals, through glial purinergic type 2Y1 receptors (P2Y1Rs). Differential regulation of synaptic plasticity occurs via presynaptic adenosine 2A receptors. In a Ca2+-dependent manner, glial cells induce a long lasting synaptic potentiation of strong but not weak terminals. Preventing glial activation by the chelation of intracellular Ca2+ or the blockade of P2Y1Rs alters the plasticity of terminals In situ and delays synapse elimination in vivo. Thus, glial cells actively and preferentially reinforce the strong input which impacts on synapse competition and elimination.
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5.2. « GLIAL CELLS DIFFERENTIALLY REGULATE SYNAPTIC PLASTICITY TO INFLUENCE SYNAPSE COMPETITION AND ELIMINATION »

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Author contributions: H.D. conducted the experiments and analyzed the data. H.D. and R.R. designed the experiments, interpreted the data and wrote the manuscript.

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5.2.1. Summary

The precise wiring of synaptic connections requires the elimination of supernumerary inputs competing for innervation of the same target cell. This competition is activity-dependent and results in the strengthening of some inputs while others are eliminated. Although glial cells have been identified as the cell type responsible for the elimination and clearance of terminals, their involvement in activity-dependent synaptic competition remains ill-defined. Here we took advantage of developing neuromuscular junctions of mice to show that perisynaptic glial cells, through type 2Y1 purinergic receptors (P2Y1Rs), decode synaptic efficacy of competing terminals in a Ca^{2+}-dependent manner. This glial activity induces a long lasting synaptic potentiation of strong but not weak terminals via presynaptic adenosine 2A receptors. Chelation of glial intracellular Ca^{2+} or the blockade of P2Y1Rs prevents this differential plasticity of terminals and delays synapse elimination *in vivo*. Hence, glial cells actively and preferentially reinforce the strong input which influences synapse competition and elimination.

5.2.2. Introduction

Connectivity of the nervous system is shaped by a critical period of synapse competition and elimination during postnatal development. At birth, there are more synaptic contacts formed than what is maintained in adulthood such that a single target cell could receive an exceeding number of inputs competing for its innervation (Chen and Regehr, 2000; Katz and Shatz, 1996; Lohof et al., 1996; Wyatt and Balice-Gordon, 2003). The presence of exuberant innervation and the necessity of synapse elimination have been observed in many areas of the central nervous system (CNS; Campbell and Shatz, 1992; Chen and Regehr, 2000; Del Rio and Feller, 2006; Hashimoto and Kano, 2013; Hooks and Chen, 2006; LaMantia and Rakic, 1990; LeVay et al., 1980; Lohof et al., 1996; Mariani and Changeux, 1981) and at the neuromuscular junction (NMJ; Redfern, 1970; Wyatt and Balice-Gordon, 2003). This refinement of connectivity relies on glial cells which are known to prune synapses in the CNS (Bialas and Stevens, 2013; Chung et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007; Zhan et al., 2014) and are responsible for the elimination of nerve terminals from the neuromuscular junction (NMJ; (Bishop et al., 2004; Smith et al., 2013; Song et al., 2008).

At birth, each NMJ is innervated by several nerve terminals originating from distinct motor neurons (Balice-Gordon and Lichtman, 1993; Cai et al., 2013; Redfern, 1970; Tapia et al., 2012; Wyatt and Balice-Gordon, 2003). These nerve terminals undergo an activity-dependent competition resulting in the elimination of all but one input (Bishop et al., 2004; Keller-Peck et al., 2001b; Tapia et al., 2012; Walsh and Lichtman, 2003; see also Darabid et al., 2014 for review). At dually-innervated NMJs, it is accepted that the more active nerve terminal, with a better synaptic efficacy (“strong terminal”), is more likely to be maintained while the less active one (“weak terminal”), is gradually eliminated (Balice-Gordon and Lichtman, 1994; Buffelli et al., 2003; Busetto et al., 2000; Colman et al., 1997; Favero et al., 2012; Ribchester and Taxt, 1983; Ridge and Betz, 1984). However, the mechanisms responsible for the regulation of synaptic activity of terminals are poorly understood. One possible candidate of such regulation is the perisynaptic glial cells. Indeed, glial cells at

mature synapses to regulate synaptic efficacy and modulate plasticity by the release of neuroactive molecules (Castonguay and Robitaille, 2001; Henneberger et al., 2010; Panatier et al., 2006; Panatier et al., 2011; Perea and Araque, 2007; Robitaille, 1998; Serrano et al., 2006; Todd et al., 2010). This regulation depends on their ability to detect synaptic activity (i.e. release of neurotransmitters) which lead to intracellular Ca^{2+}-elevations and the decoding of neuronal properties (Todd et al., 2010). Interestingly, we recently showed that, during the development of the NMJ, glial cells have also the ability to decode synaptic activity of competing inputs based on differential synaptic-induced intracellular Ca^{2+}-elevations (Darabid et al., 2013). Therefore, it is possible that this decoding ability by glial cells endows them with the ability to regulate the synaptic activity of competing terminals and, thus, influence the outcome of synaptic competition and elimination.

We propose that perisynaptic Schwann cells (PSCs), glia at the NMJ, differentially regulate synaptic activity of competing terminals favoring one input over the other, thus influencing the outcome of synaptic competition and elimination. Using simultaneous Ca^{2+}-imaging of PSCs and synaptic recordings of dually-innervated mice NMJs, we showed that strong terminals displayed a long lasting potentiation of synaptic activity in response to high frequency stimulation while the weaker competitors only displayed a small and transient one. This plasticity was mediated by presynaptic adenosine type 2A receptors (A2ARs). PSCs generated this plasticity since it was obliterated by the blockade of PSCs Ca^{2+}-dependent activity or the blockade of PSCs purinergic type 2Y1 receptors (P2Y1Rs). Importantly, *in vivo* blockade of P2Y1Rs delayed synapse elimination. Hence, we present a novel mechanism by which glial cells actively and preferentially potentiate stronger nerve terminals to influence synapse competition and elimination.

5.2.3. Results

PSCs are non-myelinating glial cells at the NMJ that participate in synapse formation and maturation (see Darabid et al., 2014 for review), actively eliminate branches from competing terminals (Bishop et al., 2004; Smith et al., 2013) and regulate synaptic activity and plasticity at mature NMJs (Castonguay and Robitaille, 2001; Robitaille, 1998; Todd et al., 2010). Ca^{2+} is a reliable reporter of glial cells activity since most described PSC-dependant regulation of synaptic properties rely on intracellular changes in Ca^{2+} concentration (Darabid et al., 2013; Jahromi et al., 1992; Robitaille, 1995; Robitaille et al., 1997; Rochon et al., 2001; Todd et al., 2010). Here we studied the role of PSCs in the regulation of synaptic activity at P7-8 dually innervated NMJs and its impact on activity-dependent synaptic competition and elimination by monitoring or perturbing PSCs intracellular Ca^{2+} signalling.

Single Cell Electroporation of PSCs and Their Decoding of Synaptic Competition

We needed first to develop an approach that would allow us to monitor PSCs Ca^{2+}-dependent activity while being able to introduce different molecules to alter their activity. To this purpose, we adapted the technique of single cell electroporation to specifically load PSCs with different types of charged molecules. This method has the advantage to rapidly and reliably load multiple cells in sequence (Figure S1; Movies S1) with no undesired effect on physiology (Nevian and Helmchen, 2007).

At P7-8, most dually-innervated NMJs at the *Soleus* muscle show the presence of one or two PSCs that cover both competing terminals (Darabid et al., 2013; Hirata et al., 1997; Kopp et al., 2000). In order to assess the activity of PSCs, we loaded the calcium indicator Fluo-4 into all visible PSCs at dually-innervated NMJs as well as the fluorescent dye Alexa Fluor 594. Importantly, we found that single cell electroporation of PSCs did not affect synaptic activity (EPP amplitude of 100.1 ±3.6% before vs 95.4 ±7.9% after 4 min vs 95.8 ±9.3% after 16 min; N=4, *One-way ANOVA, repeated measures, Tukey's multiple comparison*

post-test, p>0.05; Figure S1), nor responsiveness of PSCs to local neurotransmitter applications (See below, Figure S1 and Figure 1).

PSCs must detect and differentiate the activity of each competing nerve terminal to be able to provide an appropriate feedback (Todd et al., 2010). Although this was shown in our previous study (Darabid et al., 2013), we deemed important to confirm this observation as we used a different technique (single cell electroporation) to load PSCs. As competing terminals could have different synaptic efficacy at dually-innervated NMJs (Colman et al., 1997; Darabid et al., 2013; Kopp et al., 2000), synaptic strength of each competing input was determined by calculating its quantal content (m), using the failure method (Del Castillo and Katz, 1954), and its facilitation (F) during a paired pulse facilitation protocol (Figure S2; Darabid et al., 2013; Kopp et al., 2000). Synaptic strength allowed us to assess the disparity in synaptic efficacy of competing inputs and differentiate the "strong" from the "weak" terminal competing at the same NMJ. As previously reported, among two competing inputs at a dually-innervated NMJ, the strong terminal is characterized by the largest quantal content and the smallest paired pulse facilitation (Darabid et al., 2013; Kopp et al., 2000).

Using confocal Ca^{2+}-imaging, we studied the responsiveness of PSCs to endogenous synaptic activity from each competing input induced by high frequency stimulation of a specific ventral root (HFS) using a protocol of 50 Hz during 30 seconds. Such pattern has been reported *in vivo* during the tonic firing of motor neurons innervating the *Soleus* muscle at a similar age (Eken et al., 2008; Gorassini et al., 2000). Interestingly, at dually-innervated NMJs, the HFS of the strong nerve terminal always induced larger Ca^{2+}-responses in PSCs than its weaker competitor (Figure 1A-B, D-E; Strong: 39.53 ±13.65%ΔF/F0 vs Weak: 21.43 ±10.95%ΔF/F0; N=8, *Paired t-test*, $p<0.05$). This shows that a PSC detects neurotransmitter release from each competing nerve terminal and decodes their synaptic strength. These results, using single cell electroporation of PSCs, are similar to those described previously using bulk loading of PSCs (Darabid et al., 2013). Moreover, PSCs also responded to the local application of ATP (Figure 1C; 131.50 ±48.19%ΔF/F0; N=4) which is the main neurotransmitter that activates PSCs at developing NMJs (Darabid et al., 2013) and is commonly used to assess PSCs excitability and health (Arbour et al., 2015; Darabid et al.,

2013; Rochon et al., 2001). Altogether, these results confirm that PSCs at a dually-innervated NMJ decode synaptic competition and that single cell electroporation of PSCs is a reliable method to target specifically PSCs.

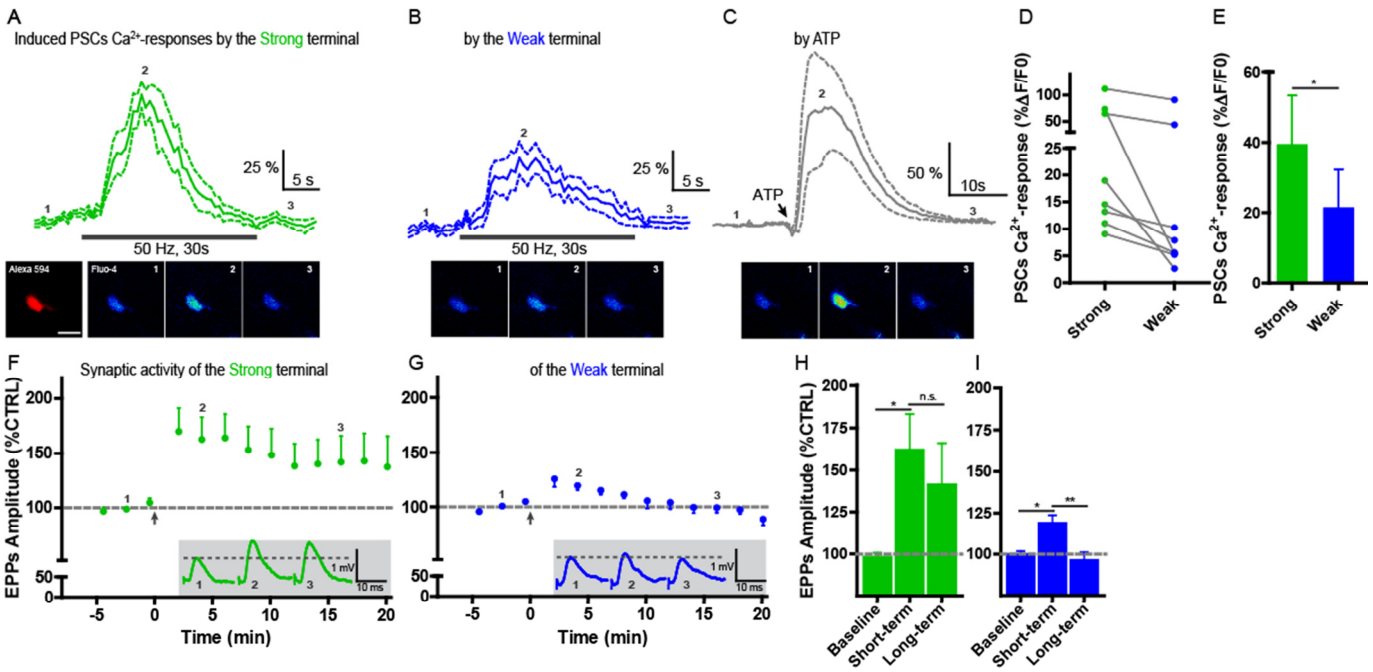

Figure 1. Competing nerve terminals induce different Ca^{2+}-responses in PSCs and show distinct synaptic plasticity to high frequency stimulation.

A-C. Average of Ca^{2+}-responses ±SEM (dotted lines) induced in PSCs by independent stimulation of the strong (green in **A**), weak (blue in **B**) competing inputs and local application of ATP (5 µM; gray in **C**). A false color confocal image of the electroporated PSC (Alexa Fluor 594 in red; Scale bar 10 µm) as well as images of changes in fluorescence of Fluo-4 illustrating Ca^{2+} levels before (1), at the peak of the response (2), and after stimulation (3) are presented for each condition. **D.** Plot of the amplitude of PSCs Ca^{2+}-responses induced by strong (green dots) and weak (blue dots) terminals. Each connected pair of strong and weak terminals are in competition at the same NMJ which induced Ca^{2+}-responses in the same PSC (1PSC/NMJ). Note that strong inputs always induced larger PSC Ca^{2+} responses than weak inputs. **E.** Histograms showing the mean amplitude of the PSC Ca^{2+}-responses ±SEM induced by strong (green), weak (blue) inputs as well as by local application of ATP (gray). **F, G.** Normalized EPP amplitude ±SEM over time showing that stimulation (gray arrow at time 0) of strong inputs resulted in a robust long lasting potentiation of neurotransmission (green in **F**), whereas stimulation of weak inputs resulted in a transient potentiation (blue in **G**). Each point represents the mean of 24 EPPs recorded at 5 sec interval. Insets show examples of EPPs

recorded before (1; Baseline), shortly after the HFS (2; at 4 min) and 16 min after the HFS. **H, I.** Histograms showing the mean amplitude of EPPs ±SEM induced by strong (green in **H**) and weak (blue in **I**) inputs during the baseline, shortly after the HFS (at 4 min) and 16 min after the HFS.

Only Strong Competing Nerve Terminals Show Long-Lasting Potentiation of Synaptic Activity

We analyzed the synaptic activity of strong and weak competing inputs in response to HFS since activity is a major determinant for synapse competition and elimination. Knowing that competing terminals could display different levels of synaptic strength (Figure S2; Colman et al., 1997; Darabid et al., 2013; Kopp et al., 2000), it is possible that they display different levels of plasticity following the HFS. Moreover, in the CNS, HFS could result in a potentiation of synaptic activity (e.g. long term potentiation; LTP) and that those synapses are more likely to be reinforced and stabilized (Matsuzaki et al., 2004; Nagerl et al., 2004), while synapses that are depressed can be weakened, leading to their elimination (Bastrikova et al., 2008; Oh et al., 2013). Hence, since strong terminals are favored to win the competition (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000), we hypothesized that these inputs are more prone to produce potentiation than weaker ones.

As shown in Figure 1(F, H), HFS of strong inputs, which induced large PSC Ca^{2+}-elevation, resulted in a long-lasting potentiation of synaptic activity such that the amplitude of EPPs was increased by more than 40%. Indeed, strong inputs showed a short term (calculated at 4 min after HFS; EPP amplitude of 162.7 ±20.4% calculated at 4 min post-HFS vs 98.7 ±2.0% at baseline; N=7, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p<0.05$*) and a long-lasting (calculated at 16 min after HFS) potentiation of synaptic activity that persisted for at least the duration of the recordings (EPP amplitude of 142.0 ±24.0% calculated at 16 min post-HFS; N=7, non-significantly different than EPP amplitude at 4 min post-HFS, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p>0.05$*). However, the HFS of weak inputs, which induced small PSC Ca^{2+}-elevation, did not induce a long lasting potentiation. Although a short term increase of EPP amplitude of weak terminals was observed (EPPs amplitude of 119.5 ±4.1% at 4 min post-HFS vs 100.6 ±1.1% during the baseline; N=5, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p<0.05$*), this increase was transient and returned to baseline after a few minutes (Figure 1G, I; 96.9 ±4.2% at 16 min post-HFS; N=5, significantly lower than EPP

amplitude at 4 min post-HFS, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p<0.05$*).

This indicates that strong and weak terminals display distinct levels of synaptic plasticity following HFS. The plasticity of strong terminals suggests a change in synaptic strength in the form of a long-lasting potentiation. This also suggests that strong inputs are more prone to potentiation and hints to the presence of mechanisms capable of selectively increasing the activity of strong but not weak inputs.

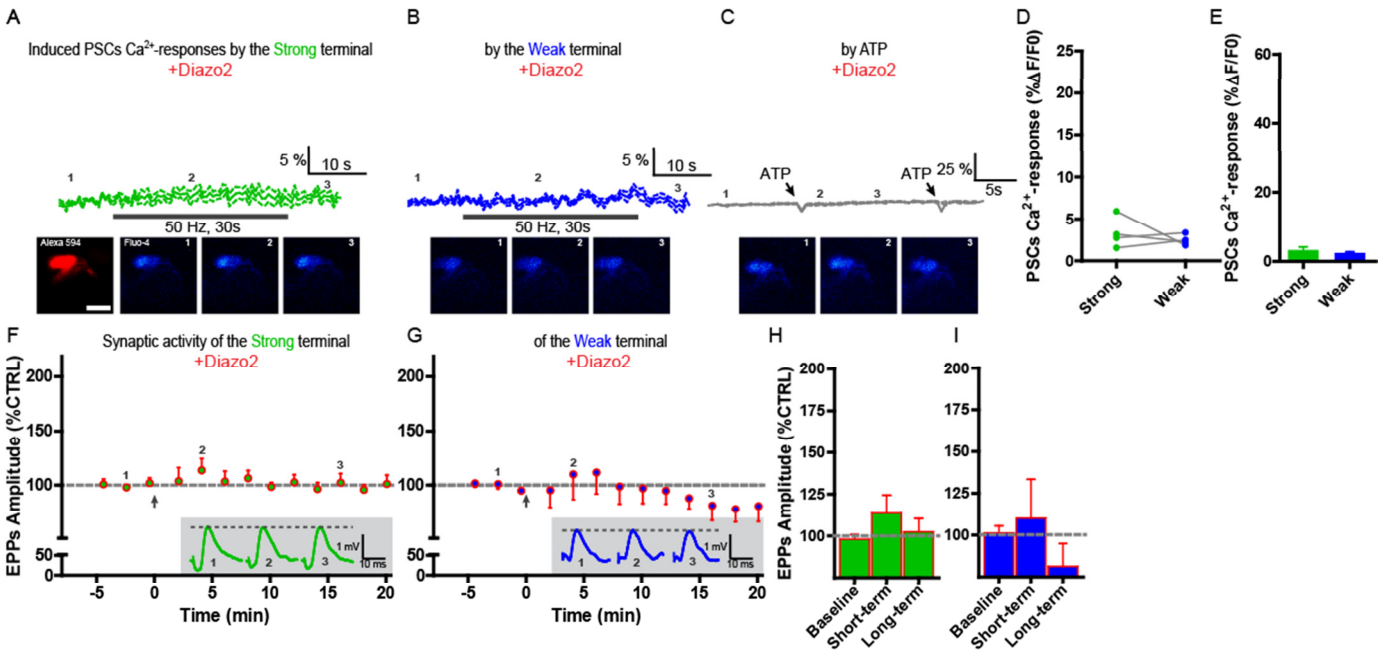

Figure 2. Blockade of PSCs Ca2+-activity alters synaptic plasticity of competing terminals. **A-C**. Photolysis of caged BAPTA molecules (Diazo2) electroporated specifically in PSCs prevents PSCs Ca^{2+}-responses. Average of Ca^{2+} activity ±SEM (dotted lines) induced in PSCs by independent stimulation of the strong (green in **A**), weak competing inputs (blue in **B**) and local application of ATP (5 µM; gray in **C**) following photo-activation of Diazo2. A false color confocal image of the electroporated PSC (Alexa Fluor 594 in red; Scale bar 10 µm) as well as images of changes in fluorescence of electroporated Fluo-4 illustrating Ca^{2+} levels before (1), at the peak of the response (2), and after stimulation (3) are presented for each condition. **D.** Plot of the amplitude of PSC Ca^{2+}-responses induced by strong (green dots) and weak (blue dots) terminals. Each connected pair of strong and weak terminals are in competition at the same NMJ whose Ca^{2+}-responses are recorded in the same PSC (1PSC/NMJ). **E.** Histograms showing the mean amplitude of the PSC Ca^{2+}-responses ±SEM induced by strong (green), weak (blue) inputs as well as by local application of ATP (gray). **F, G.** Normalized EPP amplitude ±SEM over time showing that stimulation (gray arrow at time 0) of strong (green in **F**) or weak (blue in **G**) inputs no longer resulted in changes of synaptic activity following the blockade of PSCs Ca^{2+}-responses. Insets show examples of EPPs recorded before (1; Baseline), shortly after the HFS (2; at 4 min) and 16 min after the HFS. **H,**

I. Histograms showing the mean amplitude of EPPs ±SEM induced by strong (green in **H**) and weak (blue in **I**) inputs during the baseline, shortly after the HFS (at 4 min) and 16 min after the HFS.

PSCs Ca^{2+}-activity is required for the Synaptic Plasticity of Competing Nerve Terminals

Since PSCs Ca^{2+}-activity is a major regulator of synaptic plasticity at the mature NMJ (Castonguay and Robitaille, 2001; Todd et al., 2010), we hypothesized that PSCs Ca^{2+}-activity is required for the potentiation of competing terminals following HFS. To test this hypothesis, we blocked PSCs Ca^{2+} activity using Diazo2 which was specifically electroporated in PSCs. Diazo2 is a caged BAPTA molecule with a very low affinity to Ca^{2+} at rest (Kamiya and Zucker, 1994). However, once photoactivated by exposure to 405 nm laser, its affinity to Ca^{2+} increases greatly, thus providing rapid buffering of Ca^{2+} and blockade of PSCs activity (Kamiya and Zucker, 1994; Todd et al., 2010). In the absence of Diazo2 and following blue light exposure, or when Diazo2 is present but not photoactivated, PSCs responded to endogenous transmitter release from nerve terminals and to local applications of ATP (Figure S3). However, similar to previous work at the mature NMJ (Todd et al., 2010), photoactivation of Diazo2 prevented PSCs Ca^{2+}-elevations induced by local applications of ATP and this chelation is specific to the photoactivated cells previously electroporated with Diazo2 (Figure S3). Importantly, it is an efficient method to block Ca^{2+}-activity of PSCs induced by exogenous and endogenous transmitters. As shown in Figure 2, neither the HFS stimulation of strong inputs, nor the weak ones induced Ca^{2+}-elevations in PSCs following the photoactivation of Diazo2 with a 405 nm laser (Strong: 3.35 ±0.92%ΔF/F0, N=4; Weak: 2.48 ±0.31%ΔF/F0,). Under this condition, distinguishing the strong from the weak input solely by assessing Ca^{2+}-activity was no longer possible since no responses were induced (N=4, *Paired t-test*, $p<0.05$). Moreover, local application of ATP (5 µM), which mediates PSCs activity during development and is a strong activator of glial cells (Arbour et al., 2015; Darabid et al., 2013; Jahromi et al., 1992; Robitaille, 1995; Rochon et al., 2001), did not induce any detectable Ca^{2+}-elevations in PSCs (Figure 2C; CTRL: 131.50 ±48.19%ΔF/F0, N=4 vs photoactivated Diazo2: 4.18 ±0.36%ΔF/F0,N=4; *Unpaired t-test, p=0.03*).

If PSCs Ca^{2+}-activity is necessary for the plasticity of competing nerve terminals, preventing Ca^{2+}-elevations should impair synaptic plasticity induced by HFS. Diazo-2 was photoactivated prior and during the first seconds of HFS stimulation (Figure S3A) which prevented Ca^{2+}-elevations in PSCs as described above. As shown in Figure 2 (F-H), no

persistent potentiation of the strong terminal was observed following the blockade of PSCs Ca^{2+}-activity. Indeed, the amplitude of EPPs generated by strong nerve terminals after HFS was not different from baseline (Figure 2F, H; EPP amplitude of 98.0 ±2.8% at baseline vs 113.6 ±10.9% at 4 min post-HFS vs 102.4 ±8.1% at 16 min post-HFS; N=3, non-significantly different, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p>0.05$*). Interestingly, the blockade of PSCs Ca^{2+}-responses had little effect on the plasticity of weak terminals. Indeed, as in control experiments, the amplitude of EPPs induced by the stimulation of weak terminals after 16 min post-HFS was not different from the baseline (Figure 2G, H; EPP amplitude of 101.0 ±4.4% during the baseline vs 109.9 ±23.3% at 4 min post-HFS vs 81.6 ±13.8% at 16 min post-HFS; N=3, non-significantly different, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p>0.05$*). These results reveal that the differences in synaptic activity between strong and weak competing inputs following HFS depend on PSCs Ca^{2+}-activity. Furthermore, PSCs target potentiation to strong nerve terminals in a Ca^{2+}-dependent manner while weak nerve terminals receive little modulation from PSCs even though they can elicit Ca^{2+} elevations in PSCs.

In an attempt to test whether PSCs Ca^{2+}-elevations alone are sufficient to induce a potentiation of synaptic activity, we directly activated PSCs using a caged-Ca^{2+} molecule, NP-EGTA, electroporated in PSCs. As shown in Figure S4, inducing a large Ca^{2+}-elevation in PSCs following the photolysis of NP-EGTA (153.65%ΔF/F0) was sufficient to induce a long lasting potentiation of the strong input. Yet, this only resulted in a small transient potentiation of the weak input (Figure S4A-B). However, inducing a small PSCs Ca^{2+}-elevation with NP-EGTA (23.08%ΔF/F0) had no significant effect on synaptic activity of competing terminals (Figure S4C-D).

These results are consistent with a Ca^{2+}-dependent threshold mechanism required to trigger a long lasting synaptic potentiation since the main difference in PSCs Ca^{2+}-elevations induced endogenously by HFS of strong and weak competing inputs is the amplitude of the Ca^{2+} elevation (Figure 1), and that only large photoactivation-induced PSC Ca^{2+}-elevations triggered changes in synaptic activity (Figure S4). Altogether, these results indicate that PSCs differentially regulate the expression of synaptic plasticity of strong and weak terminals.

Synaptic Potentiation is Mediated by PSCs P2Y1Rs and Presynaptic A2ARs

We next investigated the mechanisms by which PSCs differentially regulate competing inputs. While PSCs Ca^{2+}-activity is mediated by cholinergic and purinergic receptors at mature NMJs (Robitaille, 1995; Rochon et al., 2001), it solely depends on type 2Y purinergic receptors (P2YRs) activated by ATP during synaptic competition (Darabid et al., 2013). Interestingly, purinergic type 2Y1 subtype receptors (P2Y1Rs) are preferentially located on PSCs during synaptic competition, clustered near presynaptic release sites (Darabid et al., 2013). Hence, we hypothesised that ATP-dependent activation of PSCs depends on P2Y1Rs. We tested the contribution of P2Y1Rs on PSCs by performing local applications of ATP (5 µM), which is also the endogenous P2Y1Rs agonist, in the presence of MRS2179 (20 µM), a specific P2Y1Rs antagonist. Interestingly, as shown in Figure 3A-B, no Ca^{2+}-elevations were elicited in the presence of MRS2179 (10.95 ±1.90%ΔF/F0, n=20 PSCs). This blockade was reversible since Ca^{2+} elevations were induced after a 20 min washout of the drug (300.20 ± 12.00%ΔF/F0, n=20 PSCs; significantly higher than Ca^{2+} raises in the presence of MRS2179; *Paired t-test, $p<0.0001$*). This confirms that ATP-dependent activation of PSCs is mediated by P2Y1Rs.

Since synaptic plasticity depends on PSCs Ca^{2+}-activity and that PSCs activation is mediated by P2Y1Rs, blocking those receptors should alter the endogenous plasticity of terminals. Indeed, bath application of MRS2179 (20 µM) prevented the long term potentiation of strong input following HFS (Figure 3C, E) such that the amplitude of EPPs evoked by the strong input was not different from baseline (Figure 3C; EPPs amplitude of 97.5 ±3.0% during the baseline vs 86.2 ± 5.4% at 16 min post-HFS; N=6, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p>0.05$*). In addition, although weak terminals showed no long-term increase in EPP amplitude in control experiments (Figure 1), they showed a decrease in activity at 16 min post-HFS in the presence of MRS2179 (Figure 3D, F; EPP amplitude of 101.8 ±0.8% during the baseline vs 109.0 ±2.9% at 4 min post-HFS vs 81.5 ±8.9% at 16 min post-HFS; N=6, amplitude significantly smaller at 16 min than 4 min or baseline, *One-way ANOVA, repeated measures, Tukey's multiple comparison post-test, $p<0.05$*). Hence, this shows that P2Y1Rs mediate the ATP-dependent activation of PSCs and

that the endogenous potentiation of synaptic activity of strong competing terminals depends on these receptors.

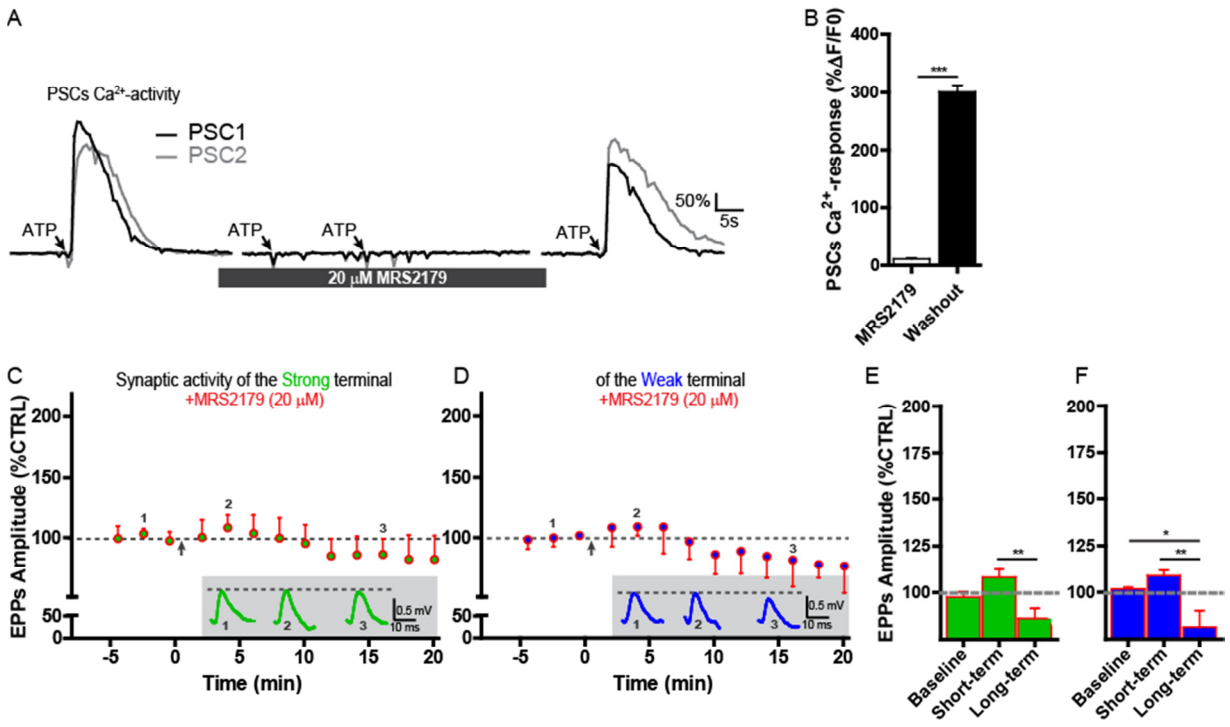

Figure 3. PSCs Ca2+-activity and synaptic plasticity of competing terminals depends on P2Y1Rs.

A. Examples of PSCs Ca^{2+}-responses to local application of ATP before (left), during (middle) and after the washout (right) of the P2Y1Rs antagonist (MRS2179 20 µM). Red and green traces represent 2 PSCs at a dually-innervated NMJ. **B.** Histograms showing the mean amplitude of the PSC Ca^{2+}-responses ±SEM induced by local application of ATP in the presence (white) or absence (black) of MRS2179. **C, D.** Normalized EPP amplitude ±SEM over time showing that stimulation (gray arrow at time 0) of strong (green in **C**) or weak (blue in **D**) inputs no longer resulted in changes of synaptic activity in the presence of MRS2179. Insets show examples of EPPs recorded before (1; Baseline), shortly after the HFS (2; at 4 min) and after the HFS (3; at 16 min). **E, F.** Histograms showing the mean amplitude of EPPs ±SEM induced by strong (green in **E**) and weak (blue in **F**) inputs during the baseline, shortly after the HFS (at 4 min) and 16 min after the HFS.

To better understand the mechanisms involved downstream of PSCs activation, we investigated which presynaptic receptors were responsible for the potentiation of neurotransmission. At mature NMJ, PSC-dependent potentiation of synaptic activity depends on the activation of presynaptic adenosine type 2A receptors (A2ARs; Todd et al., 2010). These receptors have been located on nerve terminals of adults and new born mice (Garcia et al., 2013; Tomas et al., 2014). However, there is evidence that A2ARs are also expressed by glial cells (Alloisio et al., 2004; Hettinger et al., 2001; Matos et al., 2015; Tomas et al., 2014). Unlike ATP, adenosine did not act directly on PSCs since local applications of adenosine (10 μM) failed to induce any Ca^{2+} elevation in PSCs at all tested NMJs (Figure 4A-B; adenosine: 6.5 ± 1.34%ΔF/F0 vs ATP: 101.20 ± 18.50%ΔF/F0; 8 PSCs, 5 NMJs, 5 muscles, *Paired t-test, p=0.0017*). This suggests that adenosine is not likely to directly activate adenosine receptors (including A2ARs) on PSCs.

We next tested the involvement of presynaptic A2ARs in the potentiation of neurotransmission at dually-innervated NMJs. As shown in Figure 4 (C, E), bath application of a specific antagonist of A2ARs, SCH58261 (100 nM), prevented the potentiation of the strong nerve terminal induced by HFS. Indeed, EPP amplitude following HFS of the strong input was not different from baseline (Figure 4C, E; EPPs amplitude of 100.6 ±4.85% during baseline vs 98.38 ±3.47% at 4 min post-HFS vs 90.83 ±14.39% at 16 min post-HFS; N=4, *One-way analysis of variance, Tukey's multiple comparison post-test, p>0.05*). Similarly, EPP amplitude following HFS of the weak input was not different from baseline during the bath application of SCH58261 (Figure 4D, F; EPP amplitude of 100.4 ±1.81% during baseline vs 109.5 ±7.59% at 4 min post-HFS vs 105.9 ±2.92% at 16 min post-HFS; N=4, *One-way analysis of variance, Tukey's multiple comparison post-test, p>0.05*). These data suggest that A2ARs activation is downstream to PSCs activation and is necessary for the endogenous potentiation of strong competing terminals. In addition, bath application of the specific A2ARs agonist CGS21680 (7 nM; Correia-de-Sa et al., 1996; Oliveira et al., 2004; Todd et al., 2010) was sufficient to induce a long lasting potentiation of neurotransmission from both competing terminals (Figure S5).

Altogether, these results suggest that the differential plasticity of competing nerve terminals depends on P2Y1Rs, responsible for the activation of PSCs, and on the activation of presynaptic A2ARs that potentiate neurotransmission.

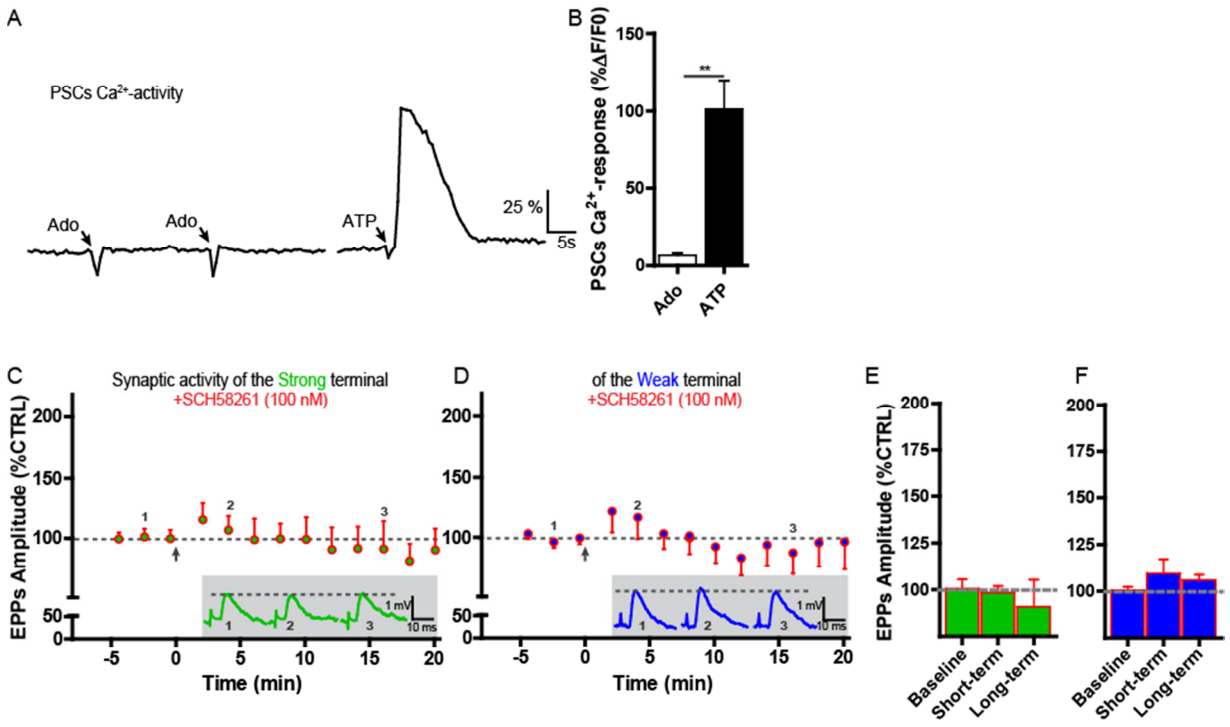

Figure 4. Synaptic plasticity of competing terminals depends on A2ARs.

A. Example of PSC Ca^{2+}-response to local application of adenosine (10 µM; left) and ATP (5 µM; right). Unlike ATP, adenosine failed to induce any Ca^{2+}-elevation in PSCs. **B.** Histograms showing the mean amplitude of the PSC Ca^{2+}-responses ±SEM induced by local application of adenosine (white) and ATP (black) of ATP. **C, D.** Normalized EPP amplitude ±SEM over time showing that stimulation (gray arrow at time 0) of strong (green in **C**) or weak (blue in **D**) inputs no longer resulted in changes of synaptic activity in the presence of the A2ARs antagonist SCH58261 (100 nM). Insets show examples of EPPs recorded before (1; Baseline), shortly after the HFS (2; at 4 min) and after the HFS (3; at 16 min). **E, F.** Histograms showing the mean amplitude of EPPs ±SEM induced by strong (green in **E**) and weak (blue in **F**) inputs during the baseline, shortly after the HFS (at 4 min) and 16 min after the HFS.

In Vivo Blockade of P2Y1Rs Delays Synapse Elimination

The results described so far propose a novel mechanism by which PSCs can differentially modulate synaptic activity of competing nerve terminals. However, it is unknown whether such PSC-dependent mechanism would influence the proper synaptic competition and elimination. Hence, we aimed to alter, *in vivo*, the activation of PSCs by blocking P2Y1Rs since they are required for PSCs activation and their differential potentiation of neurotransmission (Figure 2 and 3).

During postnatal development of the *Soleus* muscle, the proportion of poly-innervated NMJs decreases drastically from P0, where most NMJs are poly-innervated, to P14 where most NMJs are mono-innervated (Kopp et al., 2000; Personius and Balice-Gordon, 2001; Personius et al., 2007). To perturb PSCs activity and evaluate their influence on synapse elimination, we performed daily subcutaneous injections of a specific P2Y1Rs antagonist, MRS2179 (40 μM), in the hind limb area next to the mouse *Soleus* muscle from P4 to P14 (Figure 5A). Animals were then sacrificed at either P8, 10, 12 or 14. This time window was selected as it includes the period for which we described the mechanisms of differential plasticity of terminals (P7-8) and allows the analysis of synapse elimination progression. The state of innervation (as a correlate of the progression of synapse elimination) was determined by the number of mono- and poly-innervated NMJs at the *Soleus* muscle (at least 20 surface NMJs per muscle, and 7 to 8 muscles per age and condition were analyzed). This was achieved by counting the number of nerve terminals and axon profiles (labeled with antibodies against the presynaptic markers SV2 and NF-M) that contacted the endplate area of a single muscle fiber (identified by the labeling of postsynaptic nAChRs with α-bungarotoxin; Figure 5B-E).

In contralateral muscles injected with saline, the proportion of poly-innervated NMJs decreased drastically from P8, with more than 50 % of poly-innervated NMJs, to P14 where mono-innervation prevails (Figure 5B-D; 57.6 ±4.3% of poly-innervated NMJs at P8, 8 muscles, 8 mice; 26.3 ±4.9% at P10, 8 muscles, 8 mice; 11.5 ±2.2% at P12, 7 muscles, 7 mice; 1.8 ±0.9% at P14, 7 muscles, 7 mice). No differences were observed between non-injected and

saline-injected muscle (Data not shown). The proportion of poly-innervated NMJ at the different ages analyzed is in accordance with numbers reported by other groups at the same muscle and ages studied (Kopp et al., 2000; Personius and Balice-Gordon, 2001; Personius et al., 2007).

If PSCs activation via P2Y1Rs is important for proper synapse competition and elimination, blocking these receptors by injection of MRS2179 should alter the course of synapse elimination. In accordance with this hypothesis, *in vivo* blockade of P2Y1Rs altered poly-innervation at different ages (Figure 5B-E). First, no changes in the proportion of poly-innervated NMJs was observed at P8 or P10 (P8 MRS2179-injected: 60.7 ±2.7%, 8 muscles, 8 mice vs saline-injected: 57.6 ±4.3%, 8 muscles, 8 mice; *Unpaired t-test, p>0.05*; P10 MRS2179 injected: 38.6 ±5.4%, 8 muscles, 8 mice vs saline-injected: 26.3 ±4.9%, 8 muscles, 8 mice; *Unpaired t-test, p>0.05*). However, at P12, we observed a striking 3 fold increase in poly-innervated NMJs in mice injected with MRS2179 compared to saline-injected ones (MRS2179-injected: 31.0 ±3.6%, 7 muscles, 7 mice vs saline-injected: 11.5 ±2.2%, 7 muscles, 7 mice; *Mann Whitney t-test, p=0.0006*). This increase of poly-innervation was also observed at P14, an age when synapse elimination is usually mostly complete (Figure 5E; Kopp et al., 2000; Personius and Balice-Gordon, 2001; Personius et al., 2007). Precisely, 7.1 ±1.9% of NMJs/muscle were still poly-innervated at P14 MRS2179-injected mice (7 muscles, 7 mice) compared to 1.8 ±0.9 % in saline-injected ones (7 muscles, 7 mice; *Mann Whitney t-test, p=0.0365*). Thus, P2Y1Rs blockade appears to delay the process of synapse elimination. Indeed, the percentage of poly-innervated NMJs from MRS2179-injected mice at P12 is similar to the one at P10 from control animals, and the percentage of P14 MRS2179-injected is comparable to the one at P12 saline-injected mice.

Finally, we determined the presence of swollen axonal tips called “retraction bulbs”, which are indicative of nerve terminals recently retracted from the endplate area. These are signs of ongoing synapse elimination during postnatal refining of connectivity (Balice-Gordon and Lichtman, 1993; Bishop et al., 2004). In saline-injected animals, retraction bulbs were observed at P8 but not at P14 in saline-injected animals (Figure 6A) since P14 is considered the reference age for the end of synapse elimination in the *Soleus* muscle (Kopp et al., 2000;

Personius and Balice-Gordon, 2001; Personius et al., 2007). However, retraction bulbs should be observed at later stages if synapse elimination is delayed in MRS2179-injected mice. This was indeed the case as retraction bulbs were observed at all ages, including P14, in MRS2179-injected mice (Figure 6 B). Therefore, this strongly suggests that synapse elimination was not blocked by the treatment and was still ongoing, further confirming a delay in synapse elimination. Altogether, these data suggests that PSCs P2Y1Rs-dependent activation promotes the process of elimination and contribute to proper connectivity at the NMJ.

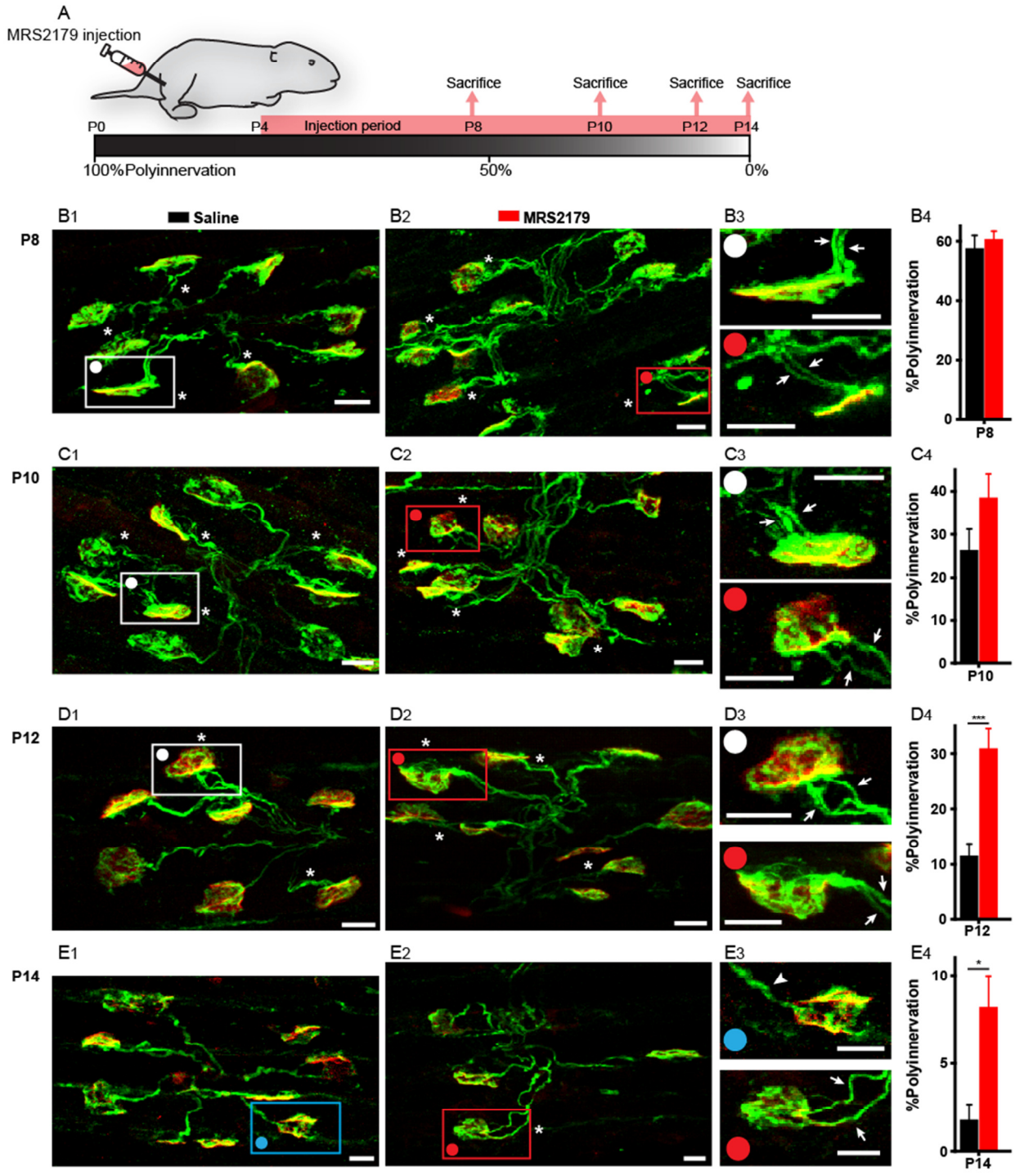

Figure 5. In vivo blockade of P2Y1Rs delays synapse elimination.

A. Schematic representation of subcutaneous MRS2179 injection. Daily subcutaneous injections of 40 µM MRS2179 were performed next to the *Soleus* muscle from P4 to P14.

Mice were scarified at P8, 10, 12 and 14. **B-E.** Confocal images presenting examples of saline- (first column; B1-E1) and MRS2179-injected *Soleus* (second column; B2-E2) labeled to observe presynaptic nerve terminals (stained with antibodies against NF-M and SV2; green) and postsynaptic endplates (nAChRs stained with α-Bungarotoxin; red). Note that more poly-innervated NMJs (asterisk) were present in MRS2179-injected mice at all ages. The state of poly-innervation was defined by the number of independent nerve terminals that innervate the same endplate. **B3-E3** (third column) shows a higher magnification of poly-innervated NMJs highlighted by a rectangle in B1-2 to E1-2. Independent inputs (green) innervating the same endplate area (red) are indicated by white arrows. White squares and dots represent the zoomed region from saline-injected mice while red square and dots represent the zoomed region from MRS2179-injected mice. Note that most P14 saline-injected mice (E1 and E3; blue square and dot) were mono-innervated by a single input marked by a white arrow head. **B4-E4.** Histograms showing the time course of synapse elimination at P8, 10, 12, 14 in saline- (black) compared to MRS2179-injected (red) mice. Note that MRS2179-injected mice had a delay in synapse elimination highlighted by the presence of more poly-innervated NMJs at P12 and P14. Scale bar: 10 μm.

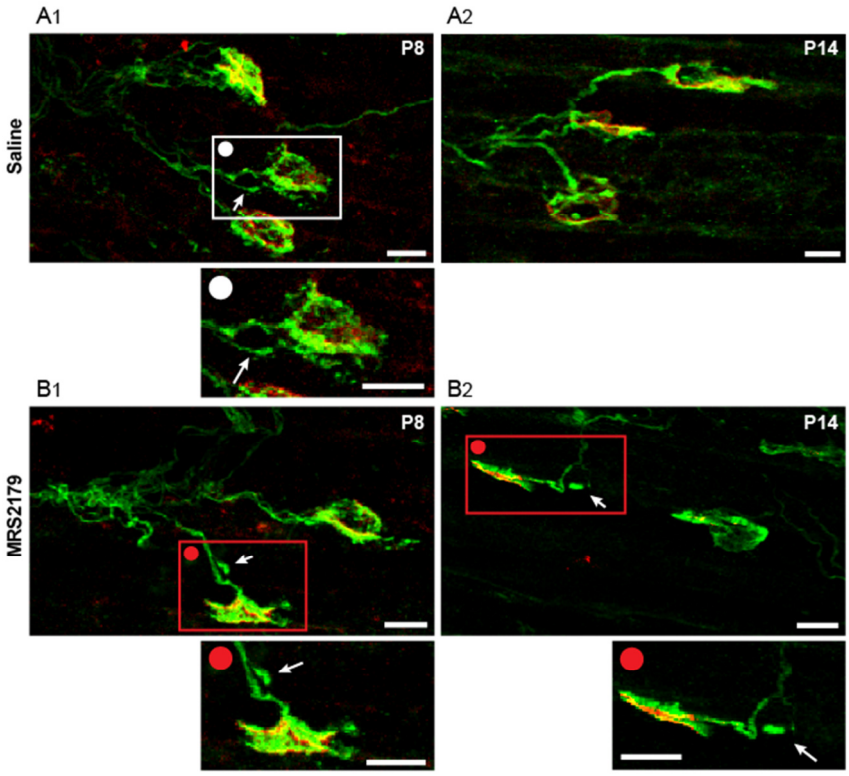

Figure 6. Morphological signs of ongoing synapse elimination at NMJs from P14 MRS2179-injected mice.

A. Confocal images of NMJs from P8 (**A1**) and P14 (**A2**) saline- and P8 (**B1**) and P14 (**B2**) MRS2179-injected *Soleus* muscles. Presynaptic nerve terminals were labeled with antibodies against NF-M and SV2 (green) and postsynaptic endplates were labeled with α-Bungarotoxin (red). Insets show higher magnification of NMJs highlighted by a rectangle in A1 and B1-2. White squares and dots represent the zoomed region from saline-injected mice while red square and dots represent the zoomed region from MRS2179-injected mice. The presence of retraction bulbs (enlargement of a disconnected nerve terminal in green) at a distance from the endplate area (red) are indicated by white arrows. Note that retraction bulbs (white arrows) were still observed in NMJs P14 MRS2179-injected mice (B2). Scale bar: 10 µm.

5.2.4. Discussion

In this study, we showed that glia at the NMJ differentially modulate the activity of competing terminals and influence synapse elimination. Perisynaptic glial cells decode the efficacy of competing terminals and preferentially potentiate the strong input while having little effect on the activity of the weak one. This differential plasticity depends on glial Ca^{2+}-activity and shapes synapse elimination. Preventing PSCs activation alters the plasticity of terminals *in situ* and results in delayed synapse elimination *in vivo*. Hence, PSCs actively reinforce the strong input which is favoured to win the competition (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000), and then influence synapse elimination. To our knowledge, this is amongst the first studies that link the activity of terminals and glial-mediated regulation of synapse competition and elimination.

Synaptic Plasticity of Competing Terminals

Here we describe a mechanism by which strong terminals show a persistent potentiation of neurotransmission in response to HFS. This potentiation suggests an increase in synaptic strength, thus an increase in disparity of synaptic efficacy between competing terminals. This implies a biased competition towards stronger inputs since it is known that disparity in synaptic strength is a predictor of the outcome of synapse elimination (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000). Importantly, the HFS pattern we used is similar to the endogenous activity recorded from newborn rodents during the tonic firing of motor units innervating the *Soleus* muscle, suggesting that such preferential potentiation of strong terminals may occur *in vivo* (Eken et al., 2008; Gorassini et al., 2000). The propensity of strong inputs to generate larger potentiation may be responsible, at least in part, for the reinforcement of stronger terminals and their maintenance, and is consistent with the large body of evidence indicating that more active and efficient nerve terminals are favoured to win the competition (Balice-Gordon and Lichtman, 1994; Buffelli et al., 2003; Busetto et al., 2000; Favero et al., 2012; Je et al., 2012; Ribchester and Taxt, 1983; Ridge and Betz, 1984; Schafer et al., 2012; Stellwagen and Shatz, 2002; Stevens et al., 2007). For instance, LTP in the CNS,

which represents a persistent increase in synaptic strength, has been associated with the enlargement and stabilization of synapses (Matsuzaki et al., 2004; Nagerl et al., 2004). Moreover, at the Drosophila NMJ, an acute increase of synaptic activity stimulates the formation of new synaptic buttons and the expansion of the synapse (Ataman et al., 2008; Fuentes-Medel et al., 2009). Thus, given the direct link between the activity, the plasticity and the structure of synapses, a differential plasticity of terminals may promote synapse growth and stabilization of strong but not weak inputs.

PSCs Govern Synaptic Plasticity of Competing Terminals

The differential potentiation of nerve terminals depends on the ability of PSCs to detect neurotransmission. Similar to our previous study (Darabid et al., 2013), we confirmed that this detection depends on the activation of ATP type 2Y1 purinergic receptors. Interestingly, these receptors are not present on the postsynaptic site, show little co-localization with presynaptic markers and are preferentially located on PSCs at the NMJ (Darabid et al., 2013). In the CNS, P2Y1Rs have been observed in the processes of astrocytes surrounding excitatory synapses of the molecular layer of the dentate gyrus (Jourdain et al., 2007). Such P2Y1Rs mediate astrocytic Ca^{2+}-elevations and allow astrocytes to control synaptic strength and certain forms of long term plasticity (Santello et al., 2011).

Activation of glial cells is important for the expression of many forms of synaptic plasticity (Henneberger et al., 2010; Navarrete and Araque, 2010; Navarrete et al., 2012; Santello et al., 2011; Serrano et al., 2006; Todd et al., 2010). Similarly, here we show that endogenous activation of PSCs leads to the potentiation of the strong competing input. However, even if the HFS of weak terminals induced Ca^{2+}-elevations in PSCs, there was little feedback on their synaptic activity and no persistent potentiation of transmission was observed (Figure 1 and 2). This suggests that Ca^{2+}-elevations in PSCs must reach a certain threshold to trigger the potentiation of terminals. Moreover, this shows that not all glial Ca^{2+} elevations lead to the same outcome on synaptic activity. This observation is consistent with a previous study at mature NMJs showing that different levels of synaptic activity induce distinct patterns of PSCs Ca^{2+} signalling, leading to distinct synaptic plasticity (Todd et al., 2010). Such

differential Ca^{2+}-activity of PSCs during development may play an important role in synapse competition since competing terminals show different levels of activity, implying that PSCs must provide an adapted feedback.

At mature NMJs, the type and level of activity define the result of adenosine-dependent plasticity. For example a continuous stimulation, similar to the one used in this study, results in the potentiation of neurotransmission through the activation of presynaptic A2ARs by adenosine (Oliveira et al., 2004; Todd et al., 2010). It is known that adenosine is a neuromodulator that is often generated from the degradation of ATP (Dunwiddie et al., 1997; Rebola et al., 2008). ATP can be released by glial cells at mature NMJs and in the CNS (Panatier et al., 2011; Pascual et al., 2005; Serrano et al., 2006; Todd et al., 2010). Thus, we propose a similar mechanism in which PSCs release purines (which would be prevented by the chelation of PSCs Ca^{2+}), producing adenosine that activates presynaptic A2ARs and cause synaptic potentiation.

Activity-dependent Synapse Elimination by PSCs

A simplified model of glial-mediated modulation of synaptic plasticity is presented in Figure S6. First, competing terminals release neurotransmitters, mainly ACh and ATP (Redman and Silinsky, 1994; Smith, 1991). Second, released transmitters are detected by glial cells trough P2Y1Rs. Third, detection of the strong and weak terminal results in large and small Ca^{2+}-elevations respectively. Fourth, glial cells release gliotransmitters (purines) that preferentially potentiate the strongest input through the activation of presynaptic A2ARs. Preventing PSCs from detecting neurotransmission by blocking P2Y1Rs or blocking Ca^{2+}-elevations in PSCs altered plasticity and influenced the outcome of synapse competition.

Most studies that describe a direct involvement of glial cells in synapse elimination genetically altered the phagocytic activity of these cells which resulted in a delayed elimination (Chung et al., 2013; Schafer et al., 2012; Stevens et al., 2007). Here we blocked Ca^{2+}-dependent glial activity, not directly targeting the elimination machinery, allowing us to reveal the requirement of the differential detection of neurotransmitter release by PSCs for

proper synapse elimination. To our knowledge, the percentage of increase in poly-innervation is among the highest reported in the literature where a delay in the activity-dependent synapse elimination was observed at a similar age (Favero et al., 2012; Personius et al., 2008). However, the blockade of PSCs P2Y1Rs resulted in a partial and transient delay of synapse elimination. This carries several important implications. First, we did not chronically or genetically block P2Y1Rs, but rather performed daily injection of MRS2179. As shown *in situ*, such antagonist is easily washable. Thus, P2Y1Rs might be blocked for only a short period of time during the day and the effect observed may underestimate the importance of these receptors. Second, other types of receptors could partially compensate for the lack of P2Y1Rs activation, as other receptors have been described to contribute to PSCs function at mature NMJs (Arbour et al., 2015; Rochon et al., 2001). Third, many other mechanisms have been described to influence synapse elimination. These mechanisms can be related to the intrinsic activity of nerve terminals (Favero et al., 2012; Personius and Balice-Gordon, 2001; Personius et al., 2007), the endogenous phagocytic activity of PSCs (Smith et al., 2013; Song et al., 2008), the direct contribution of the muscle fiber (Favero et al., 2009) as well as the availability of trophic factors such as BDNF and GDNF (Je et al., 2012; Je et al., 2013; Keller-Peck et al., 2001a; Nguyen, 1998). The presence of multiple mechanisms is not surprising and certainly highlights the importance of synapse elimination for survival. Hence, to ensure proper connectivity of the nervous system, many mechanisms act in parallel to coordinate synapse competition and elimination including the glial regulation of synaptic activity. As a perspective, it would be interesting to study the link between these different regulators of synapse elimination and to determine whether the glial machinery responsible for the detection and regulation of synaptic activity is linked to the ability of PSCs to protect or eliminate nerve terminals.

Impact on Synapse Pruning in the CNS

In the CNS, studies have also revealed the importance of glial cells, especially microglia and astrocytes, in the engulfment and elimination of synapses (Bialas and Stevens, 2013; Chung et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007; Zhan et al., 2014). Similar to the NMJ, synapse elimination in the CNS is driven by activity

such as strong inputs are more likely to be maintained (Schafer et al., 2012; Stellwagen and Shatz, 2002). For example, interfering with microglia or astrocyte pruning mechanisms resulted in delayed elimination and a higher number of weaker synapses (Chung et al., 2013; Schafer et al., 2012). In such context, an aberrant microglia-dependent elimination has been associated with social behavior defects linked to autism-like disorders (Zhan et al., 2014). It is tempting to propose that a similar mechanism to the one described in this study might be involved in synapse competition and elimination in the CNS, in particular, since astrocytes can detect and regulate activity at the single synapse level (Di Castro et al., 2011; Panatier et al., 2011).

Altogether, we show that glial cells differentially modulate the activity of competing terminals during synaptic competition at the NMJ and we present evidence that such regulation of synaptic activity is important for proper synapse elimination. Thus, we provide insights that may help understand the extended role of glial cells in synapse competition and the remodeling of connectivity in the nervous system.

5.2.5. Experimental procedures

Animals and nerve-muscle preparations:

All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Comité de déontologie animale of Université de Montréal. P7–P8 male CD-1 mice (Charles River) were anesthetized by a lethal intraperitoneal injection of ketamine (15 mg/ml)/xylazine (1 mg/ ml). The *Soleus* muscle and its innervation were dissected under oxygenated (95% O2, 5%CO2) Rees solution (in mM): 110 NaCl, 5 KCl, 1 $MgCl_2$, 25 $NaHCO_3$, 2 $CaCl_2$, 11 glucose, 0.3 glutamate, 0.4 glutamine, 5 BES, $4.34X10^{-7}$ cocarboxylase and 0.036 choline chloride. The *Soleus* muscle was dissected with its innervation up to the ventral roots and was pinned in a Sylgard-coated recording chamber. Two or three ventral roots segments were obtained and were independently stimulated by three suction-stimulation electrodes (square pulses; 0.1 mV to 2.0 V, 0.1 ms duration) using a Master-8 stimulator (AMPI). This allowed the independent stimulation of two inputs competing at the same NMJ with axons located in distinct stimulation electrodes as described previously (Darabid et al., 2013; Kopp et al., 2000).

All experiments were performed at 28°C-30°C and the temperature was constantly monitored and automatically adjusted using a TC-324B solution heater (Warner Instruments).

Intracellular recordings of synaptic transmission:

Synaptic events were recorded using an Axoclamp 2B amplifier (Axon Instruments) and further amplified (100X) and filtered at 2 kHz by a Warner Instruments amplifier. EPPs were digitized with DigiData 1322A (Axon Instruments). Data were collected and analyzed using pClamp 8.0 software (Axon Instruments).

Endplate potentials (EPPs) were evoked by nerve stimulation (square pulses of 0.2mV to 2.0V; 0.1 ms duration) using a Master-8 stimulator (AMPI) and recorded using sharp

intracellular electrodes (40-60 MΩ). Only dually-innervated NMJs were studied. They showed EPPs evoked by the independent stimulation of two of the three ventral roots. If an increase in EPP amplitude (EPP steps) occurred while increasing the stimulation intensity for a given suction electrode, the recording was discarded as this would suggest that multiple axons innervating the same NMJs were present within the same stimulation electrode (Buffelli et al., 2002; Busetto et al., 2000; Redfern, 1970).

For each NMJ, the synaptic strength of each competing terminal was determined by calculating the quantal content (m = Log_e(#nerve impulses/#failures)) (Del Castillo and Katz, 1954) and the paired pulse facilitation (Darabid et al., 2013; Kopp et al., 2000). Two stimuli were given at 10 ms interval to determine the paired pulse facilitation and was calculated as (F) = Amplitude of 2nd/ 1st EPP (including failures). All recordings of synaptic strength were obtained using a modified Ringer's solution with low Ca^{2+} (1 mM)/ high Mg^{2+} (6–7 mM) which also blocks muscle contraction. The ventral roots were stimulated at a frequency of 0.2 Hz with an intensity that was twice the threshold for eliciting EPPs. After recording synaptic activity of each input using low Ca^{2+}/ high Mg^{2+} solution to determine the synaptic strength, Rees solution with normal Ca^{2+}/Mg^{2+} concentration was perfused and used for the rest of the experiment. In this condition, muscle contractions were prevented by blocking postsynaptic nicotinic acetylcholine receptors (nAChRs) with D-tubocurarine chloride (2.0-3.5 μM, Sigma).

Nerve-muscle preparations were perfused with normal Ca^{2+}/Mg^{2+} Rees solution for 20 min and a baseline of synaptic activity was recorded for one of the two inputs by stimulating the corresponding ventral root at a frequency of 0.2 Hz for at least 10 min of stable EPPs amplitude. Then, the ventral root was stimulated at a high frequency (HFS; 50 Hz, 30 s). Following the HFS, EPPs were recorded at a frequency of 0.2 Hz for 20 min to monitor the changes in synaptic transmission in the post-tetanic period. Baseline, HFS and post-tetanic activity were then recorded for the second competing input at the same NMJ by the stimulation the second ventral root. EPP amplitude is expressed as % of amplitude change compared to baseline (EPP amplitude in mV/ mean baseline EPP amplitude in mV) X100. Each point represents the mean amplitude of 24 EPPs (2 min). All analyses, including the

calculations of synaptic strength, were performed offline once the experiment was completed. Therefore, the sequence for the stimulation of competing inputs (i.e. strong input first then the weak or vice-versa) was blind at the time of the experiment and unbiased.

In some experiments, the purinergic type 2Y1 receptors (P2Y1Rs) antagonist MRS2179 (20 μM; Abcam) or adenosine type 2A receptors (A2ARs) antagonist SCH58261 (100 nM; Sigma) was added to the extracellular solution.

Single cell electroporation of PSCs:

A glass pipette (10-12 MΩ) was filled with 500 μM of the fluorescent indicator Alexa Fluor 594 (Invitrogen) and 800 μM of the Ca^{2+}-indicator Fluo-4 Pentapotassium Salt (Invitrogen) diluted in the extracellular Rees solution. In specific sets of experiments, either 2 mM Diazo2 (Invitrogen) or 2 mM NP-EGTA (Invitrogen) was added. The pipette was mounted on a pipette holder with a platinum stimulating wire connected to a Master-8 stimulator (AMPI). The reference electrode was placed in the bath. The pipette was approached to the soma of a PSC using a micromanipulator under visual guidance. The tip of the pipette was positioned close to the PSC soma but did not touch the cell or any tissue. One to three single negative square pulses (15 V, 10 ms) were applied to open the cell membrane (Darabid et al., 2013; Nevian and Helmchen, 2007). The procedure was repeated to load other visible PSCs at the same NMJ. PSCs were easily identified with transmitted light microscopy and we previously confirmed that the electroporated cells are S100B-postive PSCs (Darabid et al., 2013). The preparation was allowed to rest for at least 20 min before further manipulations. Synaptic activity was constantly monitored by intracellular recordings from the muscle fiber to ensure no change in EPPs amplitude and thus no undesired effect of the single cell electroporation technique.

PSCs Ca^{2+}-imaging:

PSCs activity was measured using Ca^{2+}-imaging during the HFS of each competing nerve terminal and monitored with an Olympus FV1000 microscope and a 60X water-

immersion lens (0.90 NA; Olympus). The 488 and 594 nm laser lines were used for the excitation of Fluo-4 and Alexa Fluor 594 respectively. The emitted fluorescence was detected using the multispectral detection feature (bandpass filter 500-545 nm to detect the green Ca^{2+} indicator and 570-670 for the red dye). PSCs at the NMJ area were imaged at a rate of 2.1 Hz and Ca^{2+} changes were analyzed over each PSC soma and expressed as:

$$(Fluorescence - Fluorescence_{Rest} / Fluorescence_{Rest}) X100$$

In some experiments, Ca^{2+} responses in PSCs were assessed following ATP (5 or 10 μM, Sigma) or adenosine (10 μM, Sigma) local applications from a glass pipette (5-8 MΩ) positioned at proximity of cells using positive pressure pulses (15 PSI, 150 ms) with a Picospritzer II (Parker Instruments). Locally applied substances were diluted in the same extracellular solution.

Photoactivation of Diazo2 and NP-EGTA:

A 405 nm laser was used to photoactivate Diazo-2 (multiple pulses: 7% power, 1s ON/1s OFF for 20s; Figure S) or NP-EGTA (single pulse: 7% power, 500 ms). The photoactivation region was positioned to cover all PSCs somata and processes. The Olympus FV1000 SIM Lightpath function was used to allow fast and efficient photoactivation. Photobleaching was monitored and compared to the red Alexa Fluor 594 channel. Possible photodamage was constantly controlled by monitoring synaptic activity, cell morphology as well as the general aspect of PSCs and muscle fibers.

Immunohistochemistry of NMJs:

Immunohistochemical labeling was performed as described in (Darabid et al., 2013). In brief, freshly dissected *Soleus* muscles were pinned in a Sylgard-coated dish and fixed for 10 min in 4% formaldehyde at room temperature and then permeabilized in 100% cold methanol for 6 min at -20°C. Nonspecific labeling was minimized by incubation in 10% normal donkey serum (NDS) and 0.01% Triton X-100 solution for 20 min. Preparations were incubated overnight at 4°C with a rabbit anti-S100B (1:250, Dako, with 0.01% Triton X-100 and 2%

NDS). Muscles were then incubated in chicken anti-NF-M (1:1000; Rockland Immunochemicals) and mouse IgG1 anti-SV2 (1:1500; Developmental Studies Hybridoma Bank) antibodies for 90 min. Preparations were incubated with Alexa Fluor 488 donkey anti-chicken, Alexa Fluor 647 donkey anti-rabbit and goat anti-mouse IgG1 DL488 secondary antibodies (all at 1:500) for 60 min at room temperature. Finally, muscles were incubated with α-bungarotoxin (Alexa Fluor 594, 0.75 µg/ml) for 45 min. After each step, muscles were washed in PBS containing 0.01% Triton X-100 (3 times, 5 min). The preparations were then mounted in the Prolong Gold antifade reagent containing DAPI (Invitrogen) and all labels were observed in two phases and using the spectral detection feature of an Olympus FV1000 confocal microscope. An approximate airy disk value of 1 was obtained by adjusting the Pinhole. Images were not manipulated after the acquisition.

In vivo injections:

Daily subcutaneous injections of MRS2179 (40 µM in 0.9% NaCl; 5-20 µl) were carefully performed next to the *Soleus* muscle from P4 to P13. The contralateral leg was injected with normal 0.9% NaCl saline solution. No differences were observed between non-injected and saline-injected muscles (data not shown). As expected by a subcutaneous injection, no signs of muscle damage were observed (data not shown).

Mice were sacrificed at P8, P10, P12 or P14. The state of polyinnervation was evaluated by counting the number of independent inputs (labeled by anti-neurofilament-M (NF-M) and anti-synaptic vesicular protein 2 (SV2) antibodies) that contacted a single NMJ endplate (nAChR area labeled by α-bungarotoxin). Each NMJ was then classified as mono- or poly-innervated. At least 20 surface NMJs were analyzed per muscle.

Statistical analysis:

Results are presented as mean ±SEM, and N represents the number of PSCs in Ca^{2+}-imaging experiments or the number of recorded NMJs in electrophysiological experiments. Unless stated otherwise, only one PSC/NMJ was kept for further analyses and only one

dually-innervated NMJ was recorded per muscle. Paired t tests were performed when comparing PSCs Ca^{2+}-responses induced by the two competing terminals during the same experiment. When data were found not to conform to normality, Mann–Whitney U tests were used. Unpaired t tests were performed to compare two different conditions from different experiments. One-way ANOVA, repeated measures, with Tukey's multiple comparison tests were used to compare three groups or more. Analyses were deemed significant at $p<0.05$.

5.2.6. References

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5.2.7. Supplemental data

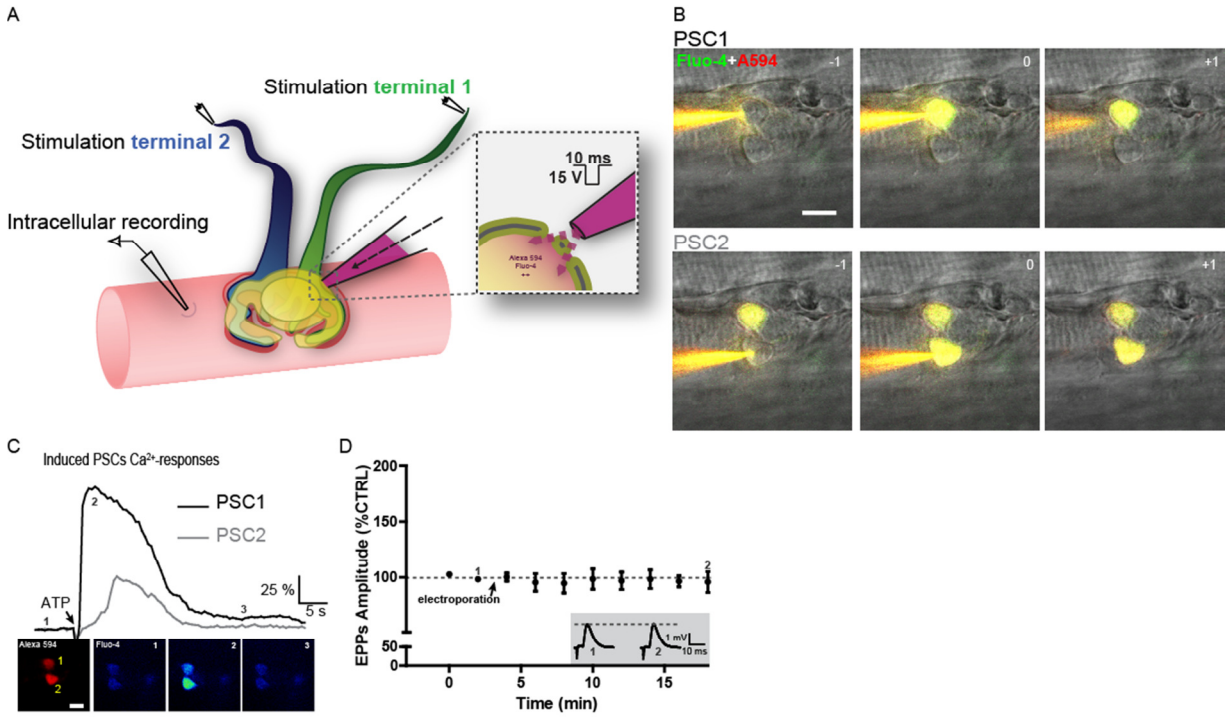

Figure S1. Single cell electroporation of PSCs.

A. Schematic drawing of a PSC electroporated at a dually-innervated NMJ. A high resistance pipette containing molecules to be electroporated is placed next to the soma of a PSC. A high voltage pulse (15V; 10 ms duration) passed through the electroporation pipette allows to transiently open the membrane and transport charged molecules from the pipette to the target PSC (inset). **B.** Example of sequential electroporation of 2 PSCs at the same NMJ. Each PSC (top PSC1 and bottom PSC2) is electroporated with Alexa Fluor 594 and Fluo-4. False color images show PSCs before (-1), during (0) and after (+1) the high voltage pulse. Using bright-field illumination, PSCs are easily visible cells as they show oval shaped soma slightly elevated over a given NMJ (Darabid et al., 2013, Rochon et al., 2001) **C.** Ca^{2+} activity of electroporated PSCs shown in B in response to local application of ATP (5 µM). A false color confocal image of the electroporated PSC (Alexa Fluor 594 in red; Scale bar 10 µm) is presented as well as changes in fluorescence of electroporated Fluo-4 illustrating Ca^{2+} levels before (1), at the peak of the response (2), and after ATP application (3). **D.** Normalized EPP

amplitude ±SEM over time showing that single cell electroporation of PSCs did not affect neurotransmission. The insets show examples of EPPs recorded before (1) and after the electroporation (2).

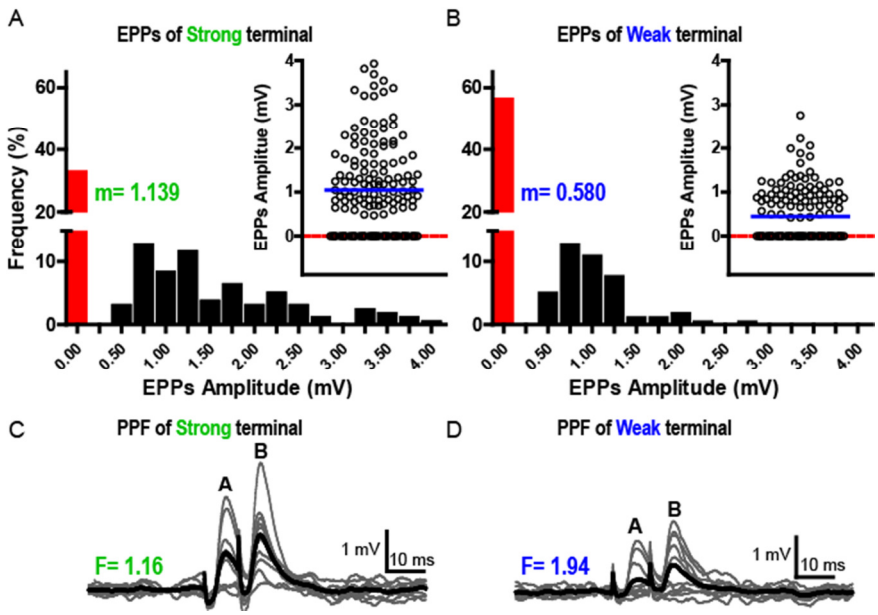

Figure S2. Assessment of synaptic efficacy of each terminal in competition at a dually-innervated NMJ.

A, B. Histograms showing the distribution of EPP amplitude induced by stimulation of the strong (**A**) and weak terminal (**B**), including failures (red). Inset: distribution of EPP amplitude from each terminal. Each circle represents a single synaptic event. Red bar regroups events that failed to induce an EPP and m represents the calculated quantal content for each competing terminal. **C, D.** Average (dark line) of 10 superimposed EPPs (gray) induced by the stimulation of the strong (**C**) and weak (**D**) input. Facilitation values (F) were obtained from the paired-pulse (10 ms interval) independent stimulation of strong and weak inputs. Note that the strong input had a higher quantal content (m) and lower paired pulse facilitation (F).

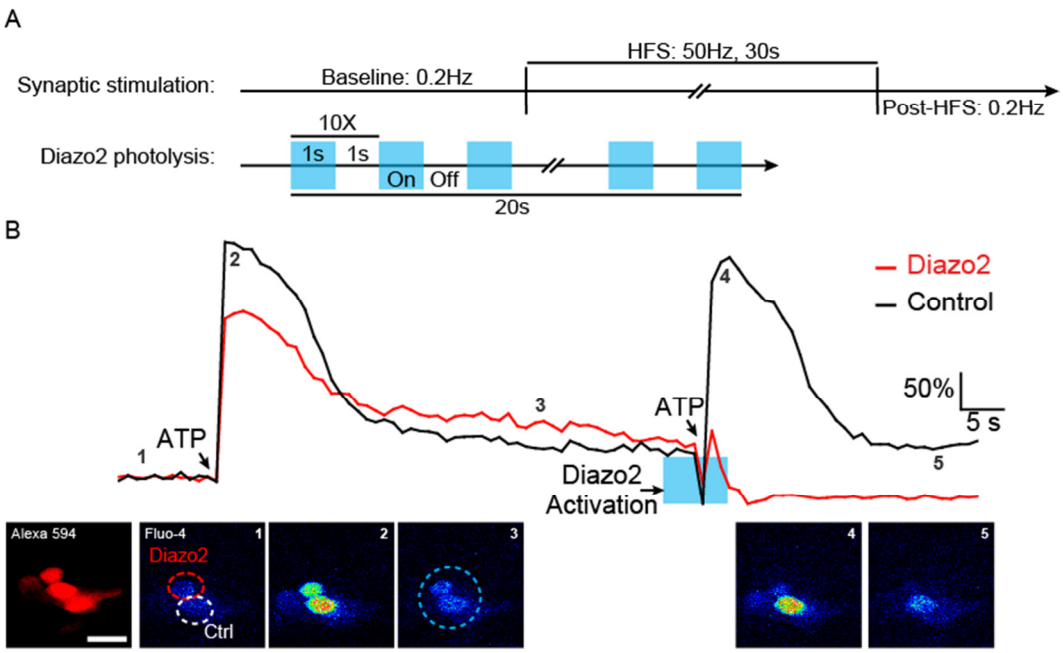

Figure S3. Blockade of PSCs Ca^{2+}-responses with photoactivation of Diazo2.

A. Synaptic stimulation protocol is presented at the top. It shows a period of baseline recording of EPPs at 0.2 Hz, continuous high frequency stimulation (HFS; 50 Hz) and the post-HFS period (at 0.2 Hz). Diazo2 photolysis protocol is presented at the bottom. The photoactivation of Diazo2 consisted of 1 second 405 nm laser activation (On; Blue square) followed by 1 second rest (Off) repeated 10 times. The photoactivation sequence was started before and overlaps with the HFS. **B.** Ca^{2+} activity of electroporated PSCs in response to local application of ATP (5 µM). A false color confocal image of the electroporated PSC (Alexa Fluor 594 in red; Scale bar 10 µm) as well as images of changes in fluorescence of electroporated Fluo-4 illustrating Ca^{2+} levels before, at the peak of the response, and after ATP application. The top PSC (outlined in red) was electroporated with Diazo2 while the PSC in bottom (outlined in white) was electroporated without Diazo2. Note that both cells show Ca^{2+}-elevations in response to ATP before photoactivation of Diazo2. Following 405 nm laser activation of both cells (blue dotted circle delineate the photoactivation area), the PSC electroporated with Diazo2 no longer shows Ca^{2+}-elevations.

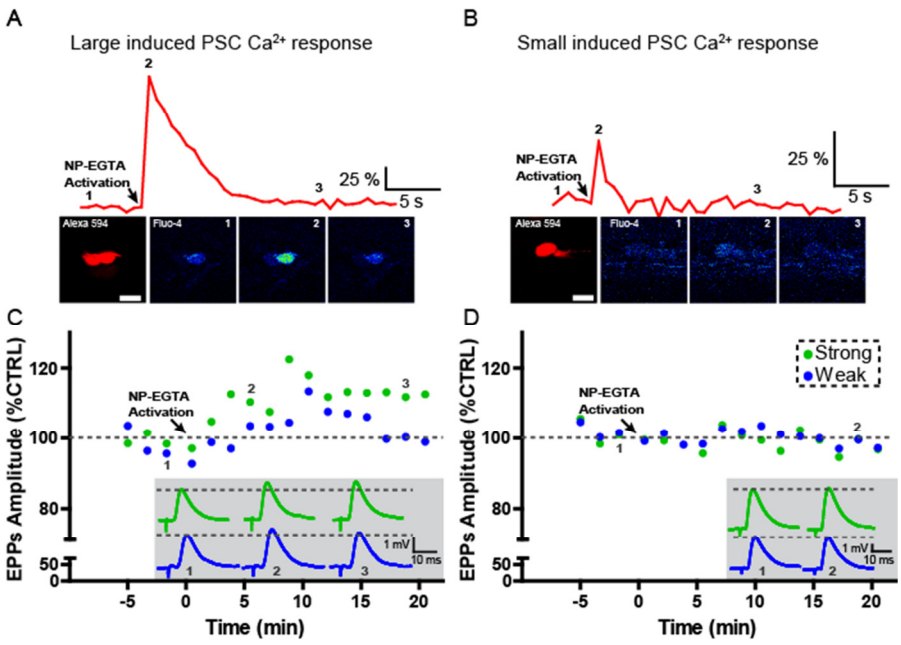

Figure S4. Direct induction of a large, but not small, PSC Ca2+-elevation induces synaptic plasticity.

A, B. Example of a large (red in **A**) or a small (red in **B**) induced PSC Ca^{2+}-response induced by the photoactivation of caged Ca^{2+} (NP-EGTA; photoactivated by a single 405 nm laser pulse of 500 ms duration) electroporated specifically in PSCs. A false color confocal image of the electroporated PSC (Alexa Fluor 594 in red; Scale bar 10 µm) as well as images of changes in fluorescence of electroporated Fluo-4 illustrating Ca^{2+} levels before, at the peak of the response, and after photoactivation of NP-EGTA are presented. **C, D.** Normalized EPP amplitude over time showing that a large (**C**), but not a small (**D**) PSC Ca^{2+}-response, induced by photoactivation of NP-EGTA, resulted in the plasticity of strong (green) and weak (blue) inputs. Note that the strong input in C shows a long lasting potentiation whereas the weak one displays only a transient potentiation. Insets show examples of EPPs recorded before (1; Baseline), shortly after the HFS (2; at 4 min) and 16 min after the HFS.

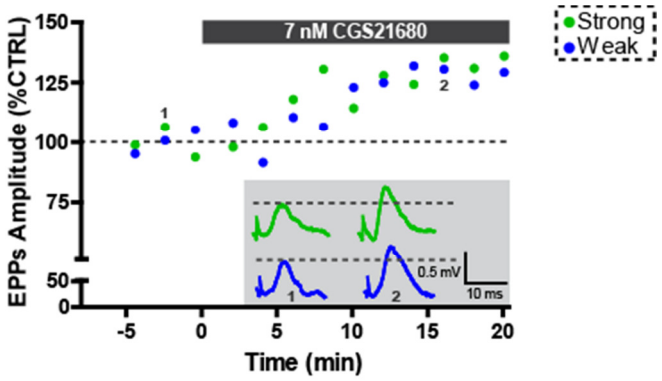

Figure S5. Exogenous activation of A2ARs is sufficient to induce a potentiation of neurotransmission from competing terminals.

Normalized EPP amplitude recorded over time from a dually-innervated NMJ showing the effect of bath application of the specific A2ARs agonist CGS21680 (7 nM; Gray bar). Note that both strong (green) and weak (blue) competing terminals show a long lasting potentiation of neurotransmission following CGS21680 application. Insets show examples of EPPs recorded before (1) and after CGS21680 bath application (2).

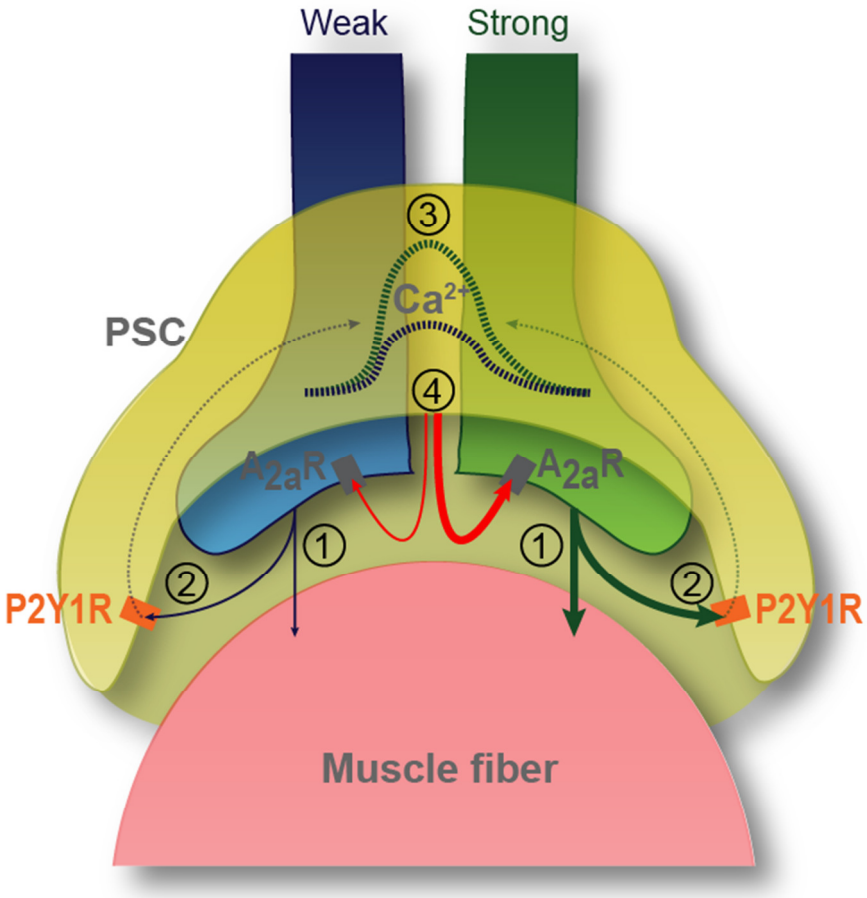

Figure S6. Simplified model of glial-mediated modulation of synaptic plasticity during synaptic competition.

1. Competing terminals release neurotransmitters, mainly ACh and ATP. ACh activates postsynaptic muscle fiber. **2.** Released ATP is detected by the PSC (yellow) through P2Y1Rs (orange). **3.** Detection of the strong (green) and weak (blue) terminal results in large and small Ca^{2+}-responses respectively (green and blue dotted traces in PSC). **4.** Glial cells release gliotransmitters (purines) that preferentially potentiate the strongest input through the activation of presynaptic A2ARs (gray).

6. Discussion générale

Dans cette thèse, j'ai abordé deux idées principales en ce qui concerne l'implication des cellules gliales dans la compétition et l'élimination synaptique. Plus précisément, j'ai montré que : 1) les cellules gliales décodent avec précision l'efficacité synaptique des terminaisons en compétition, et 2) les cellules gliales régulent différemment l'activité et la plasticité synaptique de ces terminaisons ce qui influence l'issue de l'élimination et la connectivité synaptique. Ainsi, nous décrivons un nouveau mécanisme par lequel les cellules gliales renforcent non seulement l'activité de la terminaison forte, mais régulent la compétition et l'élimination synaptique. À ma connaissance, ceci est la première étude qui montre un lien direct entre l'activité des terminaisons, la régulation par les cellules gliales et la connectivité synaptique lors du développement du système nerveux.

Ces résultats peuvent avoir plusieurs impacts à la fois dans le domaine des interactions neurone-glie et de la plasticité synaptique, mais aussi celui de la régulation de la connectivité par les cellules gliales. En parallèle, cette étude relève plusieurs aspects qui méritent d'être discutés. Dès lors, je discuterai du décodage des propriétés synaptiques par les CSPs et les mécanismes impliqués, de la modulation différentielle de l'activité des terminaisons et de la gliotransmission ainsi que de l'implication fonctionnelle et structurale d'une telle plasticité différentielle. De plus, je discuterai de la régulation *in vivo* de l'élimination synaptique et la connectivité par les CSPs. Pour cette partie, je proposerai un modèle mis à jour de la compétition et de l'élimination synaptique qui met en perspective l'implication des résultats de cette thèse avec les données de la littérature. Finalement, je montrerai comment ces nouvelles données pourraient aider à mieux comprendre l'élimination synaptique dans le SNC ainsi que les implications pour le système nerveux mature en conditions physiologiques et pathologiques.

6.1. Décodage des propriétés synaptiques par les CSPs

Dans la première partie de cette thèse, nous avons montré qu'une seule CSP détecte les neurotransmetteurs libérés par chacune des terminaisons en compétition à une même JNM. Les élévations Ca^{2+} induites dans une CSP par l'activité des terminaisons sont proportionnelles à l'efficacité relative de chaque terminaison. Les différences d'amplitudes des réponses Ca^{2+} ne peuvent pas être expliquées uniquement par la quantité de neurotransmetteurs libérés. En effet, même si nous avons montré qu'une terminaison forte libère plus de neurotransmetteurs que sa compétitrice faible, les propriétés gliales jouent aussi un rôle important dans le décodage de l'efficacité. Parmi ces propriétés, nous avons décrit la distribution spatiofonctionnelle des récepteurs P2YRs. Il semble qu'une CSP organise ses récepteurs en différents groupes fonctionnels qui font face à chacune des terminaisons. Un tel arrangement pourrait faciliter une détection fine et efficace de la relâche de neurotransmetteurs et permettrait de distinguer les terminaisons en compétition (Figure 6.1). De cette façon, les CSPs peuvent intégrer les propriétés synaptiques en évaluant constamment l'efficacité des terminaisons.

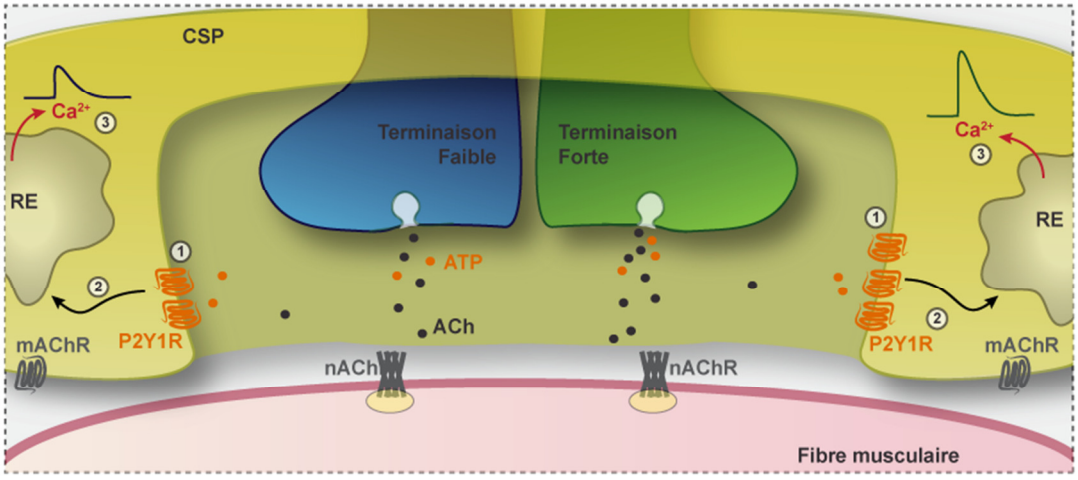

Figure 6.1. Décodage de la compétition synaptique par les CSPs.

Il semble y avoir une distribution spatio-fonctionnelle des récepteurs gliaux P2Y1, dédiés à la détection de l'activité de chaque terminaison, présents à proximité des sites de libérations de neurotransmetteurs **(1)**. Ces récepteurs induisent une augmentation de la concentration

intracellulaire de Ca^{2+} dans les CSPs **(2)**. Cette distribution pourrait faciliter le décodage de l'efficacité des terminaisons en compétition par les CSPs **(3)** qui ne dépend pas uniquement de la quantité de neurotransmetteurs libérés. De cette façon, les CSPs pourraient intégrer les propriétés synaptiques en évaluant constamment l'efficacité des terminaisons.

Perspectives :

1. Est-ce qu'il y aurait une plus grande densité de P2Y1Rs proche d'une terminaison forte qu'une terminaison faible proportionnellement à la quantité de neurotransmetteurs libérés? Quels sont les mécanismes qui permettent une telle ségrégation de récepteurs?
2. Il serait important d'étudier les conséquences de l'activation des récepteurs P2Y1Rs en plus du décodage de l'activité. Quelques aspects sont décrits dans la Figure 6.2.
3. Quelles sont les sources du Ca^{2+}? Étant donné la nature des P2Y1Rs comme récepteurs couplés aux protéines G_q, il est fort probable que cette augmentation dépende de l'IP3 et de la libération du Ca^{2+} du RE, réticulum endoplasmique (Robitaille, 1995; Waldo and Harden, 2004).

6.1.1. Signalisation purinergique vs cholinergique

Dans les deux études présentées dans cette thèse, nous avons montré que la détection de la neurotransmission ainsi que le décodage de l'efficacité synaptique des terminaisons en compétition par les CSPs dépend de l'activation des récepteurs purinergiques P2Y1Rs. Ces récepteurs ne sont pas présents sur la fibre postsynaptique, montrent très peu de colocalisation avec les marqueurs présynaptiques et sont préférentiellement situés sur les CSPs à la JNM (Darabid et al., 2013).

De manière intéressante, le type de récepteurs gliaux activés par l'activité synaptique endogène diffère entre la JNM adulte et immature. En effet, à la JNM mature, les CSPs détectent la neurotransmission par une combinaison de récepteurs purinergiques et muscariniques (Arbour et al., 2015; Rochon et al., 2001). Lors de la compétition synaptique, nous avons montré que cette détection dépend uniquement des récepteurs purinergiques de la famille P2Y bien que les récepteurs muscariniques soient présents et fonctionnels. Tel que discuté dans la première étude, l'absence d'une contribution muscarinique dans la détection de l'activité endogène pourrait s'expliquer par la distribution diffuse des récepteurs muscariniques qui, contrairement aux récepteurs P2Y1Rs, ne sont pas concentrés près des zones actives, sites préférentiels de la libération de neurotransmetteurs. Dès lors, la prédominance de la signalisation purinergique lors du développement et l'apparition tardive d'une contribution muscarinique sous-tendrait un changement des propriétés et de la fonction des CSPs lors de la maturation.

L'une des hypothèses attrayantes à propos de l'importance de la balance purinergique/ muscarinique concerne la régulation de l'expression de protéines telles que la GFAP. En effet, la GFAP est un filament intermédiaire du cytosquelette présent dans certaines cellules gliales du système nerveux, incluant les astrocytes et les CSPs, et son expression est régulée à la baisse dans les CSPs suite à l'activation des récepteurs mAChRs (Georgiou et al., 1999). La GFAP est nécessaire pour l'élaboration de nouveaux prolongements des CSPs qui sont formés lors du remodelage synaptique suite à une dénervation (Georgiou et al., 1999). Donc, la

diminution ou l'absence d'une signalisation muscarinique des CSPs durant la compétition synaptique devrait faciliter la formation de prolongements des CSPs et le remodelage de la JNM. À la JNM mature, le blocage de différents sous types de mAChRs, incluant ceux associés aux CSPs, induit un remodelage de la synapse similaire à celui observé au cours du développement (Wright et al., 2009). Dans cette perspective, il est possible que les récepteurs muscariniques favorisent la stabilisation des CSPs alors que le système purinergique facilite les changements morphologiques. Ceci serait cohérent avec les processus de compétition et d'élimination synaptique où il y a un remodelage constant de la synapse. De plus, ceci est également en accord avec une étude montrant que les CSPs aux JNMs immatures sont très dynamiques, alors que très peu de motilité est observée aux JNMs matures (Brill et al., 2011).

Dans le même ordre d'idées, la prédominance d'une signalisation purinergique peut faciliter le remodelage synaptique tout en favorisant la maturation des CSPs puisqu'il est connu que les récepteurs P2YRs promeuvent la survie et la prolifération des cellules gliales ainsi que la formation de prolongements (Abe and Saito, 1999; Verkhratsky et al., 2009). Ainsi, une faible contribution muscarinique et une forte signalisation purinergique seraient permissifs pour le remodelage et la croissance synaptique. Si tel est vraiment le cas, les CSPs devraient changer la balance muscarinique-purinergique lors de conditions qui nécessitent un remodelage de la JNM. Une récente étude de notre laboratoire montre que, lors de la reformation synaptique suite à des lésions nerveuses, les CSPs se reconvertissent vers un état immature (Perez-Gonzalez et al., 2016). En effet, une augmentation de la contribution purinergique est observée en parallèle avec une diminution de la contribution muscarinique. Ceci est en accord avec les besoins de la JNM en contexte de reformation synaptique, où les CSPs participent à éliminer les débris, guider les terminaisons nerveuses, étendre la synapse et remodeler la JNM (Kang and Lichtman, 2013; Kang et al., 2014; Kang et al., 2003). D'ailleurs, l'importance de l'état de maturité des cellules gliales pour créer un environnement permissif au remodelage des connexions synaptique a été proposée il y a déjà plusieurs années. En effet, une étude en 1989 montre que la transplantation d'astrocytes immatures, isolés du cortex visuel de jeune chattons, dans le cortex de chats adultes, bien après la période de compétition et d'élimination synaptique, permet d'induire de nouveau la plasticité des colonnes de dominance oculaire (Muller and Best, 1989).

De manière générale, l'état de maturité des cellules gliales, incluant la balance de la signalisation purinergique et muscarinique des CSPs à la JNM, est important pour créer un environnement propice à la maturation synaptique (Figure 6.2). De plus, cette balance purinergique-muscarinique ne régulerait pas uniquement les CSPs, mais serait importante pour l'ensemble de la JNM puisque les purines régulent différents aspects de la synaptogenèse incluant l'expression de l'acetylcholinesterase et des AChRs sur la fibre musculaire (Choi et al., 2001; Choi et al., 2003; Tung et al., 2004).

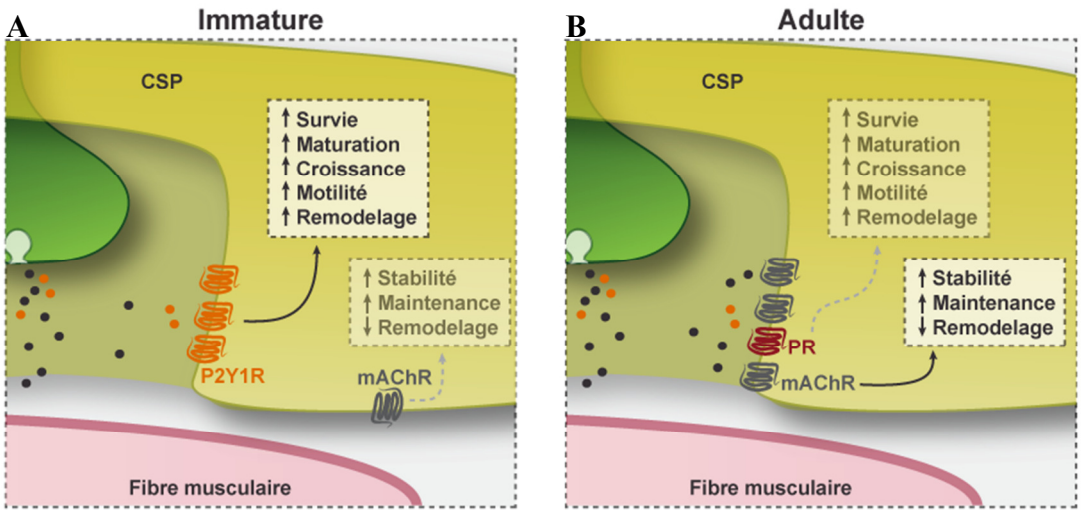

Figure 6.2. Balance entre la signalisation purinergique et cholinergique des CSPs.

A. Il semblerait que la prédominance d'une signalisation gliale purinergique à la JNM immature favorise un environnement propice au remodelage synaptique en favorisant la survie, la maturation, la croissance ainsi que la motilité et la formation de prolongements des cellules gliales. **B.** À la JNM adulte, l'augmentation de la contribution cholinergique gliale et la diminution de la contribution purinergique (PR : récepteurs purinergiques) préviendraient le remodelage synaptique et favoriseraient la stabilité et la maintenance de la JNM.

Perspectives :

1. Il serait important d'étudier le rôle de la signalisation cholinergique aux JNM immatures puisque des récepteurs mAChRs sont présents et fonctionnels, mais ne sont pas activés par la libération endogène de neurotransmetteurs.
2. Dans le même ordre d'idées, quel serait le rôle de la signalisation gliale purinergique aux synapses matures? Tel que discuté dans l'introduction à la section 2.4.1.2.1., la présence d'une diversité de récepteurs gliaux pourrait permettre à la cellule gliale de mieux détecter et décoder les propriétés synaptiques selon un contexte précis.

6.1.2. Décodage du niveau d'activité par la CSPs

L'une des observations fondamentales présentées dans cette thèse est que les CSPs sont capables de décoder l'efficacité et le niveau d'activité des terminaisons en compétition. L'activité des CSPs pourrait se limiter uniquement à différencier la terminaison forte de la terminaison faible. Si tel est le cas, il serait possible d'imaginer un scénario dans lequel toutes les terminaisons fortes induisent des réponses Ca^{2+} gliales d'une amplitude donnée alors que les terminaisons faibles induiraient des réponses Ca^{2+} d'une plus petite amplitude. Un tel scénario impliquerait que l'activité Ca^{2+} des CSPs serait de type binaire "tout ou rien" qui sert simplement à distinguer une terminaison forte d'une autre faible. Cependant, cette hypothèse ne tient pas compte de la différence de la force synaptique entre les terminaisons. En effet, l'activité des terminaisons en compétition peut varier et la disparité des terminaisons peut constamment changer (Colman et al., 1997; Kopp et al., 2000). Le mécanisme que nous proposons n'est pas de type binaire "tout ou rien", mais reflète la disparité entre l'activité des terminaisons en montrant une gradation des réponses Ca^{2+} des CSPs équivalente aux différences de l'efficacité synaptique. Puisque cette disparité est un déterminant majeur de l'élimination synaptique (Balice-Gordon and Lichtman, 1994; Stellwagen and Shatz, 2002), les résultats que nous présentons sont en accord avec ce processus. La relation entre l'activité Ca^{2+} des CSPs et la disparité des terminaisons est étroite de façon à ce que de très petites différences de force synaptique entre les terminaisons puissent être perçues par les CSPs. Encore une fois, la distribution spatiale et la densité des récepteurs P2Y1Rs à proximité des sites de libération pourraient faciliter la capacité des CSPs à percevoir ces petites différences d'efficacité synaptique entre les terminaisons.

Nos données suggèrent que toutes les élévations Ca^{2+} des CSPs ne mènent pas forcément à un même impact sur l'activité synaptique, mais peuvent véhiculer une information spécifique sur les propriétés des terminaisons. À la JNM adulte, il est connu que différents niveaux d'activité synaptique (reflétés par différents patrons de stimulation : continue vs phasique) induisent des signalisations Ca^{2+} distinctes des CSPs (patron continu : grande amplitude Ca^{2+} de courte durée vs patron phasique : faible amplitude calcique oscillatoire de

longue durée) et mènent à des plasticités synaptiques opposées (potentialisation vs dépression synaptique persistante; Todd et al., 2010). Cependant, les mécanismes impliqués dans le décodage des patrons d'activité neuronale par les CSPs à la JNM adulte pourraient être différents du décodage de l'efficacité synaptique des terminaisons en compétition à la JNM immature. En effet, à la JNM mature, le décodage de différents patrons d'activité par les CSPs serait facilité par une activation temporelle précise des CSPs. Par exemple, une décharge tonique de la terminaison nerveuse activerait la CSP de manière continue alors qu'une décharge phasique activerait les CSPs de manière transitoire. Dans cette situation, l'activation temporelle des CSPs permettrait de différencier les patrons d'activité neuronale et de fournir un feedback approprié à chaque condition. À la JNM immature, la situation serait différente puisque les patrons d'activité des deux terminaisons en compétition seraient peu différents et, dans les deux cas, l'activité nerveuse serait de type tonique (Eken et al., 2008). Ainsi, une CSP doit différencier l'activité des terminaisons basée uniquement sur leur efficacité synaptique. Dans cette situation, le décodage dépendrait de la quantité de neurotransmetteurs libérés par chaque terminaison, mais serait aussi facilité par les propriétés gliales intrinsèques telles que la présence de récepteurs dédiés à chaque terminaison. Ainsi, nous montrons un autre niveau de sophistication du décodage glial de l'activité synaptique basé sur l'efficacité synaptique. Par conséquent, les CSPs pourraient utiliser des stratégies différentes et adaptées au contexte synaptique pour décoder les propriétés synaptiques.

Nos données renforcent l'idée que l'activité Ca^{2+} des cellules gliales est un code qui reflète les propriétés synaptiques et entraine une modulation de la transmission ajustée à l'état actuel de la synapse (Araque et al., 2014). Des études précédentes montrent aussi l'importance du décodage par les astrocytes dans des conditions particulières en montrant une activité calcique unique à différentes connexions synaptiques (Perea and Araque, 2005). La présente étude montre un autre niveau de sophistication du décodage gliale où des différences subtiles de niveau d'activité sont perçues par une même cellule gliale. Cette activation différentielle des CSPs peut jouer un rôle important durant la compétition synaptique puisque la CSP possède la machinerie nécessaire pour décoder l'activité des terminaisons et doit donc fournir un rétrocontrôle approprié.

6.2. Régulation de la plasticité synaptique par les CSPs

6.2.1. Modulation différentielle de l'activité des terminaisons

Bien que l'activité synaptique ait souvent été décrite comme un déterminant de l'issue de la compétition synaptique, très peu d'études documentent l'activité et surtout la plasticité des terminaisons en compétition. Nous montrons que non seulement les terminaisons en compétition expriment différents niveaux d'activité et de plasticité synaptique, mais que cette plasticité différentielle dépend des cellules gliales. En effet, les CSPs potentialisent l'activité de la terminaison forte alors qu'elles n'ont que peu d'effet sur l'activité de la terminaison faible (Figure 6.3). La potentialisation de la terminaison forte reflète une amélioration de sa force synaptique. Puisque l'issue de la compétition est grandement dépendante de la disparité entre l'efficacité synaptique des terminaisons en compétition, l'augmentation de la force synaptique de la terminaison forte créerait davantage de disparité et biaiserait la compétition vers le maintien de la terminaison forte (Balice-Gordon and Lichtman, 1994; Stellwagen and Shatz, 2002). Naturellement, la terminaison avec la meilleure efficacité devrait être favorisée, car elle serait mieux adaptée aux besoins du système et serait plus apte à assurer une communication synaptique robuste et en phase avec sa cible (Buffelli et al., 2003; Favero et al., 2012; Favero et al., 2013; Wyatt and Balice-Gordon, 2003).

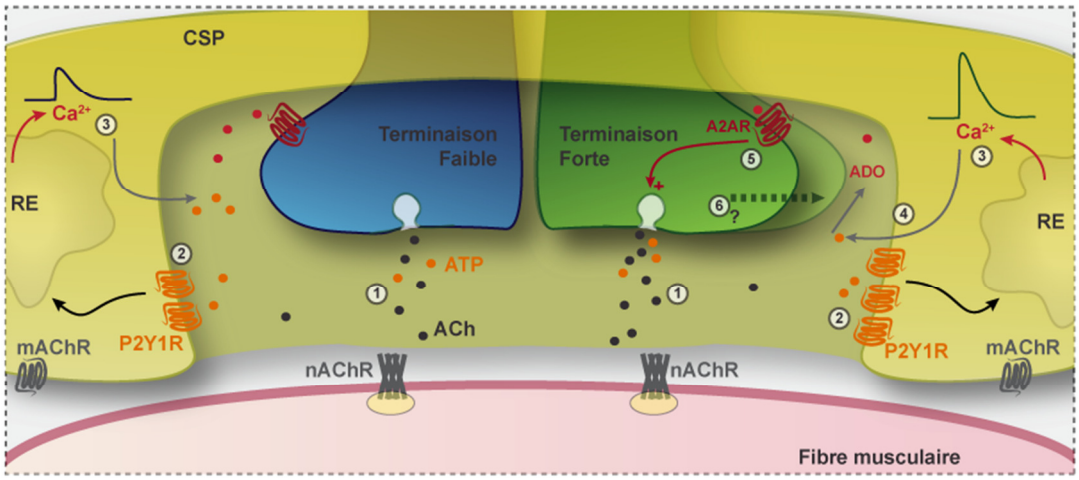

Figure 6.3. Modèle de la potentialisation préférentielle de la terminaison forte par les CSPs.

1. Les terminaisons en compétition libèrent des neurotransmetteurs.
2. La CSP détecte l'activité des terminaisons par l'entremise de l'activation des P2Y1Rs.
3. Les CSP décodent l'efficacité synaptique des terminaisons en compétition de façon à ce que la terminaison forte induise une plus grande réponse Ca^{2+} gliale que la terminaison faible
4. En retour, la CSP libérerait des gliotransmetteurs qui potentialisent préférentiellement l'activité synaptique de la terminaison forte. Les CSPs libèreraient de l'ATP qui est convertie en adénosine (Todd et al., 2010). Les CSPs n'ont que très peu d'effets sur la terminaison faible.
5. La potentialisation de la terminaison forte dépend de l'activation des récepteurs présynaptiques A2ARs.
6. La potentialisation de la terminaison forte pourrait favoriser son maintien et son renforcement.

Perspectives :

1. Il serait important d'étudier les mécanismes de libération des gliotransmetteurs. Alors que la gliotransmission est plus étudiée dans les SNC, les modes de libération de gliotransmetteurs à la JNM sont très peu connus. Par exemple, est-ce qu'il s'agit d'une

libération vésiculaire dépendante du complexe SNARE ou d'une libération par canaux de type connexines/pannexines comme proposé dans le SNC (Perea et al., 2009)?

2. Les CSPs libèrent de l'ATP qui est rapidement convertie en adénosine par des enzymes ectonucleosidases à la JNM mature. Est-ce que ces enzymes et leur concentration sont similaires lors de la compétition synaptique?
3. Quel est l'effet de l'activation des A2ARs présynaptiques? À la JNM mature, l'activation des récepteurs A2A entrainerait le recrutement des canaux Ca^{2+} de type L ce qui augmenterait l'entrée de Ca^{2+} dans la terminaison présynaptique induisant ainsi une plus grande relâche de neurotransmetteurs (Oliveira et al., 2004). L'activation de ces récepteurs pourrait aussi promouvoir la survie neuronale (Wiese et al., 2007).
4. Est-ce qu'une amélioration de l'activité synaptique entraîne un renforcement et une expansion de la terminaison forte? Une expérience où il serait possible de potentialiser l'activité d'une terminaison et d'observer le territoire occupé permettrait de mieux comprendre le lien entre l'activité et l'organisation synaptique lors de la compétition.

6.2.2. Implication de la gliotransmission

Plusieurs données montrent que les cellules gliales régulent la plasticité synaptique par la relâche de gliotransmetteurs. L'une des familles de gliotransmetteurs les mieux étudiée est celle des purines. En effet, l'ATP et l'adénosine d'origine gliale sont connues pour potentialiser l'activité de synapses uniques (Panatier et al., 2011), participer à la plasticité hétérosynaptique (Serrano et al., 2006), et réguler la potentialisation à long terme (Pascual et al., 2005). De plus, les purines sont connues pour réguler la potentialisation ainsi que la dépression persistante de la neurotransmission à la JNM mature (Todd et al., 2010). La modulation purinergique de la neurotransmission par les cellules gliales est souvent attribuée à l'activation des récepteurs neuronaux à l'adénosine A1 qui réduisent la relâche de neurotransmetteurs ou à l'activation des récepteurs A2A qui augmentent la neurotransmission (Panatier et al., 2011; Pascual et al., 2005; Serrano et al., 2006; Todd et al., 2010).

Dans le contexte de la présente thèse, l'activation des CSPs par la terminaison forte entraîne la relâche de gliotransmetteurs de manière dépendante du Ca^{2+}, ce qui potentialise la neurotransmission. La potentialisation de la terminaison forte dépend de l'activation des A2ARs. Tel que décrit dans l'introduction, l'activation de ces récepteurs solliciterait le recrutement des canaux calciques de type L (Oliveira et al., 2004). Ceci induirait une plus grande entrée de Ca^{2+} dans la terminaison présynaptique et une relâche plus importante de neurotransmetteurs.

6.2.2.1. Récepteurs A2ARs vs A1Rs

Il est connu que l'adénosine peut augmenter ou réduire la neurotransmission par l'activation respective des A2ARs ou A1Rs présynaptiques (Oliveira et al., 2004; Silinsky, 2004; Todd et al., 2010). La potentialisation de la terminaison forte dépend uniquement des A2ARs alors que la terminaison faible montre très peu de changements. Un autre scénario possible est qu'il y ait une potentialisation de la terminaison forte, dépendante des A2ARs,

mais aussi une dépression de la terminaison faible de manière dépendante des A1Rs. Un tel scénario permettrait d'augmenter davantage la disparité entre l'activité des terminaisons. Cette situation est peu probable puisqu'aucune dépression de la terminaison faible n'a été observée dans les conditions contrôles. De plus, ceci ne serait pas cohérent avec l'activité endogène de la JNM immature qui serait plus propice à une potentialisation qu'à une dépression. En effet, durant le développement postnatal de la JNM, une activité soutenue à haute fréquence est prédominante dans le muscle SOL et serait associée au maintien de la posture (Eken et al., 2008). À la JNM, une stimulation soutenue, similaire à celle utilisée dans cette étude, entraîne une potentialisation dépendante des A2ARs (Oliveira et al., 2004; Todd et al., 2010). En parallèle, une activité de type phasique entraîne une dépression synaptique dépendante des A1Rs (Correia-de-Sa et al., 1996; Todd et al., 2010). Cette activité phasique se développe de manière endogène à des stades plus avancés de la maturation du SOL et serait associée à la fonction locomotrice de ce muscle (Eken et al., 2008; Gorassini et al., 2000; Hennig and Lomo, 1985). Ces données sont importantes dans le contexte de la présente étude puisqu'elles suggèrent que, durant la compétition synaptique, la JNM est plus propice à une potentialisation de la neurotransmission en raison de l'activité soutenue prédominante et donc l'activation préférentielle des A2ARs. Si tel est le cas, l'expression des récepteurs A2ARs et A1Rs devrait être régulée lors du développement. En effet, les A2ARs sont plus abondants durant le développement alors que les A1Rs sont plus abondants à la JNM mature (Garcia et al., 2013). Ceci est cohérent avec des études physiologiques qui montrent que les purines potentialisent la neurotransmission durant le développement de la JNM de têtards (Fu and Poo, 1991) alors qu'un effet opposé est observé à la JNM mature de grenouilles (Giniatullin and Sokolova, 1998).

En plus de la potentialisation de l'activité de la terminaison forte par les CSPs, l'activation des A2ARs pourrait avoir un effet direct sur la survie neuronale. En effet, il est connu que l'activation des A2ARs peut trans-activer la signalisation des récepteurs tyrosine kinase « Tropomyosin receptor kinase B (TrkB) » et promouvoir la survie des motoneurones (Wiese et al., 2007). Dès lors, si un mécanisme similaire est présent durant la compétition synaptique, il permettrait la protection et la survie de la terminaison forte.

6.2.2.2. Relâche d'ATP par les CSPs et origine de l'adénosine

Aux JNMs matures, la potentialisation de l'activité qui dépend de l'adénosine et des CSPs est le résultat de la dégradation de l'ATP libérée par les CSPs (Todd et al., 2010). Cette dégradation est possible par l'action d'enzyme ectonucleotidases, principalement l'ecto-ATPase et l'ecto-5′-nucleotidase, qui génèrent l'adénosine à partir de l'ATP (Dunwiddie et al., 1997; Rebola et al., 2008). Dès lors, nous proposons qu'un mécanisme similaire soit impliqué lors de la compétition synaptique dans lequel l'activation des CSPs entraîne la relâche de purines qui potentialisent la neurotransmission par l'activation des récepteurs presynaptique A2ARs (Figure 6.4). Il aurait été intéressant de tester directement la libération de l'ATP par les cellules gliales ainsi que l'accumulation d'adénosine en utilisant des biosenseurs électrochimiques capables de détecter l'ATP et les produits de sa conversion enzymatique (Halassa et al., 2009; Schmitt et al., 2012). De plus, il est possible de bloquer la dégradation de l'ATP en inhibant l'activité des ectonucleotidases pour confirmer les mécanismes impliqués et l'importance de l'adénosine dans la potentialisation synaptique.

En général, il est connu que l'ATP est un gliotransmetteur libéré par les cellules gliales de manière dépendante du Ca^{2+} (Halassa et al., 2009; Panatier et al., 2011; Pascual et al., 2005; Serrano et al., 2006; Todd et al., 2010). Par conséquent, le blocage de l'activité Ca^{2+} des CSPs empêcherait la libération de l'ATP et l'activation des récepteurs présynaptiques A2ARs par l'adénosine. Les mécanismes impliqués dans la relâche d'ATP par les CSPs demeurent inconnus. Cependant, dans le SNC, il semblerait que la relâche d'ATP par les astrocytes serait dépendante du complexe SNARE (Panatier et al., 2011; Pascual et al., 2005) . En effet, interférer avec le complexe SNARE, impliqué dans l'exocytose vésiculaire, semble diminuer la libération d'ATP (Panatier et al., 2011; Pascual et al., 2005)

6.2.2.3. L'ATP : gliotransmetteur et neurotransmetteur

L'ATP pourrait aussi influencer directement l'activité synaptique et l'issue de la compétition puisque plusieurs rôles lui ont été attribués. Sans être dégradée, l'ATP peut affecter la neurotransmission à la JNM (Giniatullin et al., 2005; Giniatullin and Sokolova, 1998; Grishin et al., 2005; Moores et al., 2005). De plus, l'ATP régule l'expression de plusieurs gènes ainsi que la survie cellulaire, ce qui peut avoir un impact important sur la synaptogenèse (Choi et al., 2001; Choi et al., 2003; Tung et al., 2004; Verkhratsky et al., 2009). Tel que mentionné plus haut, l'ATP favorise la survie et la prolifération des cellules gliales ainsi que la formation de prolongements par l'activation de récepteurs P2Y (Abe and Saito, 1999; Verkhratsky et al., 2009). L'ATP peut aussi agir au niveau postsynaptique pour faciliter l'action de l'ACh en régulant la sensibilité des récepteurs cholinergiques (Ribeiro, 1977), leur expression ainsi que l'activité des cholinestérases (Choi et al., 2003). D'ailleurs, durant le développement précoce de la JNM, l'ATP peut agir sur les récepteurs purinergiques postsynaptiques de type 2X, qui seraient importants pour la prolifération et/ou la différentiation du muscle squelettique (Urano et al., 1997). De plus, l'ATP régule l'expression de facteurs de transcription impliqués dans la croissance de neurones dans le cortex ainsi que dans la survie neuronale (Arthur et al., 2006; McKee et al., 2006). Dès lors, les différents rôles de l'ATP, autres que ceux décrits dans cette thèse, pourraient s'additionner pour expliquer les résultats obtenus, mais toujours dans le même but qui consiste à former une synapse mature et efficace.

La régulation différentielle de l'activité des terminaisons par la relâche d'ATP est délicate puisque l'ATP est à la fois un neurotransmetteur libéré par les terminaisons et un gliotransmetteurs relâché par les CSPs. L'ATP est un co-transmetteur libéré avec l'ACh par les terminaisons nerveuses (Redman and Silinsky, 1994; Smith, 1991). Il est aussi possible que la libération présynaptique d'ATP, et sa dégradation en adénosine, puisse contribuer à la plasticité différentielle des terminaisons en compétitions.

Cependant, les résultats obtenus suggèrent fortement que la régulation de l'activité dans ce contexte serait d'origine gliale. En effet, l'activité calcique des CSPs est à la fois nécessaire et suffisante pour induire la potentialisation différentielle des terminaisons en compétition à une même JNM. Cela suggère que des mécanismes sophistiqués seraient en place pour limiter l'action de l'ATP des différentes sources vers des cibles spécifiques. À notre connaissance, de tels mécanismes ne sont pas encore connus, mais l'organisation synaptique de la JNM ainsi que la distribution des récepteurs purinergiques pourraient participer à la régulation de son effet.

Pourquoi la dégradation de l'ATP d'origine neuronale n'activerait-elle pas les récepteurs A2ARs présynaptique? Une possibilité est que les A2ARs ne soient pas localisés près de la fente synaptique. Ceci rendrait leur activation difficile par des purines d'origine présynaptique libérées dans fente synaptique. En effet, quelques études montrent la présence des A2ARs dans des sites extra-synaptiques (Fuxe et al., 2012; Fuxe et al., 1998). Puisque les CSPs enveloppent les terminaisons (Tapia et al., 2012; Zuo and Bishop, 2008), la libération de purines par les CSPs pourrait plus facilement activer ces récepteurs extra-synaptiques. Un modèle qui englobe ces hypothèses et qui pourrait expliquer nos résultats est présenté à la Figure 6.4.

Le type d'activité synaptique jouerait un rôle dans la sélection de la cible et l'activation de différents types de récepteurs. Selon le contexte, l'activité synaptique, la concentration de purines produite et l'affinité des récepteurs purinergiques, l'activation d'un type de récepteur particulier serait privilégiée (Correia-de-Sa et al., 1996; Sebastiao and Ribeiro, 2000; Todd et al., 2010). Tout cela suggère qu'une signalisation purinergique complexe est impliquée lors de la compétition et l'élimination synaptique ce qui suggère une organisation spécifique de la synapse dans ce contexte.

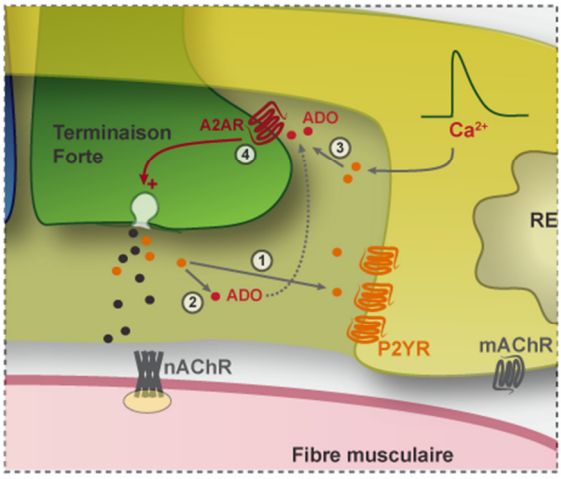

Figure 6.4. Origine et rôle des purines dans la potentialisation de la terminaison forte.

Cette figure montre une hypothèse qui explique pourquoi les purines d'origine gliale, et non neuronale, serait responsable de la potentialisation de la terminaison forte. **1.** L'ATP libérée par les terminaisons activerait facilement les récepteurs P2Y1Rs gliaux vu leur proximité des sites de relâche. **2.** Cette ATP pourrait être convertie en adénosine. Cependant, si les A2ARs présynaptiques ne sont pas à proximité de la fente et se trouveraient plutôt à des sites extra-synaptiques, l'adénosine d'origine présynaptique aurait moins de chance d'activer ces récepteurs. **3.** L'adénosine convertie de l'ATP libérée par les CSPs à des sites extra-synaptiques aurait plus de chance d'activer les A2ARs présynaptiques.

Perspectives :

1. Y aurait-il une distribution spécifique des récepteurs A2ARs dans les sites synaptiques et extra-synaptiques?
2. Est-ce que les CSPs ciblent la libération de purines vers des sites particuliers (synaptiques vs extra-synaptiques)?

La distribution spatiale des récepteurs gliaux P2Y1Rs et leur proximité des sites de relâche présynaptiques pourraient assurer une activation rapide des CSPs par l'ATP libérée de la terminaison présynaptique (Darabid et al., 2013). De plus, les récepteurs P2Y sont connus pour avoir une très grande affinité pour l'ATP ce qui suggère que de très petites concentrations libérées par les terminaisons seraient suffisantes pour activer les CSPs (Araque et al., 2014; Waldo and Harden, 2004). De manière générale, l'action de l'ATP est très localisée puisque, une fois libérée dans le milieu extracellulaire, elle ne peut parcourir que de courtes distances sous cette forme avant d'être dégradée (Dunwiddie et al., 1997; Zimmermann, 1999). Dès lors, l'ATP libérée par les terminaisons présynaptiques activerait rapidement les récepteurs P2Y1 gliaux avant d'être dégradée en adénosine. De la même manière, l'ATP d'origine gliale peut avoir un effet paracrine en activant ses propres P2Y1Rs. Finalement, les purines d'origine gliale activeraient les A2ARs à des sites extra-synaptiques ce qui expliquerait la potentialisation persistante de la neurotransmission par la terminaison forte.

6.2.3. Les terminaisons faibles sont-elles programmées à être éliminées?

L'absence de potentialisation de l'activité synaptique de la terminaison faible est une observation importante qui reste difficile à expliquer, mais qui peut aider à mieux comprendre le rôle des cellules gliales dans la compétition synaptique. Tel que discuté dans le deuxième article de recherche inclus dans cette thèse, l'induction d'une petite réponse calcique dans la CSP par la terminaison faible ne serait peut-être pas suffisante pour induire la libération de gliotransmetteurs, ce suggère qu'un seuil d'élévation Ca^{2+} dans les CSPs est nécessaire. Cette possibilité est appuyée par les expériences d'activation directe des CSPs par la photolyse de NP-EGTA. En effet, l'induction d'une grande, mais pas une petite, réponse calcique des CSPs est suffisante pour induire une potentialisation de la terminaison forte. Cependant, cette même grande activation calcique des CSPs ne potentialise pas l'activité de la terminaison faible de manière persistante. Cela suggère que la terminaison faible n'est pas dotée d'un mécanisme endogène capable d'induire une potentialisation robuste. Par exemple, il est possible que la

CSPs dirige les gliotransmetteurs libérées vers la terminaison forte. Puisqu'une terminaison forte occuperait un plus grand territoire (Walsh and Lichtman, 2003), elle serait davantage en interaction avec la CSPs que la terminaison faible et recevrait un support plus important qu'une terminaison faible occupant un plus faible territoire (Bishop et al., 2004; Brill et al., 2011). En parallèle, il est aussi possible qu'une terminaison faible exprime moins de récepteurs A2ARs et que malgré la disponibilité de l'adénosine, son activité ne soit que très peu modifiée. Un résumé des hypothèses qui peuvent expliquer l'absence d'une potentialisation persistante de la terminaison faible est présenté à la Figure 2.5.

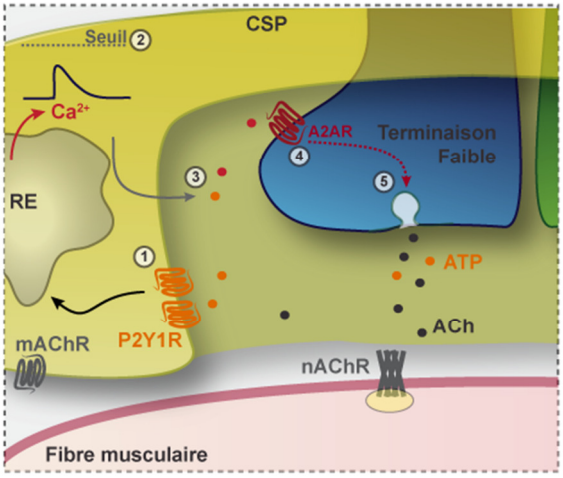

Figure 6.5. Hypothèses et perspectives sur l'absence de potentialisation persistante des terminaisons faibles.

Cette figure montre quelques hypothèses qui pourraient expliquer l'absence d'une potentialisation persistante des terminaisons faibles :

1. Même si les CSPs détectent les neurotransmetteurs libérés par la terminaison faible, il est plausible que la CSP n'exprime pas une assez haute densité de P2Y1Rs dédiés pour la terminaison faible. Il est aussi possible que la terminaison faible ne libère pas assez de neurotransmetteurs, mais ceci est peu probable puisque nous avons montré qu'augmenter, de manière transitoire, la quantité de

neurotransmetteurs libérés par la terminaison forte n'augmente pas les réponses Ca^{2+} gliales induites.

2. En lien avec l'activation de la CSP, il est probable que la réponse Ca^{2+} induite par la terminaison faible n'atteigne pas le seuil nécessaire pour la libération de gliotransmetteurs.
3. Il est aussi possible que la CSP ne libère pas assez de gliotransmetteurs (ex : ATP) ou ne les dirigent pas vers la terminaison faible.
4. Même si la CSP libère assez de gliotransmetteurs, il est envisageable que la terminaison faible n'exprime pas assez de A2ARs.
5. Dans la même lignée, une autre possibilité est que la terminaison faible ne possède pas la machinerie nécessaire pour potentialiser la neurotransmission de manière persistante.

.....

Dans ce contexte, est-ce qu'une terminaison faible est forcément destinée à être éliminée? Il est connu que l'activité relative et les territoires synaptiques de chaque terminaison peuvent changer constamment (Colman et al., 1997; Kopp et al., 2000; Walsh and Lichtman, 2003). Même une terminaison destinée à l'élimination, occupant une très petite région synaptique, pourrait regagner du territoire et devenir la terminaison unique innervant la JNM (Turney and Lichtman, 2012; Walsh and Lichtman, 2003). Ceci appui l'hypothèse qu'une terminaison faible n'est pas programmée à être éliminée. Si son efficacité est améliorée ou si la terminaison forte est affaiblie ou endommagée, la terminaison initialement faible pourrait être potentialisée et renforcée par les CSPs. D'ailleurs, une étude récente montre qu'une terminaison en voie d'élimination peut regagner du territoire si l'autre terminaison est endommagée suite à une ablation (Turney and Lichtman, 2012). Un tel mécanisme serait important pour assurer que chaque fibre musculaire soit innervée à la fin des processus de compétition et d'élimination synaptique.

6.3. Implications de la plasticité synaptique différentielle des terminaisons lors de la compétition synaptique

La plasticité synaptique aurait d'importantes implications pour un système immature en plein remodelage, spécialement durant la compétition et l'élimination synaptique. Ces implications peuvent être d'ordre fonctionnel, structurel, ou même être interdépendantes.

6.3.1. Implication fonctionnelle de la potentialisation synaptique de la terminaison forte

Plusieurs fonctions importantes sont attribuées à la plasticité synaptique. Entre autres, la plasticité synaptique pourrait être un mécanisme facilitateur qui assure une libération suffisante de neurotransmetteurs. Dans le cas où deux terminaisons sont en compétition pour l'innervation d'une même cible, celle qui est capable de s'adapter à des changements d'activité serait favorisée afin d'assurer la fonction synaptique. Puisque la terminaison forte montre une capacité de potentialisation et d'amélioration de son efficacité synaptique, elle devrait logiquement être avantagée. Par exemple, suite à une activité soutenue de longue durée, similaire à la stimulation à haute fréquence utilisée dans ces études, l'augmentation de la neurotransmission par la terminaison forte soutiendrait une libération suffisante de neurotransmetteur pour assurer une réponse postsynaptique propice et donc un risque plus faible d'échouer à induire une contraction musculaire. En contrepartie, un manque d'adaptation, telle que suggérée par l'absence de plasticité persistante de la terminaison faible, augmenterait le risque d'échecs de la contraction musculaire. D'ailleurs, il est connu qu'une terminaison qui n'a pas une activité en phase avec sa cible postsynaptique est plus propice à être éliminée (Favero et al., 2012). Par exemple, une terminaison qui ne libère pas assez de neurotransmetteurs ou qui n'active pas régulièrement sa cible pourrait ne pas recevoir une rétroaction adéquate de la fibre musculaire ou même des CSPs et ne sera donc pas renforcée

(Darabid et al., 2014). Tout ceci semble être compatible avec l'un des buts de la compétition et de l'élimination synaptique qui est de s'assurer que la terminaison la plus adaptée soit maintenue pour avoir une synapse mature capable d'assurer la contraction musculaire.

6.3.2. Implication structurelle de la potentialisation synaptique de la terminaison forte

En plus de son importance physiologique, la plasticité synaptique peut avoir un impact direct sur l'organisation de la synapse. Tel que mentionnée dans l'introduction, les synapses du SNC qui sont potentialisées sont renforcées et stabilisées (Matsuzaki et al., 2004; Nagerl et al., 2004) alors que les synapses qui subissent une dépression synaptique peuvent être affaiblies et éliminées (Bastrikova et al., 2008; Oh et al., 2013). Durant la compétition synaptique à la JNM, il est possible d'imaginer un processus capable de renforcer les terminaisons potentialisées par une expansion du territoire synaptique ou l'ajout de branches présynaptiques. De manière intéressante, une augmentation transitoire de l'activité synaptique peut induire la formation de nouveaux boutons synaptiques et mener à l'expansion de la synapse à la JNM de drosophile (Ataman et al., 2008; Fuentes-Medel et al., 2009). Donc, il semble y avoir un lien direct entre l'activité, la plasticité et l'organisation synaptique. En effet, quelques données appuient cette hypothèse, mais les mécanismes impliqués sont inconnus (Buffelli et al., 2003; Turney and Lichtman, 2012; Wyatt and Balice-Gordon, 2003). Une expérience qui permettrait à la fois d'étudier l'activité synaptique ainsi que le territoire occupé par chaque terminaison permettrait de mieux comprendre le lien entre l'activité, la plasticité et l'organisation synaptique durant le processus de compétition synaptique.

6.3.3. Lien entre l'activité et l'organisation structurale des terminaisons

L'expansion d'une terminaison impliquerait un remodelage constant et nécessiterait la disponibilité d'une multitude de facteurs de croissance. Si une augmentation de l'activité synaptique provoque une expansion du territoire synaptique (Ataman et al., 2008; Fuentes-Medel et al., 2009) alors l'interaction entre la terminaison forte, la fibre musculaire et les CSPs pourrait être renforcée. Cette terminaison renforcée pourrait recevoir un meilleure feedback des autres éléments de la synapse et profiterait davantage de la disponibilité de facteurs trophiques comme le GDNF et BDNF connus pour réguler l'expansion et l'élimination synaptique (Je et al., 2012; Je et al., 2013; Keller-Peck et al., 2001a; Nguyen, 1998). De manière générale, une terminaison qui est mieux adaptée montrerait une meilleure efficacité synaptique, un territoire synaptique plus important et serait donc dans une meilleure position pour bénéficier des facteurs de survie.

De plus, les CSPs relâchent des facteurs qui favorisent la maturation de la JNM (Feng and Ko, 2008; Fuentes-Medel et al., 2012; Kerr et al., 2014; Reddy et al., 2003; Yang et al., 2001), sont responsables de l'élimination des débris et des terminaisons nerveuses (Bishop et al., 2004; Smith et al., 2013), sont essentielles pour le maintien de la structure synaptique (Reddy et al., 2003) et, tel que démontré dans cette thèse, peuvent réguler l'activité synaptique des terminaisons en compétition. Ainsi, il est attrayant de proposer que la cellule gliale est le lien central entre l'activité des terminaisons nerveuses et les changements structuraux observés lors du remodelage synaptique de JNMs immatures (Ko and Robitaille, 2015). Dans cette lignée, il serait alors intéressant de savoir si la machinerie gliale responsable de la détection et la régulation de l'activité des terminaisons serait liée à la capacité des cellules gliales à libérer des facteurs de croissance et éliminer les terminaisons redondantes lors de la maturation de la JNM. Une telle interdépendance de ces mécanismes permettrait à la cellule gliale de cibler son action en fournissant, par exemple, des facteurs de croissance aux terminaisons fortes tout en éliminant les terminaisons faibles. D'ailleurs, un modèle revisité de la compétition et l'élimination synaptique à la JNM est présenté dans la section suivante.

6.4. Régulation *in vivo* de l'élimination synaptique et la connectivité par les CSPs

6.4.1. Importance de la détection de l'activité synaptique par les CSPs dans l'élimination synaptique *in vivo*

La majorité des études qui montrent un impact direct des cellules gliales dans l'élimination synaptique ont utilisé des approches génétiques qui altèrent la capacité phagocytaire des microglies ou des astrocytes (Chung et al., 2013; Schafer et al., 2012; Stevens et al., 2007). Ces perturbations mènent à des déficits d'organisation synaptique et à un retard de l'élimination synaptique. Dans la présente étude, nous avons effectué une manipulation plus sélective de l'activité gliale. En effet, bien que la machinerie impliquée dans l'élimination n'ait pas été directement ciblée, nous observons que la capacité des CSPs à détecter la libération de neurotransmetteurs par les terminaisons en compétition est essentielle pour l'élimination adéquate de synapses, tel que suggérée par le blocage chronique des CSPs qui entraine un retard de l'élimination synaptique.

En effet, le blocage des récepteurs gliaux P2Y1 augmente le nombre de JNMs polyinnervées. Dans le contexte de la compétition et de l'élimination synaptique, le blocage de ces récepteurs empêcherait les CSPs de décoder et différencier l'activité des terminaisons en compétition. Puisque le décodage de l'activité synaptique par les cellules gliales est un prérequis pour une régulation adéquate de la neurotransmission et la plasticité synaptique (Henneberger et al., 2010; Navarrete et al., 2012; Takata et al., 2011; Todd et al., 2010), empêcher les CSPs de détecter l'activité des terminaisons entraverait à la plasticité différentielle des terminaisons. Par conséquent, ceci altèrerait la potentialisation et le renforcement de la terminaison forte tel que suggéré par les expériences *in situ*. Du même coup, l'augmentation de la disparité de l'efficacité synaptique entre les terminaisons ne serait pas aussi efficace ce qui expliquerait le retard dans l'élimination synaptique.

Nous rapportons presque 3 fois plus de JNMs polyinnervées chez les souris injectées à P12 et présque 4 fois à P14. À notre connaissance, ces pourcentages sont parmi les plus élevés rapportés dans la littérature où un retard dans l'élimination synaptique à la JNM a été observé à un âge similaire. Favero et al. (2012) décrivent environ 2 fois plus de polyinnervation lorsqu'une activité synchrone des motoneurones est imposée. Personius et al. (2008) montrent 1,5 fois plus de JNMs polyinnervées à P7-8 et environ 2,5 fois à P14-15 lorsque le couplage électrique entre les motoneurones est maintenu. Cependant, tout comme ces études, le blocage des P2Y1Rs des CSPs n'entraîne qu'un blocage partiel et transitoire de l'élimination synaptique. Ceci peut s'expliquer par différentes hypothèses et aurait d'importantes implications. Premièrement, nous n'avons pas bloqué génétiquement les P2Y1Rs, mais plutôt effectué des injections quotidiennes de MRS2179. Puisque l'effet de cet antagoniste est facilement réversible tel que montré par nos expériences *in situ*, les P2Y1Rs pourraient n'être bloqués que pour une courte période de temps durant la journée. Dès lors, l'effet observé reflète probablement une sous-estimation de l'importance de ces récepteurs. Deuxièmement, d'autres récepteurs purinergiques, et même muscariniques pourraient compenser en partie au manque d'activation des P2Y1Rs puisque d'autres récepteurs sont connus pour contribuer à la fonction des CSPs à la JNM adulte (Arbour et al., 2015; Robitaille, 1995; Rochon et al., 2001). Troisièmement, d'autres mécanismes importants sont connus pour influencer l'élimination synaptique à la JNM. Ces mécanismes peuvent être liés à l'activité intrinsèque des terminaisons nerveuses (Favero et al., 2012; Personius and Balice-Gordon, 2001; Personius et al., 2007; Personius et al., 2008), l'activité phagocytaire endogène des CSPs (Smith et al., 2013; Song et al., 2008), la contribution directe de la fibre musculaire (Favero et al., 2009) ainsi que la disponibilité des facteurs trophiques tels que BDNF et GDNF (Je et al., 2012; Je et al., 2013; Keller-Peck et al., 2001a; Nguyen, 1998). La présence de mécanismes multiples n'est pas surprenant et réitère la grande importance de l'élimination synaptique lors du développement pour la survie de l'organisme. En effet, si un mécanisme particulier est perturbé, plusieurs autres agissent en parallèle pour coordonner la compétition et l'élimination ainsi que la maturation adéquate de la synapse.

6.4.2. Actualisation du modèle de la compétition et élimination synaptique à la JNM

À la lumière des découvertes présentées dans cette thèse, nous croyons que le modèle de la compétition et de l'élimination synaptique à la JNM doit être révisé. Avant nos études, il était présumé que le rôle des cellules gliales était limité uniquement à l'engloutissement de branches terminales et à la phagocytose des débris (Bishop et al., 2004; Smith et al., 2013; Song et al., 2008). Nous montrons que les CSPs décodent l'activité des terminaisons en compétition ce qui permet la régulation de la plasticité et l'élimination synaptique.

6.4.2.1. Modèle de la régulation de l'activité synaptique par les CSPs lors de la compétition synaptique

Dans cette thèse, nous avons montré que les CSPs, cellules gliales à la JNM, décodent l'efficacité synaptique des terminaisons en compétition et potentialisent préférentiellement la terminaison forte alors qu'elles n'ont que très peu d'effet sur la terminaison considérée faible. Un modèle simplifié de cette modulation par les CSPs est présenté à la Figure 6.3 et se résume comme suit. Premièrement, les terminaisons en compétition libèrent des neurotransmetteurs, principalement l'ACh et l'ATP (Redman and Silinsky, 1994; Smith, 1991). Deuxièmement, l'ATP libérée par les terminaisons est détectée par les CSPs via l'activation des P2Y1Rs. Troisièmement, la détection de l'activité de la terminaison forte et faible se traduit respectivement par une grande et une petite réponse calcique des CSPs. Quatrièmement, les CSPs libèrent des gliotransmetteurs, principalement des purines, qui potentialisent préférentiellement l'activité de la terminaison forte en activant les récepteurs A2ARs présynaptiques.

Nous décrivons un mécanisme nouveau par lequel les CSPs, non seulement régulent la plasticité synaptique, mais pourraient biaiser l'issue de la compétition en favorisant le renforcement de la terminaison forte. Ceci pourrait expliquer en partie pourquoi les

terminaisons fortes sont privilégiées pour gagner la compétition (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000). Ce nouveau rôle des CSPs s'insère aisément dans le modèle de la compétition et l'élimination synaptique qui mérite d'être actualisé.

La compétition synaptique à la JNM peut être divisée en principalement deux périodes lors du développement postnatal. Une période « précoce » (~P0-4) durant laquelle l'activité des unités motrices des terminaisons en compétition est synchrone, suivie d'une période « avancée » (~P5-14) caractérisée par une activité asynchrone qui se développe graduellement (Buffelli et al., 2002; Darabid et al., 2014; Personius and Balice-Gordon, 2001). Les unités motrices sont complètement asynchrones à la fin de la compétition et l'élimination synaptique (~P14 chez la souris) (Buffelli et al., 2002; Personius and Balice-Gordon, 2001).

Durant le développement postnatal « précoce », plusieurs motoneurones innervent la même JNM. Une JNM peut recevoir plus d'une dizaine de terminaisons, dépendamment du muscle, qui occupent des territoires entremêlés (Smith et al., 2013; Tapia et al., 2012). Cette période coïncide avec l'arrivée des premières CSPs à la JNM (Hirata et al., 1997; Love and Thompson, 1998). Les CSPs sont en contact avec toutes les terminaisons d'une même JNM (Smith et al., 2013; Tapia et al., 2012). De plus, ces terminaisons en compétition déchargent de manière synchrone (c.-à-d. une corrélation temporale de l'activité des terminaisons qui activent la fibre musculaire en même temps) (Buffelli et al., 2002; Personius and Balice-Gordon, 2001). Dès lors, la capacité de décodage des CSPs ne serait peut-être pas aussi importante puisqu'il serait difficile de différencier l'activité des terminaisons qui déchargent en phase. De plus, il a été proposé que les CSPs durant cette période n'ont pas une préférence particulière et peuvent éliminer aléatoirement des branches de différentes terminaisons ce qui entraîne une réduction graduelle du nombre de terminaisons (Smith et al., 2013).

Lorsque seulement un petit nombre de terminaisons survivent (2 ou 3 approximativement à l'âge P7; Kopp et al., 2000; Personius and Balice-Gordon, 2001), celles-ci occupent des territoires délimités (Keller-Peck et al., 2001b; Walsh and Lichtman, 2003). Durant cette phase « avancée », l'activité des terminaisons en compétition devient asynchrone (c.-à-d. les terminaisons activent la fibre musculaire par alternance) (Buffelli et al., 2002;

Personius and Balice-Gordon, 2001). Puisque les terminaisons ne sont plus synchronisées, il serait plus facile pour les CSPs de différencier leur activité. Dans ces conditions, les CSPs pourront décodent l'efficacité relative des terminaisons et ainsi réguler la neurotransmission en renforçant l'activité de la terminaison forte. De plus, leur capacité à décoder l'efficacité des terminaisons pourrait leur permettre de déclencher l'élimination des terminaisons faibles. De cette façon, les CSPs s'assurent que la terminaison la plus efficace et la mieux adaptée soit maintenue.

Une telle interaction active des CSPs avec les terminaisons en compétition nécessiterait une organisation synaptique spécifique. Lors des stades « avancés » de la compétition synaptique, chaque terminaison occupe son propre territoire et fait face à des récepteurs postsynaptiques dédiés (Bishop et al., 2004; Gan and Lichtman, 1998; Keller-Peck et al., 2001b; Walsh and Lichtman, 2003; Wyatt and Balice-Gordon, 2003). Une idée intéressante est la possibilité que la CSP montre une organisation équivalente et renfermerait des compartiments distincts dédiés à l'interaction avec chacune des terminaisons en compétition. De tels compartiments pourraient être des structures morpho-fonctionnelles qui partagent des similitudes aux éléments pré- et postsynaptiques et pourraient être équipés de la machinerie nécessaire pour détecter, décoder et réguler l'efficacité de chaque terminaison. Par exemple, tout comme l'organisation spatiofonctionnelle des P2Y1Rs, il est possible que ces compartiments contiennent aussi les réserves de Ca^{2+} nécessaires pour la signalisation de la CSP ainsi qu'une densité de gliotransmetteurs, similaire à celles des vésicules de la terminaison présynaptique, à proximité de la cible. Si tel est le cas, ceci requiert la présence de mécanismes qui forment et maintiennent cette organisation telle que des protéines d'échafaudage pour stabiliser les récepteurs en place, maintenir la machinerie nécessaire pour la synthèse la relâche et la recapture de gliotransmetteurs ainsi que la machinerie pour une élimination locale des branches presynaptiques.

Certainement, un tel arrangement pourrait faciliter une détection fine et efficace de la relâche de neurotransmetteurs, permettrait de distinguer les terminaisons en compétition et de produire une rétroaction adéquate. Dans le SNC, plusieurs pistes suggèrent qu'une telle compartimentalisation existe. En effet, un même astrocyte est organisé en de multiples

domaines morphologiques distincts proches de chaque synapse (Bushong et al., 2002; Grosche et al., 1999). La détection de l'activité de synapses uniques par l'astrocyte se fait dans des domaines isolés à proximité de la synapse ce qui permet la régulation de l'efficacité de la neurotransmission (Di Castro et al., 2011; Panatier et al., 2011). Ces domaines contiendraient les transporteurs nécessaires à la recapture de neurotransmetteurs et sont confinés à proximité des synapses (Murphy-Royal et al., 2015). De plus, il est connu que la molécule d'adhésion Ephrin-A3 est exprimée dans des structures isolées de l'astrocyte et fait face à son récepteur postsynaptique EphA4. La signalisation Ephrin-A3/EphA4 régule l'organisation postsynaptique et l'expression des transporteurs glutamatergiques gliaux (Carmona et al., 2009; Murai et al., 2003).

De manière intéressante, une nouvelle étude montre que Ephrin-A3 est aussi exprimé à la JNM et que son récepteur, dans ce cas-ci EphA8, est exprimé exclusivement par les CSPs (Stark et al., 2015). L'interaction Ephrin-A3/EphA8 serait importante pour définir l'identité et la maturation de la JNM lors du développement et de la réinnervation (Stark et al., 2015). Tout cela suggère qu'une organisation de l'astrocyte en différents compartiments serait à la base de l'interaction des cellules gliales avec des synapses uniques. Finalement, considérant les analogies entre le SNC et la JNM, une telle organisation pourrait aussi être présente dans les CSPs et serait en accord avec le processus de compétition et d'élimination synaptique durant lequel une seule CSP aurait à interagir avec plusieurs terminaisons indépendantes.

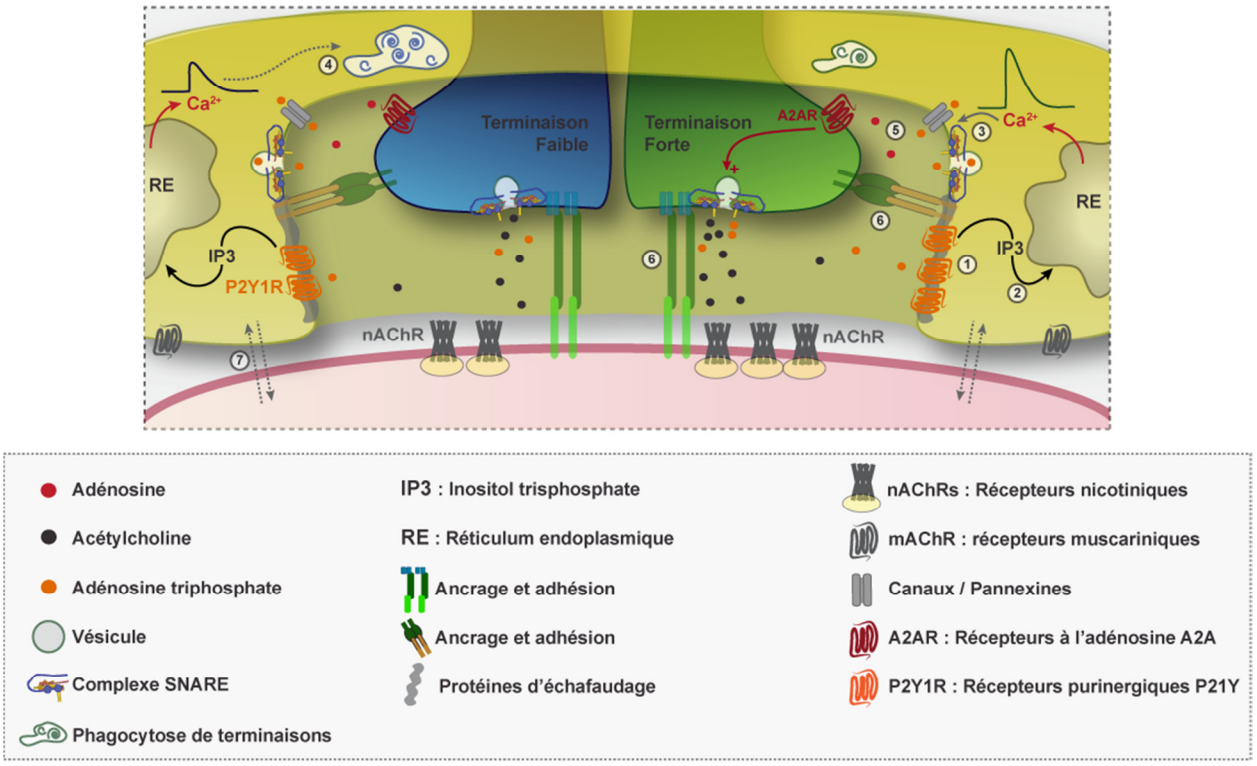

Figure 6.6. Modèle d'une organisation compartimentalisée de la CSP pour interagir avec chacune des terminaisons en compétition.

Nous proposons que la CSP pourrait être organisée en des compartiments distincts dédiés à l'interaction avec chacune des terminaisons en compétition. De tels compartiments pourraient être des structures morpho-fonctionnelles équipées de la machinerie nécessaire pour détecter, décoder et réguler l'efficacité de chaque terminaison. Chaque compartiment pourrait inclure:

1. Des protéines d'échafaudage pour stabiliser les récepteurs et la machinerie gliale en place.
2. Les réserves de Ca^{2+} nécessaires pour la signalisation de la CSP.
3. Une densité de gliotransmetteurs ainsi que la machinerie nécessaire pour leur synthèse et relâche localisée.
4. La machinerie pour une élimination locale des branches présynaptiques. Cette élimination pourrait être ciblée préférentiellement vers la terminaison faible étant donné la capacité de la CSP à décoder l'efficacité synaptique des terminaisons.

5. Des mécanismes de ciblage des gliotransmetteurs vers des sites synaptiques ou extra-synaptiques.
6. Des molécules d'ancrage, d'adhésion et de signalisation transmembranaire similaires à l'interaction pré- et postsynaptique [ex : interaction des intégrines (sur la fibre musculaire) – laminines (dans la matrice extracellulaire) – canaux Ca^{2+} (sur la terminaison présynaptique)]. Aussi, une nouvelle étude montre qu'à la JNM, Neuregulin1, exprimée par les terminaisons axonales, interagit avec son récepteur erbB2/3 sur les CSPs pour réguler l'élimination synaptique par les CSPs (Lee et al., 2016).
7. Une interaction entre la fibre musculaire et les CSPs ainsi qu'une contribution musculaire dans la régulation de la compétion, l'élimination et la maturation synaptique. Par exemple, dans le SNC, la signalisation Ephrin-A3 (exprimée par les astrocytes) avec EphA4 (récepteur postsynaptique) régule l'organisation postsynaptique et l'expression des transporteurs glutamatergiques gliaux (Carmona et al., 2009; Murai et al., 2003).

6.5. Impact sur l'élimination synaptique dans le SNC

La JNM partage plusieurs similitudes avec les synapses du SNC (Figure 2.1). Le fonctionnement du système nerveux ne dépend pas uniquement des éléments pré- et postsynaptiques, mais requiert la contribution des cellules gliales. De manière générale, il devient de plus en plus évident que les cellules gliales jouent un rôle crucial pour le fonctionnement du système nerveux et sont impliquées dans différents aspects incluant la synaptogenèse, le raffinement des circuits neuronaux et la régulation de l'activité synaptique.

Tout comme les CSPs à la JNM, les microglies et les astrocytes dans le SNC participent activement au raffinement de la connectivité. Une altération des mécanismes des microglies ou des astrocytes impliqués dans l'élimination synaptique entraîne la présence d'un nombre plus important de synapses (Bialas and Stevens, 2013; Chung et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012). Cependant, la manière dont l'élimination est dirigée vers des synapses spécifiques alors que d'autres sont épargnées demeure inconnue.

Il est possible qu'un mécanisme de décodage similaire à celui décrit pour la JNM soit impliqué dans le remodelage des connexions synaptiques du SNC. Par exemple, la capacité des astrocytes à détecter l'activité de synapses uniques pourrait leur permettre d'améliorer l'efficacité de synapses spécifiques (Di Castro et al., 2011; Panatier et al., 2011; Perea and Araque, 2007). Les astrocytes pourraient ainsi renforcer l'activité des synapses les plus actives et influencer le processus d'élimination. Dans la région du gyrus dentelée de l'hippocampe, l'activation des récepteurs P2Y1 des astrocytes contrôle leur signalisation calcique et permet aux astrocytes de réguler la force des synapses avoisinantes ainsi que des formes spécifiques de plasticités synaptiques à long terme (Jourdain et al., 2007; Santello et al., 2011). De plus, une capacité de détection de l'efficacité synaptique pourrait permettre aux astrocytes de déclencher l'élimination des synapses les plus faibles (Chung et al., 2013; Schafer et al., 2012; Stevens et al., 2007).

Ainsi, étudier le rôle des cellules gliales dans la compétition et l'élimination synaptique dans un modèle plus simple comme la JNM peut aider à comprendre l'implication générale des cellules gliales dans la synapsegenèse et le remodelage de la connectivité dans le SNC.

6.6. Implication pour le système mature

Les données présentées dans cette thèse peuvent avoir une portée au-delà de la synaptogenèse et du développement du système nerveux. En effet, plusieurs observations peuvent être transposées dans le système nerveux mature en conditions physiologiques normales ou suite à des atteintes particulières.

6.6.1. Implication en conditions physiologiques normales

6.6.1.1. Élimination synaptique dans le système nerveux mature

L'élimination synaptique est aussi importante dans le système nerveux mature dans lequel l'expérience sensorielle peut entraîner une suppression des synapses de manière dépendante de l'activité pour permettre une adaptation des circuits neuronaux (Trachtenberg et al., 2002). Par exemple, l'expérience sensorielle ainsi que des tâches cognitives reliées à l'apprentissage et à la mémoire peuvent induire des changements de la connectivité et le remodelage des synapses (Bastrikova et al., 2008; Matsuzaki et al., 2004; Nagerl et al., 2004; Oh et al., 2013; Trachtenberg et al., 2002). Pour cette raison, il a été suggéré que les mécanismes présents lors du développement peuvent être impliqués dans le remodelage synaptique des synapses matures. Il est donc possible que l'implication gliale soit aussi nécessaire dans ces conditions pour assurer une adaptation appropriée des circuits neuronaux (Clarke and Barres, 2013).

6.6.1.2. Interactions neurone-glie et synapse tripartite

Les données présentées dans cette thèse permettent de mieux comprendre l'importance et le fonctionnement des interactions neurone-glie dans le système nerveux. Dans le cas qui nous concerne, une question importante à propos de la régulation synaptique par les cellules gliales dans le SNC est de savoir si un même astrocyte peut réguler indépendamment l'activité de différentes synapses. Il est difficile de répondre à cette question dans le SNC étant donné la grande densité synaptique et le grand nombre de synapses couvertes par un seul astrocyte (Bushong et al., 2002; Grosche et al., 1999). Sachant qu'une seule CSP peut décoder et réguler différemment l'activité d'au moins deux terminaisons indépendantes innervant la même cible, il est possible qu'un même astrocyte régule indépendamment différentes synapses. Certainement, ceci serait d'une importance particulière dans des phénomènes physiologiques comme l'apprentissage et la mémoire durant lesquelles certaines synapses doivent être potentialisées alors que d'autres déprimées (Bastrikova et al., 2008; Escobar and Derrick, 2007; Matsuzaki et al., 2004; Nagerl et al., 2004; Whitlock et al., 2006).

L'étude des interactions neurone-glie à la JNM en développement permettrait aussi une meilleure compréhension de l'intégration de l'information synaptique par les cellules gliales. Entre autres, cela permet de confirmer que l'activité calcique des cellules gliales est un code qui reflète les propriétés synaptiques ce qui pourrait mener à différents effets sur la neurotransmission (Araque et al., 2014; Perea and Araque, 2005; Todd et al., 2010). Aussi, nous montrons qu'une diversité de récepteurs peut contrôler l'activation et la signalisation calcique des cellules gliales. De plus, ces récepteurs peuvent être régulés de manière développementale de façon à ce qu'un type particulier soit prédominant à un stade (p.ex. : balance purinergique-muscarinique). Une observation similaire montre que l'expression des récepteurs glutamatergiques métabotropes des astrocytes, impliqués dans la détection de l'activité synaptique (Panatier et al., 2011; Wang et al., 2006), est régulée durant le développement (Sun et al., 2013). Cependant, il est important de prendre en considération la distribution et la localisation des récepteurs. Nous montrons que les récepteurs mAChRs, quoique présents et fonctionnels, ne sont pas impliqués dans la détection de l'activité

synaptique endogène par les cellules gliales. Il est alors possible qu'une situation similaire soit présente aux synapses matures dans lesquelles certains types de récepteurs montreraient une expression faible (Sun et al., 2013), mais auraient une distribution spatiale spécifique et une contribution importante à la signalisation gliale. Appuyant cette idée, il a été montré que la « quantité » de certains types de récepteurs ne définit pas leur importance et la « qualité » de la réponse induite (Han et al., 2012).

À la lumière de ces observations, il est possible que l'activité, l'organisation en domaines et la présence de différents types de récepteurs des cellules gliales soient adaptées aux besoins synaptiques. En effet, l'organisation et l'activité synaptique varient d'une région du système nerveux à l'autre et changent lors de conditions spécifiques telles que la maturation synaptique. Pour que la cellule gliale soit un partenaire adéquat, elle doit être en harmonie avec les synapses associées. Elle doit présenter une organisation appropriée au contexte et exprimer la machinerie de détection et de régulation nécessaire pour remplir sa fonction.

6.6.2. Implications lors d'une atteinte du système nerveux

Les données de la présente thèse peuvent aider à mieux comprendre les mécanismes impliqués dans certaines atteintes du système nerveux. Par exemple, la réinnervation à la JNM suite à des lésions axonales partage plusieurs similarités avec la formation synaptique lors du développement. D'abord, lors de la réinnervation, plusieurs terminaisons axonales innervent la même fibre musculaire (Kang and Lichtman, 2013; Magill et al., 2007; Rich and Lichtman, 1989). Ces terminaisons suivent un processus de compétition et d'élimination qui est façonné par l'activité synaptique (Favero et al., 2012; Laskowski et al., 1998). Ainsi, il semble que la reformation synaptique suite à des lésions nerveuses suit les mêmes étapes que la formation synaptique lors du développement du système nerveux. Il est donc possible que les cellules gliales changent leur activité pour s'adapter aux besoins de la reformation des synapses. Une récente étude de notre laboratoire montre que la balance purinergique-

muscarinique bascule vers un état immature (diminution de la signalisation muscarinique et augmentation de la signalisation purinergique) ce qui permettrait de créer un environnement propice à la reformation, l'expansion et le remodelage synaptique (Perez-Gonzalez et al., 2016).

Dans le SNC, les astrocytes endommagés retournent vers un état immature et expriment de nouveau des molécules impliquées dans la formation synaptique. Par exemple, la signalisation purinergique régule à la hausse l'expression des thrombospondins, molécules clés libérées par les astrocytes, qui favorisent la formation et l'intégrité structurale des synapses excitatrices (Christopherson et al., 2005; Tran and Neary, 2006). Une régulation similaire peut aussi se produire *in vivo* à la suite de lésions et serait nécessaire pour la récupération fonctionnelle après une lésion cérébrale traumatique, l'ischémie ou un accident vasculaire cérébral (Liauw et al., 2008; Lin et al., 2003).

Comprendre le rôle des cellules gliales dans l'élimination synaptique pourrait aider à mieux comprendre les maladies neuro-développementales et neurodégénératives. Par exemple le syndrome de Guillain-Barre (Ilyas et al., 1992), le syndrome de Miller-Fisher (Chiba et al., 1992; Slater, 2001) et la neuropathie motrice multifocale (Pestronk et al., 1988) sont des pathologies de la JNM associées à la dégénérescence des motoneurones. D'ailleurs, dans la sclérose latérale amyotrophique, la rétraction périphérique des motoneurones de la JNM est l'un des phénomènes précoces observés et qui précède la perte neuronale (Fischer et al., 2004). Il est donc possible que la rétraction et la dégénérescence dans ces maladies partagent des mécanismes cellulaires avec l'élimination synaptique lors du développement, incluant des mécanismes gliaux. De manière intéressante, une récente étude montre que des déficits de la signalisation des CSPs sont évidents bien avant le déclenchement de la maladie et la rétraction des motoneurones (Arbour et al., 2015). Ainsi, il est possible que cette altération précoce des CSPs contribue au déclenchement de la maladie. De plus, si cette altération gliale est aussi présente lors du développement, il est possible que la régulation de l'activité des terminaisons en compétition par les CSPs soit altérée.

Dans le SNC, une élimination aberrante par la microglie a été récemment associée à des déficits de comportements sociaux liés à des troubles semblables à ceux observés dans l'autisme (Zhan et al., 2014). De la même manière, une élimination précoce de contacts synaptiques a été décrite dans différentes maladies neurodégénératives telles que la schizophrénie où une dérégulation de l'activité microgliale a été décrite (voir revue Bilimoria and Stevens, 2015 et McGlashan and Hoffman, 2000) et la maladie d'Alzheimer, liée à diverses perturbations de la fonction gliale (voir revue Lian and Zheng, 2015).

En somme, de nombreux aspects des maladies neurodégénératives pourraient être expliqués par un dérèglement de l'élimination synaptique. Dès lors, comprendre les processus d'élimination synaptique lors du développement et l'implication des cellules gliales pourrait aider à comprendre les dysfonctions qui sous-tendent certaines de ces maladies.

7. Conclusions

Dans cette thèse, je présente un nouveau rôle des cellules gliales dans l'un des processus fondamentaux à la base du développement du système nerveux. En effet, je montre que, lors de la compétition et de l'élimination synaptique, le rôle des cellules gliales à la JNM ne se limite pas uniquement à l'élimination des terminaisons excédentaires, mais elles (1) décodent avec précision l'efficacité synaptique des terminaisons en compétition, (2) régulent différemment l'activité des terminaisons en compétition en potentialisant préférentiellement l'activité de la terminaison forte et (3) influencent l'issue de l'élimination synaptique. Nous décrivons un mécanisme particulier par lequel les CSPs pourraient biaiser l'issue de la compétition en favorisant le renforcement de la terminaison forte. Cela pourrait expliquer en partie pourquoi les terminaisons fortes sont privilégiées pour être maintenues et gagner la compétition (Buffelli et al., 2003; Colman et al., 1997; Kopp et al., 2000).

Nous décrivons un nouveau rôle des cellules gliales qui pourrait être crucial pour la régulation de la synaptogènese. En effet, le décodage de la compétition synaptique pourrait permettre aux cellules gliales de fournir des facteurs synaptogèniques appropriés, réguler adéquatement l'activité des terminaisons en compétition et diriger la phagocytose vers les terminaisons mal adaptées. Il devient alors clair que les cellules gliales occupent une fonction importante lors du développement puisque toutes ces actions font partie de la capacité multipotente des cellules gliales.

En somme, ces résultats permettent de mieux comprendre l'importance de la régulation de l'activité synaptique par les cellules gliales et la connectivité du système nerveux. Ceci pourrait aider à mieux comprendre les interactions neurone-glie et la plasticité synaptique, le remodelage de la connectivité par les cellules gliales qui sous-tendent différentes tâches cognitives ainsi que les mécanismes potentiellement impliqués dans certaines atteintes du système nerveux.

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Annexe I

Cellular/Molecular

Perisynaptic Glia Discriminate Patterns of Motor Nerve Activity and Influence Plasticity at the Neuromuscular Junction

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In the nervous system, the induction of plasticity is coded by patterns of synaptic activity. Glial cells are now recognized as dynamic partners in a wide variety of brain functions, including the induction and modulation of various forms of synaptic plasticity. However, it appears that glial cells are usually activated by stereotyped, sustained neuronal activity, and little attention has been given to more subtle changes in the patterns of synaptic activation. To this end, we used the mouse neuromuscular junction as a simple and useful model to study glial modulation of synaptic plasticity. We used two patterns of motor nerve stimulation that mimic endogenous motor-neuronal activity. A continuous stimulation induced a post-tetanic potentiation and a phasic Ca^{2+} response in perisynaptic Schwann cells (PSCs), glial cells at this synapse. A bursting pattern of activity induced a post-tetanic depression and oscillatory Ca^{2+} responses in PSCs. The different Ca^{2+} responses in PSCs indicate that they decode the pattern of synaptic activity. Furthermore, the chelation of glial Ca^{2+} impaired the production of the sustained plasticity events indicating that PSCs govern the outcome of synaptic plasticity. The mechanisms involved were studied using direct photo-activation of PSCs with caged Ca^{2+} that mimicked endogenous plasticity. Using specific pharmacology and transgenic knock-out animals for adenosine receptors, we showed that the sustained depression was mediated by A1 receptors while the sustained potentiation is mediated by A_{2A} receptors. These results demonstrate that glial cells decode the pattern of synaptic activity and subsequently provide bidirectional feedback to synapses.

Introduction

Neuronal information processing in the brain is not only coded by the frequency of neuronal activity but also by the pattern of action potential firing in neurons. This frequency and pattern coding greatly influences synaptic plasticity of CNS synapses as well as the neuromuscular junction (NMJ) (Magleby and Zengel, 1976; Guyonneau et al., 2004; Harris, 2005; Nicoll and Schmitz, 2005).

In addition to the neuronal elements of the brain, it is now increasingly recognized that glial cells are dynamic partners in a wide variety of brain functions including the induction and modulation of various forms of synaptic plasticity (Pascual et al., 2005; Haydon and Carmignoto, 2006; Perea and Araque, 2007; Barres, 2008; Gordon et al., 2009; Henneberger et al., 2010). Glial cells respond to different neurotransmitters with Ca^{2+} release from internal stores. These Ca^{2+} elevations represent the excitability of glial cells and are dependent on the frequency of neuronal and synaptic activity; that is, the responses are more reliable and larger at higher frequencies of stimulation (Pasti et al., 1997). Also, the activation of glial cells is regulated by a frequency-dependent threshold, below which no Ca^{2+} response has been detected so far. In turn, glial cells modulate neuronal activity and synaptic plasticity by releasing neuroactive substances called gliotransmitters (Haydon and Carmignoto, 2006).

Although glial cell activation varies according to different frequencies of stimuli (Pasti et al., 1997), it is still unclear whether glial cells can decode different patterns of neuronal activity (e.g., differences in burst duration), a critical property that would allow them to fully integrate neuronal information and, in return, accordingly adjust their feedback modulation to neuronal and synaptic activity. Furthermore, it is unknown whether the outcome of glial cell regulation of neuronal activity is dependent upon the property of the glial activation. Therefore, we set out to investigate the impact of different patterns of neuronal activity on glial cells and their subsequent modulation of synaptic transmission.

To this end, we took advantage of the NMJ, which is a simple synapse, easily accessible, and covered by perisynaptic Schwann

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cells (PSCs; ~5 cells per NMJ). These are nonmyelinating glial cells that surround the synapse and have similar roles to astrocytes in the CNS (Auld and Robitaille, 2003).

We found that endogenously evoked synaptic post-tetanic potentiation and depression relied on different glial calcium signals. Therefore, our results demonstrate that glial cells detect differences in ongoing synaptic activity and decode these differences to provide specific feedback to the synapse. These glial calcium signals result in the differential activation of potentiating A_{2A} adenosine receptor and depressing A_1 receptors presynaptically. This feedback provides a critical level of modulation to regulate a balance of potentiating and depressing influences on the synapse.

Materials and Methods

Animals and preparation. All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Animal Care Committee at the Université de Montréal. Juvenile [postnatal day (P) 21–P28] male CD-1 mice (Charles River) were killed by decapitation under deep anesthesia (0.1 ml/g midazolam and hypnorm dissolved in distilled water, administered intraperitoneally). Soleus muscles, with the tibial nerve intact, were removed and pinned in Rees saline solution (Rees, 1978) as follows (in mM): NaCl 110; KCl 5; MgCl2 1; NaHCO3 25; CaCl2 2; glucose 11; glutamate 0.3; glutamine 0.4; BES buffer, 5; cocarboxylase 0.4 μM; and choline chloride 36 μM and bubbled with 95% O_2/5% CO_2. Experiments were performed at 30–32°C under continuous perfusion of saline.

A_1 and A_{2A} knock-out mice. The A_1 −/− mice were a kind gift from Bertil Fredholm (Karolinska Institute, Stockholm, Sweden) and Stephen Tilley (University of North Carolina, Chapel Hill, NC). These mice have previously been described (Giménez-Llort et al., 2002). A_{2A} −/− mice have been characterized by Chen et al. (1999) and kindly provided by Jiang-Fan Chen (Boston University, Boston, MA) and Michael Schwarzschild (Massachusetts General Hospital, Boston, MA).

Electrophysiological recordings. The tibial nerve was stimulated through a suction electrode filled with extracellular saline. Muscle contractions were prevented with partial blockade of the postsynaptic ACh receptors using D-tubocurarine chloride (2.9–4.4 μM, Sigma). Intracellular recordings of postsynaptic potentials (PSPs) were performed using glass microelectrodes (1.0 mm OD; WPI) pulled to 50–70 MΩ (filled with 3M KCl) with a Brown–Flaming micropipette puller (Sutter Instruments). Recordings were amplified (200×) using an AM Systems 1200 amplifier connected to a WPI external amplifier, digitized using a National Instruments BNC 2110 board and acquired using WinWCP software (John Dempster, Strathclyde University, Glasgow, UK). It is noteworthy that the choice of the nicotinic receptor antagonist has no impact on synaptic plasticity events studied at the NMJ (Robitaille, 1998; Silinsky, 2005; Bélair et al., 2010).

The continuous stimulation paradigm consisted of 1800 pulses delivered at 20 Hz. The bursting stimulation paradigm (Fig. 1*A*) consisted of 30 repetitions of 20 pulses at 20 Hz repeated every 2 s. This was repeated three times with 20 s of rest between repetitions. Synaptic efficacy was monitored with test pulses delivered at a frequency of 0.2 Hz, a frequency known to have no effect on synaptic efficacy. Control baselines were generated by stimulation at 0.2 Hz for 20 min for comparison with treatments. For comparison with photolysis experiments, baseline controls were performed where a UV flash was given in the absence of caged compound and the 0.2 Hz stimulation was continued for 15 min after, as with treatments. PSP amplitude before, during high-frequency stimulation, and after were normalized to the PSP amplitude of test pulses obtained during the baseline period, before 20 Hz stimulation. In some experiments, stimulation was performed using a protocol of two stimuli at 10 ms interval elicited at 0.2 Hz to measure paired-pulse facilitation (PPF). Recordings were discarded when the holding potential changed by >5 mV. Throughout, *n* indicates the number of NMJs studied. Only one NMJ was studied per muscle.

Calcium imaging and analysis in PSCs. Dissected soleus muscles were incubated in 10 μM Fluo-4AM (Invitrogen) containing 0.02% pluronic acid (Invitrogen) for 1.5 h at room temperature. NMJs were located on the surface of muscle fibers using bright-field optics. Evoked calcium responses were obtained by stimulating the tibial nerve with one of the stimulation paradigms described above. Epifluorescent images were acquired on a Nikon E600N upright microscope fitted with a Princeton Instruments CCD-1300 camera. Digital acquisition was performed using MetaFluor software (Molecular Devices, a division of MDS Analytical Technologies) driving a Lamda 10-2 shutter wheel (Sutter Instruments). Images were acquired at a rate of one image per second with an integration time of 400 ms. Fluorescence was quantified by subtracting the background fluorescence from the neighboring muscle fiber and then performing the calculation $(F - F_0/F_0)*100$ to give the percentage $\Delta F/F_0$. Experiments were discarded when bleaching or focus drift occurred.

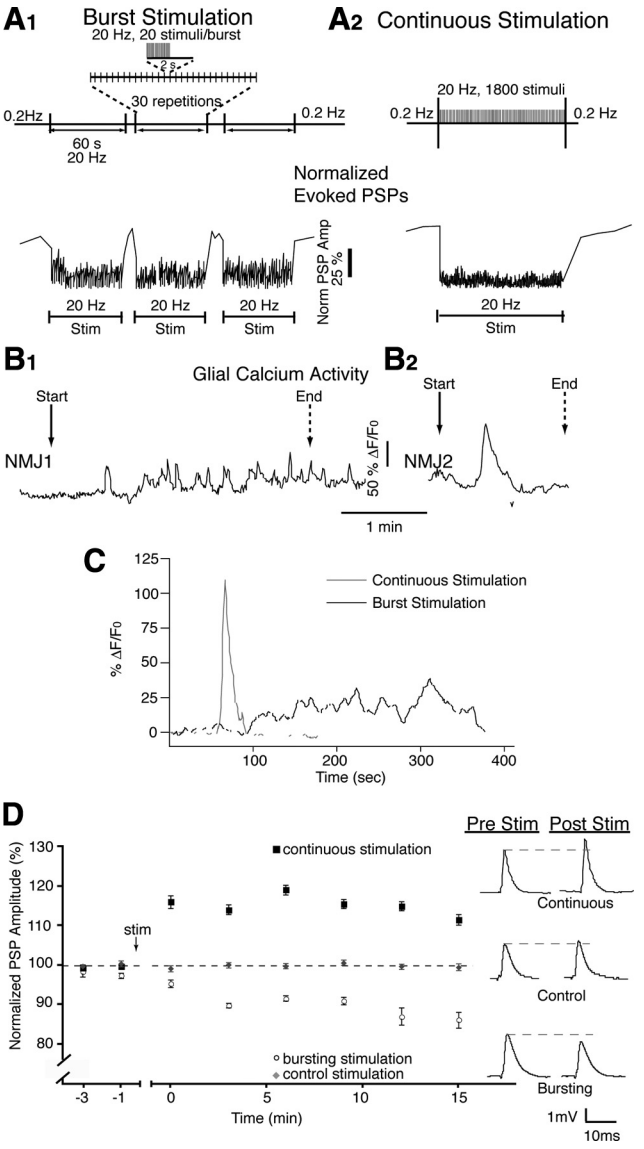

Figure 1. Different stimulation paradigms induce different glial calcium signals and post-tetanic plasticity. ***A1***, Bursting stimulation protocol mimicking endogenous activity (top) induced repeated periods of synaptic depression (bottom), which recovered at the cessation of high-frequency activity. ***A2***, Continuous stimulation protocol (top) induced a prolonged period of synaptic depression (bottom), which also recovered at the cessation of high-frequency activity. ***B1***, ***B2***, Multiple (***B1***) and single (***B2***) calcium elevations were correlated with bursting and continuous stimulation respectively. ***C***, Average of calcium responses recorded in response to bursting and continuous stimulation. Responses were aligned on first rise. ***D***, Normalized PSP amplitude over time showing that bursting stimulation caused post-tetanic depression, whereas continuous stimulation caused post-tetanic potentiation. PSPs are representative averages of 30 events taken from a single recording at the 15 min time point. Pre Stim, Before stimulation; Post Stim, after stimulation.

Photo-activation of caged molecules. Caged compounds [diazo-2-AM, o-nitrophenyl (NP)-EGTA-AM; Invitrogen] were loaded with the following protocol. Muscles were incubated first with Fluo-4-AM (10 μM) alone for 30 min and then with both Fluo-4-AM and caged compound (20 μM) for 45 min followed by another period of 45 min with fresh solution. For direct injection of NP-EGTA in muscle fibers, Ringer solution was used as the intracellular solution containing NP-EGTA (5 mM). Iontophoretic injections of NP-EGTA were performed using current pulses (−10 nA, 200 ms) generated using a Grass S88 stimulator at a frequency of 2 Hz for 10 min.

Photolysis of NP-EGTA was performed using a Laser Science Nitrogen Pulsed UV laser (337 nM) delivering pulses of 4 ns duration. The UV pulses were aimed at the preparation using a fiberoptic probe with a diameter of ~30 μm inserted into a pipette for guidance. The alignment of the fiberoptic probe was performed using visible red light emitted by an HeNe laser passing through the same optic fiber.

For multiple uncaging events mimicking Ca^{2+} response observed during bursting activity, 10 pulses generated at 60 Hz for 2 ms were used repeatedly. For a single large Ca^{2+} event observed during continuous stimulation, 15 pulses of 10 ms at 50 Hz were used. Two or more PSCs were targeted for photolysis during all experiments. Photolysis of NP-EGTA in the presynaptic terminal and muscle fiber as well as diazo-2 was induced using a regime of 15 pulses of 10 ms at 50 Hz. Calculated efficacy of the optic fiber is shown in supplemental Figure 1 (available at www.jneurosci.org as supplemental material).

A protocol was designed to specifically target PSCs taking advantage of the morphological organization of the NMJs at soleus muscles. In the first situation, we took advantage that PSCs were often ectopic, with their somata located off limit of the endplate area while their processes covered the nerve terminal. Also, NMJs were often found on the side of the muscle fiber (Fig. 2*A*, side NMJs). Owing to these two characteristics, we could find side NMJs with ectopic PSC somata lying on the surface of the muscle fiber. This provided us with a morphological situation whereby the PSC somata could be selectively exposed to UV light without affecting the presynaptic terminal (for details, see Fig. 2). Second, we chose surface NMJs where the PSCs somata were clearly off limit from the endplate area and could be aimed selectively by the laser beam. This was possible because the alignment and positioning of the fiberoptic was performed using visible red light emitted by a red Diode laser and passed through the fiberoptic such that the cone of light covered at least two PSCs and not the endplate area.

Some uncaging experiments with diazo-2 were performed using an Olympus FV1000 (see Fig. 9 and supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The 405 line of a UV laser was used to induce the photo-activation of diazo-2 with the tornado feature and the SIM scanner to simultaneously activate diazo-2 while imaging calcium. The power of the UV laser was set at 30%, which represents a final power of 8.3% since only 25% of the laser light is carried through the SIM scanner. An ROI was placed on top of at least two PSCs and scanned for up to 5 s to optimally activate diazo-2. Exposure to 405 laser light alone does not affect PSCs excitability as shown by their unaltered ability to respond to local agonists applications (data not shown).

Immunohistochemistry. Dissected soleus muscles were pinned in a Sylgard-coated dish containing PBS and fixed for 10 min in 4% formaldehyde, at room temperature. Muscles were permeabilized in 100% cold methanol for 6 min, at −20°C. Nonspecific antibody labeling was prevented by incubating the muscles in 10% normal donkey serum (NDS) with 0.1% Triton X-100 for 20 min. Rabbit, anti-S100β (1:250, Dako) with 0.01% Triton X-100 and 2% NDS was incubated overnight at 4°C, then washed in normal serum muscles, to be then incubated in goat, anti-neurofilament (1:250; SC16143, Santa Cruz Biotechnology) for 90 min. Secondary antibodies Alexa 488 (α-goat) and 647 (α-rabbit, 1:500) were incubated together for 60 min at room temperature. After washout, muscles were incubated with α-bungarotoxin (Alexa 594, 0.75 μg/ml) for 30 min. After each step, soleus muscles were washed in PBS plus 0.01% Triton X-100 for 3 × 5 min. The preparations were then mounted in the Prolong Kit (Invitrogen) and observed using an Olympus FV1000 microscope. The three channels were observed simultaneously using the spectral detection feature of the confocal system. Pinholes were set to

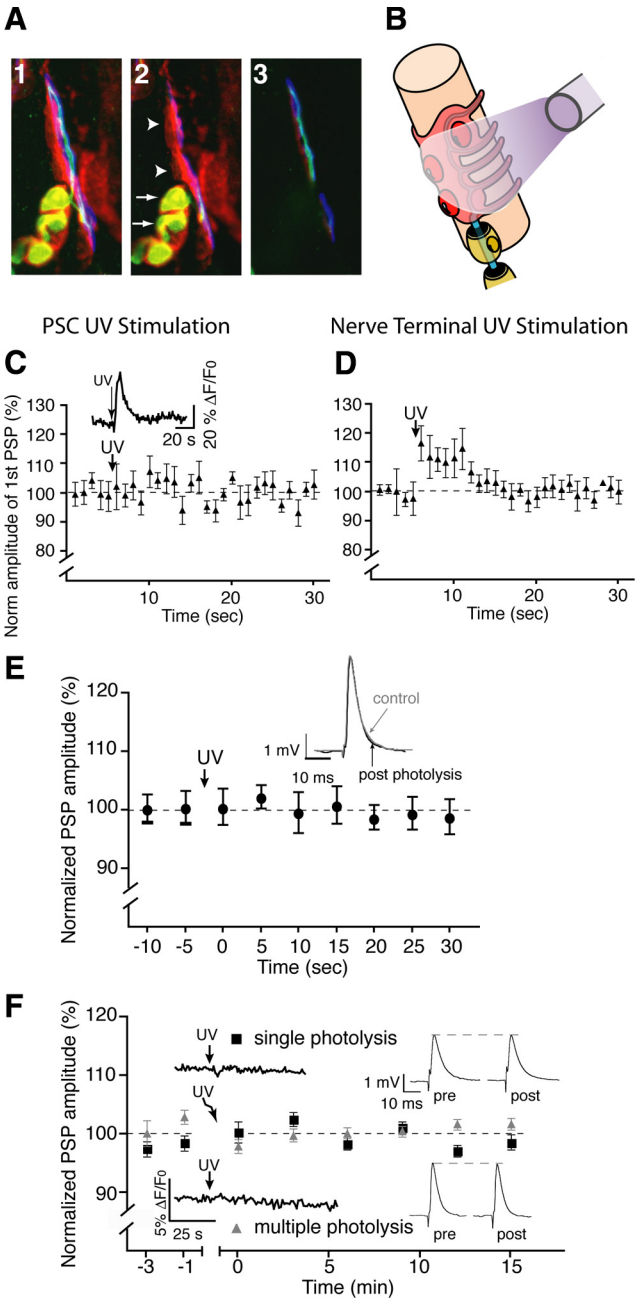

Figure 2. Selective photo-activation of PSCs. ***A1***, A 9.6 μm *Z*-stack of immunofluorescent staining from a mouse NMJ with PSCs labeled red with anti-S100β, green anti-neurofilament in the presynaptic terminal, and blue α-bungarotoxin labeling postsynaptic nAChr receptors. ***A2***, A single optical section at the surface of the junction with the myelinating Schwann cells colocalized with the presynaptic terminal (arrows). PSCs are seen separated from the last myelinating segment overlying the presynaptic terminal (arrowheads). ***A3***, The NMJ at 5.6 μm below the surface. PSC somata are located at the surface, while the presynaptic terminal is clearly visible at this depth. ***B***, Arrangement of the fiberoptic used for photolysis and its position in relation to the synaptic components. ***C***, Normalized average PSP amplitude showing that photo-activation of NP-EGTA in a PSC-targeted situation did not induce a rapid, transient increase in PSP amplitude indicative of a lack of direct effect on the presynaptic terminal. A PSC calcium elevation was detected (inset). ***D***, Normalized average PSP amplitude showing that presynaptic photo-activation of NP-EGTA resulted in a rapid and transient increase in PSP amplitude. ***E***, Changes of PSP amplitude before and after glial-targeted photo-activation of diazo-2. No rapid changes (up to 30 s after photolysis) were observed in PSP amplitude indicating that photolysis of diazo-2 had not direct presynaptic effect. ***F***, Normalized changes in PSP amplitude showing that injection of cell impermeant NP-EGTA salt into muscle fibers had no effect on plasticity after photolysis. Insets show traces of calcium level measurements in PSCs (left) and PSP averages from representative individual experiments (left, $n = 4$).

obtain an airy disk value of 1. Figures were not manipulated in any way after the acquisition.

Chemicals. All chemicals were purchased from Sigma-Aldrich except for ARL 67156, CGS 21680, and PSB-36 (Tocris Bioscience).

Statistical analysis. PSP values were compared with the control PSP amplitude with Student's *t* tests at the 15 min time point. When data were found not to conform to normality, Mann–Whitney U tests were used. Analyses were deemed significant at $p \leq 0.05$.

Results

Glia decode the pattern of synaptic activity

We chose two physiological patterns of stimulation for the soleus muscle, similar to *in vivo* motoneuronal activity, which produced two forms of post-tetanic plasticity. This provided us with two different patterns of stimulation (bursting and continuous) at the same frequency (20 Hz) using the same number of pulses.

The first stimulation paradigm consisted of bursts of activity at 20 Hz with a total of 1800 pulses that induced three periods of synaptic depression each separated by a brief period of recovery (Fig. 1*A1*), and closely replicates a form of endogenous activity (Hennig and Lømo, 1985). The second pattern used continuous delivery of 1800 pulses at 20 Hz, causing a sustained depression during stimulation (Fig. 1*A2*). This is the frequency normally seen at the soleus muscle (Hennig and Lømo, 1985), and a pattern typical for studies of synapse–glia interactions at the NMJ (Rochon et al., 2001).

Transmitter release was evoked by stimulating the tibial motor nerve using different stimulation paradigms while we simultaneously recorded PSPs and monitored glial calcium responses using Fluo-4 AM (during and for 1 min after the 20 Hz stimulation). Interestingly, during burst stimulation, oscillatory calcium activity was observed in PSCs at each synapse where several small transient calcium responses (duration, 11.2 ± 1.6 s; amplitude, 47.2 ± 6.3% $\Delta F/F_0$) occurred in 27 of 29 cells. In addition, this stimulation induced an underlying increase in basal calcium that lasted on average 94.5 ± 6.6 s (Fig. 1*B1*). Unlike bursting, continuous stimulation always elicited one to two calcium responses that lasted on average 27.3 ± 4.8 s with an average amplitude of 115.4 ± 28.7% $\Delta F/F_0$ (Fig. 1*B2*). The individual responses were longer ($p = 0.003$, two-tailed *t* test) and had greater amplitude ($p = 0.001$, two-tailed *t* test) than the transient responses evoked with burst stimulation. However, as shown in Figure 1*C*, on average, Ca^{2+} responses elicited by continuous stimulation had a faster rise time while the bursting motor nerve stimulation produced a slower and more sustained response.

In addition, the two patterns of motor nerve stimulation produced different types of post-tetanic plasticity. Indeed, as shown in Figure 1*D*, the bursting stimulation induced post-tetanic depression of PSP amplitude that develops over a period of 10 min. Post-tetanic depression was 86.2 ± 2.0% of control at 15 min compared with control PSPs evoked using test pulses delivered at 0.2 Hz (Fig. 1*D*, gray circles) ($n = 5$, $p < 0.0001$, two-tailed *t* test). However, rather than a post-tetanic depression, we found that continuous stimulation induced a post-tetanic potentiation of 111.5 ± 1.3% of control at 15 min compared with control stimulation (Fig. 1*D*) ($n = 8$; $p < 0.0001$; two-tailed *t* test).

Hence, the two stimulation patterns induced different PSC activation and post-tetanic plasticity. More importantly, the difference in PSC calcium elevations induced by the two stimulation protocols indicates that they are differentially activated by these patterns, and thus, that glial cells decode the pattern of neuronal activity.

Selective PSC modulation with caged molecules

Next we wanted to directly manipulate glial cells to test whether the different calcium elevations induced by the two patterns determined the outcome of the two post-tetanic plasticities. However, owing to the complex structure of the mature mouse NMJ, we needed a protocol that would allow us to reliably manipulate multiple PSCs at the same time. To do this, we used loading of membrane-permeant, caged compounds combined with specific light activation of PSCs.

This technique allowed us to target PSCs overlying the presynaptic nerve terminal by using precise and specific placement of the fiberoptic probe, and by selecting NMJs with a favorable morphological conformation, as indicated in the Methods section (Fig. 2*A*,*B*). Additionally, synapses were selected only when multiple PSCs were visible on the surface. The alignment of the fiberoptic probe was performed using visible red light emitted by an HeNe laser and passed through the optic fiber such that the cone of light covered at least two PSC somata and not the presynaptic terminal.

We controlled for possible direct presynaptic effects in each experiment by monitoring rapid changes (within seconds) (Kamiya and Zucker, 1994) in transmitter release and PPF. This approach is based on the rapid and readily reversible effects observed when manipulating presynaptic calcium levels using photolysis experiments. Finally, a brief calcium elevation in presynaptic terminals is known to rapidly and transiently increase transmitter release. However, UV exposure in conditions where PSCs were targeted had no rapid effect on transmitter release (Fig. 2*C*) or on paired-pulse facilitation (PPF was 0.69 ± 0.06 in control and 0.72 ± 0.06 after uncaging, $p > 0.05$). This is consistent with the latency of the slower time course of the glial modulation compared with a direct presynaptic effect. In addition, no changes in PSP kinetics were observed (10–90% rise time, 2.41 ± 0.33 ms; before, 2.07 ± 0.17 ms; after; 10–90% decay 14.30 ± 3.25 ms; before, 14.97 ± 3.66 ms; after, significance at $p < 0.05$, $n = 4$). The photolysis was efficient since a Ca^{2+} elevation was observed in the PSCs (Fig. 2*C*, inset). On the other hand, when photolysis was performed on an NMJ where the presynaptic terminal could be targeted, we observed a rapid and short-lived rise in transmitter release (Fig. 2*D*) concomitant with a reduction in paired-pulse facilitation (0.89 ± 0.05 in control and 0.78 ± 0.04 after uncaging, $p < 0.05$).

Furthermore, the addition of a calcium chelator into the presynaptic terminal should effectively reduce transmitter release (Delaney et al., 1989, 1991; Adler et al., 1991; Robitaille and Charlton, 1992; Robitaille et al., 1993). This is known to be a sensitive assay for presynaptic effect. Importantly, the introduction of a calcium chelator will have only transient effects on transmitter release unless its concentration is continuously maintained (Adler et al., 1991). Indeed, a transient reduction of transmitter release was observed when the presynaptic terminal was targeted as indicated above (data not shown). However, such a transient reduction of transmitter release was never observed in our experiments when caged BAPTA (diazo-2) was photo-activated in a PSC-specific manner (Fig. 2*E*). Hence, these control experiments indicate that the experimental strategy of choosing NMJs allowed us to selectively target PSCs without affecting the presynaptic terminal. Experiments were discarded when direct presynaptic effects were observed.

Finally, we controlled for possible postsynaptic effects by directly injecting the impermeant form of NP-EGTA in the muscle fiber. We found no evidence for a postsynaptic, muscle fiber contribution to synaptic transmission or plasticity when PSCs

were exposed to our photolysis protocols (Fig. 2*F*), as indicated by the lack of effect on PSP amplitude and the absence of Ca^{2+} elevation in PSCs.

Selective regulation PSC Ca^{2+}

Using the approach described above for NMJ selection and specific PSC activation, we determined how to elicit different Ca^{2+} responses using different photo-activation protocols with NP-EGTA. Additionally, we determined how to block PSC activation by chelating Ca^{2+} using diazo-2, the caged BAPTA compound.

As shown in Figure 3*A*, a calcium elevation in PSCs was induced by photo-activation of the caged calcium molecule NP-EGTA after its loading with the membrane permeant form (20 μM). These responses can be repeated on the same cells (Fig. 3*B*). Additionally, we were able to control the properties of the calcium photolysis by adapting the pattern and duration of the UV exposure so that multiple, smaller calcium elevations in glial cells could be induced (Fig. 3*C*). Conversely, activating caged BAPTA after loading the cells with membrane-permeant form (Diazo2-AM, Invitrogen) blocked Ca^{2+} responses elicited in PSCs. Indeed, as shown in Figure 3*D*, ATP induced a first calcium response with average amplitude of 39.4 ± 8.5% $\Delta F/F_0$ (top), whereas the response was completely abolished after diazo-2 photo-activation (average change of 0.4 ± 0.2% $\Delta F/F_0$, bottom). Also, calcium responses in PSCs evoked by endogenous synaptic activity were no longer observed (Fig. 3*E*). These results indicate that PSCs can be activated or blocked using photo-activation of caged molecules.

Importantly, direct exposure to UV light had no effect on PSCs or synaptic transmission. Indeed, in the absence of any caged compound, sustained and large UV photolysis had no direct effect on the resting fluorescence of PSCs (Fig. 3*F*) ($n = 4$). Cell viability and excitability were confirmed by the ability of ATP to induce calcium responses similar to the ones elicited without prior exposure to UV flashes. In addition, PSPs evoked by paired-pulse stimulation in control were unaffected after UV photolysis in the absence of any caged compound ($n = 4$; PPF was 0.79 ± 0.06 in control and 0.77 ± 0.06 after uncaging, $p > 0.05$).

Endogenous glial calcium elevations determine the outcome of plasticity

After the validation of our technique, we next tested whether the pattern of glial activity caused the selective expression of short-term post-tetanic plasticity. If this is the case, we predict that preventing the glial Ca^{2+} rise should impair the outcome of the post-tetanic plasticity. We used the caged calcium chelator diazo-2 AM to rapidly buffer calcium upon photo-activation with UV light (Kamiya and Zucker, 1994). The UV fiberoptic probe was positioned to specifically target glial cells, as indicated above. The caged calcium chelator diazo-2 was then photo-activated in PSCs before nerve stimulation to effectively block PSC activity through inhibition of calcium elevations. As shown in Figure 4*A*, chelation of glial cell calcium during bursting stimulation no longer produced post-tetanic depression, but rather resulted in a potentiation in PSP amplitude. PSP amplitude was 122.8 ± 1.8% at 15 min compared with control stimulation ($n = 8$; $p < 0.0001$; two-tailed *t* test). Conversely, photolysis of diazo-2 before continuous stimulation prevented post-tetanic potentiation and induced depression (Fig. 4*B*) (94.9 ± 1.3%; $n = 7$; $p < 0.0001$; two-tailed *t* test).

These results clearly indicate the importance of glial activation (via calcium) in the expression of appropriate endogenous synaptic plasticity. Notably, these data suggest that glial cell calcium elevations are not all-or-none events, but contain specific information concerning the ongoing activity of the synapse. Furthermore, these data indicate that glial cells govern the outcome of

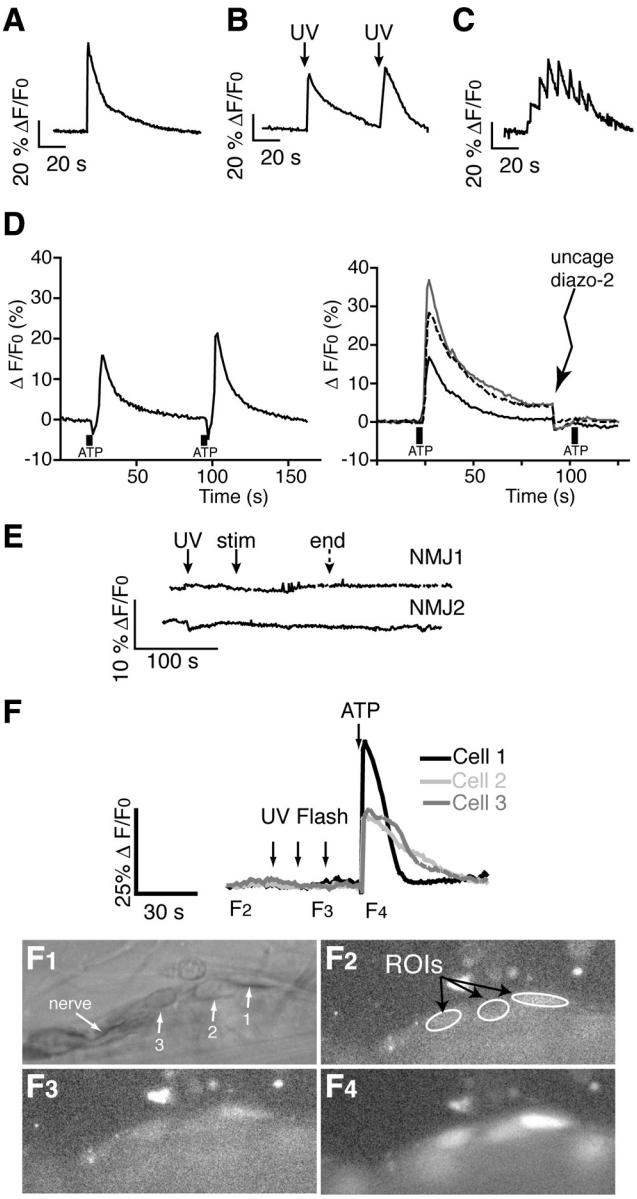

Figure 3. Manipulation of calcium in PSCs with caged compounds does not affect neuronal function. ***A***, ***B***, Single (***A***) or repeated (***B***) calcium elevations can be elicited in a single PSC using photo-activation of NP-EGTA. ***C***, Multiple, consecutive calcium elevations can be evoked in the same PSC by modifying the protocol of photo-activation of NP-EGTA. ***D***, Left, ATP-induced calcium responses in PSCs show little desensitization and are reproducible. Right, After a first ATP-induced calcium response with average amplitude of 39.4 ± 8.5% $\Delta F/F_0$, diazo-2 was photo-activated and ATP locally applied again on the same cells (shown are 3 responses from different NMJs), resulting in an average change of 0.4 ± 0.2% $\Delta F/F_0$. The second response was completely prevented when diazo-2 was uncaged before local application. ***E***, Calcium elevations evoked with endogenous nerve stimulation were abolished by prior photolysis of diazo-2 in PSCs. ***F***, Top, Large UV photolysis had no direct effect on the resting fluorescence of PSCs in the absence of any caged compound. Cell viability was confirmed by the ability of ATP to induce calcium responses that were similar to the ones elicited without prior exposure to UV flashes ($n = 4$). Bottom, Bright field and fluorescent images of the same neuromuscular junction as depicted graphically with cells 1, 2, and 3 indicated (***F1***). ***F2***, Fluorescent image acquired during the baseline period before the UV flashes. Measured regions of interest (ROI) are indicated. ***F3***, Fluorescent image acquired immediately after the second UV flash. ***F4***, Fluorescent image acquired immediately after local application of ATP.

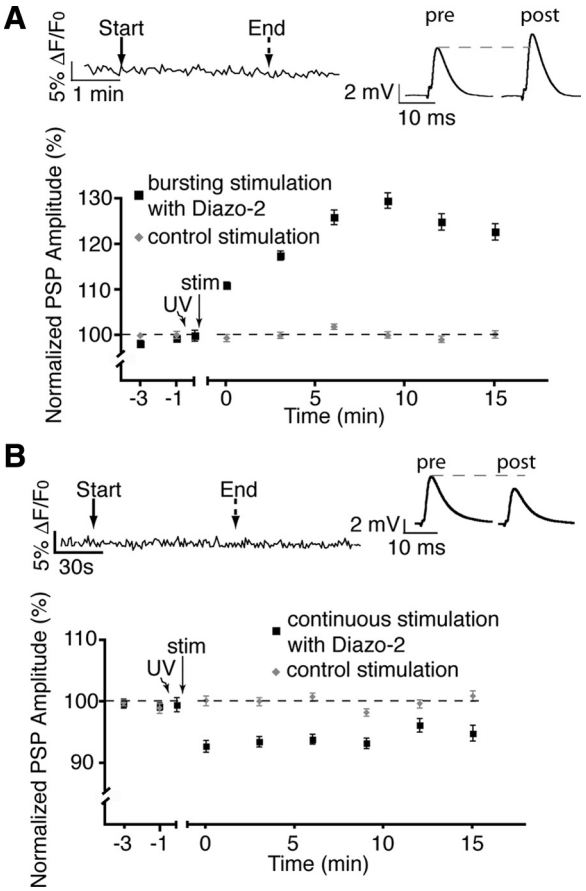

Figure 4. Inhibition of glial calcium elevations alters plasticity. ***A***, Normalized PSP amplitude over time showing that post-tetanic depression was prevented by the photolysis of diazo-2 (caged BAPTA) in glial cells during burst stimulation revealing a potentiation. The top left inset shows the absence of a detectable Ca^{2+} rise in the PSCs. The top right inset shows representative PSPs (averages of 30 events) taken from a single recording at the 15 min time point. ***B***, Photolysis of diazo-2 in glial cells during continuous stimulation blocked all post-tetanic potentiation. The top left inset shows that no detectable Ca^{2+} elevations were observed in glial cells after photolysis of diazo-2. The top right inset shows representative PSPs.

synaptic plasticity in a context-dependent manner. Hence, not only do glial cells decode neuronal activity, but their differential activation results in an adjusted feedback relevant to the current state of the synapse.

Glial calcium elevations are sufficient to induce plasticity in a pattern-dependent manner

To determine the importance of the distinct glial calcium signals observed during different patterns of synaptic activity, we designed protocols using photolysis of caged calcium compounds to mimic endogenously evoked calcium responses and, more importantly, the synaptic plasticity. Furthermore, direct glial activation would allow us to tease apart the underlying mechanisms, a task that would be difficult with endogenous activity alone since both the presynaptic terminal and PSCs bear many of the same types of receptors (e.g., A_1 and muscarinic ACh receptors) (Hamilton and Smith, 1991; Correia-de-Sá et al., 1996; Grafe et al., 1999; Galkin et al., 2001; Rochon et al., 2001; Oliveira et al., 2002, 2004; De Lorenzo et al., 2004; Silinsky, 2004; Baxter et al., 2005; Moores et al., 2005; Todd and Robitaille, 2006; Dudel, 2007).

Based on the control experiments presented in Fig. 3, we established two different photolysis protocols to elicit either multiple calcium responses, thus mimicking the oscillatory responses seen with bursting stimulation, or a single calcium response, similar to that seen with continuous stimulation (Fig. 5*A*, insets). Individual responses occurring during the burst activity elicited by the repeated photolysis protocol were not significantly different from endogenously evoked responses in amplitude or duration ($p > 0.05$, two-tailed t test). On average, each transient calcium response had an amplitude of 38.5 ± 5.1% $\Delta F/F_0$ and a duration of 8.2 ± 1.0 s ($n = 25$ events, from 8 PSCs and 8 NMJs). Similarly, calcium responses elicited by a single photo-activation were larger and longer (average amplitudes of 122.9 ± 14.2% $\Delta F/F_0$ and average duration of 51.9 ± 9.7 s, $n = 11$ PSCs from 11 NMJs) then the transient responses occurring during the bursting activity but were not different from the responses elicited by endogenous activity ($p > 0.05$, Student's t test) (Fig. 1*B2*).

Furthermore, the photo-activated responses in PSCs mimicked the endogenous synaptic plasticity events. Indeed, PSP amplitudes were depressed (88.6 ± 1.5%, $p < 0.0003$, two-tailed t test, $n = 7$) compared with controls after multiple photo-activations (Fig. 5*A*), and were not statistically different from those evoked by endogenous 20 Hz burst stimulation ($p > 0.05$; Student's t test). In contrast, evoking a single calcium response induced a potentiation of PSP amplitude compared with controls (106.4 ± 1.2%, $p = 0.0006$, two-tailed t test, $n = 8$), similar to that seen after continuous stimulation ($p > 0.05$; Student's t test).

Importantly, not only did direct glial activation elicit plasticity events that were similar to the endogenous ones, but the two methods of glial activation occluded each other. Indeed, when nerve stimulation was followed by direct activation of glia, no further potentiation (108.2 ± 1.4% at 15 min, $n = 6$) or depression (81.9 ± 3.4% at 15 min, $n = 6$) was observed (Fig. 5*B*). Similarly, photolysis-induced plasticity followed by stimulation did not result in greater plasticity (Fig. 5*C*). Therefore, the two methods of glial activation and induction of synaptic plasticity (photolysis or endogenous) occlude each other indicating that photolysis-induced activation mimics endogenous activation of glial cells and the resulting changes in synaptic efficacy. More importantly, these results demonstrate that direct, differential activation of glial cells is sufficient to selectively induce post-tetanic potentiation and depression.

Purine receptors regulate PSC-induced plasticity

Knowing that glial cells were responsible for controlling bidirectional plasticity, we next investigated the mechanisms involved. A number of receptor systems offered the possibility of an opposing regulation of synaptic efficacy, such as muscarinic and purine receptor systems (Correia-de-Sá et al., 1996; Dudel, 2007). Since ATP is a prominent gliotransmitter involved in a number of neuronal regulations by glial cells (Fields and Burnstock, 2006), we tested the involvement of purines in our model of glial regulation of synaptic activity. We began by using the 5′-ectonucleotidase inhibitor ARL 67156 (50 μM) (Rebola et al., 2008) that prevents the degradation of ATP into its metabolites. As shown in Figure 6*A*, rather than a depression of PSP amplitude, we observed a potentiation of 113.5 ± 1.8% compared with controls ($n = 4$; $p < 0.0001$, two-tailed t test) in the presence of ARL 67156 after the multiple photolysis protocol. Conversely, when we performed a single photolysis of caged calcium in the presence of ARL (Fig. 6*B*), which normally produced potentiation of PSP amplitude, we observed no post-tetanic plasticity (102.0 ± 1.2%; $n = 4$; $p > 0.05$). These changes confirm that indeed a purine-dependent system is necessary for the glial-dependent modulation. However, it is unlikely that these results can be explained by the accu-

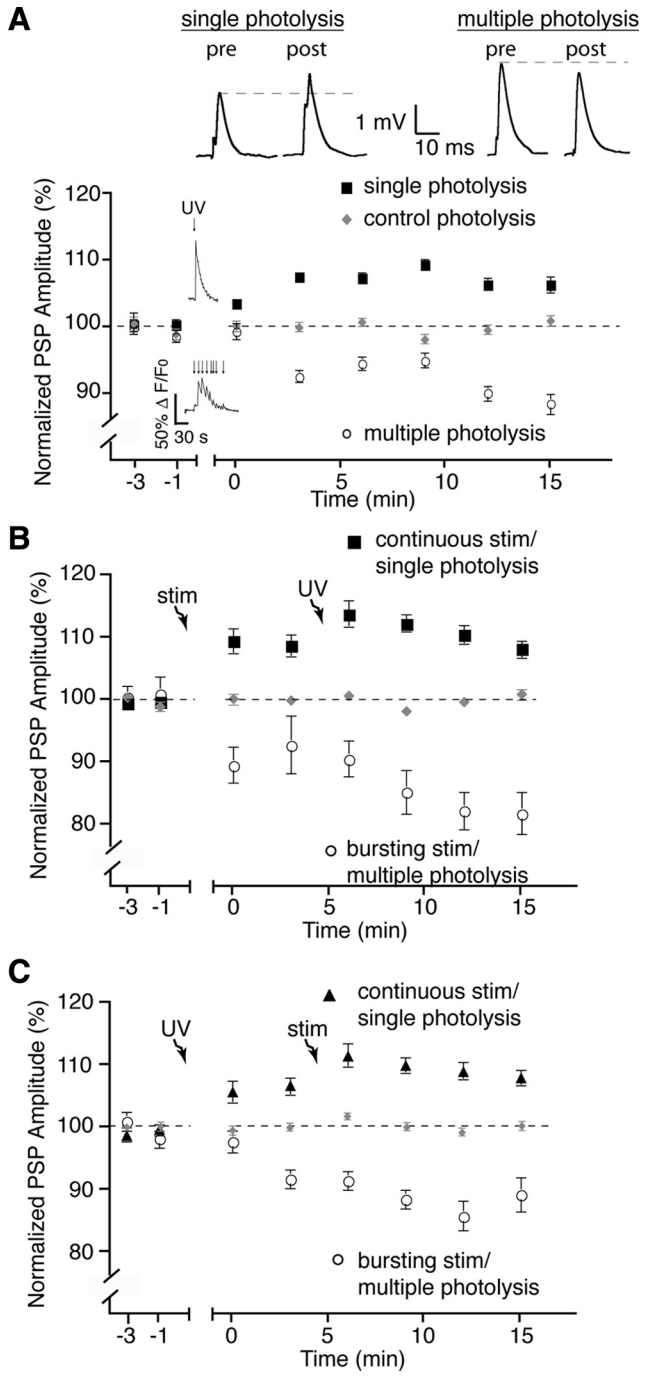

Figure 5. Direct induction of glial calcium elevations is sufficient for plasticity. ***A***, Normalized PSP amplitude over time showing that multiple photolysis of NP-EGTA (caged calcium) in glial cells induced a prolonged elevation of glial calcium and post-tetanic depression similar to burst stimulation, whereas a single calcium elevation induced with photolysis of NP-EGTA in glia resulted in a post-tetanic potentiation similar to continuous stimulation. Insets in the graph illustrate a typical PSC Ca^{2+} responses elicited by photo-activation of NP-EGTA. Insets on the top show representative PSPs (averages of 30 events) taken from a single recording at the 15 min time point. ***B***, Normalized PSP amplitude over time showing that photolysis of NP-EGTA performed after nerve stimulation did not induce further plasticity after that induced by the endogenous activity. ***C***, Normalized PSP amplitude over time where photolysis of NP-EGTA was performed before nerve stimulation. No additional effect on post-tetanic plasticity was observed. Photolysis and stimulation-induced plasticity occluded each other. pre, Before; post, after; stim, stimulation.

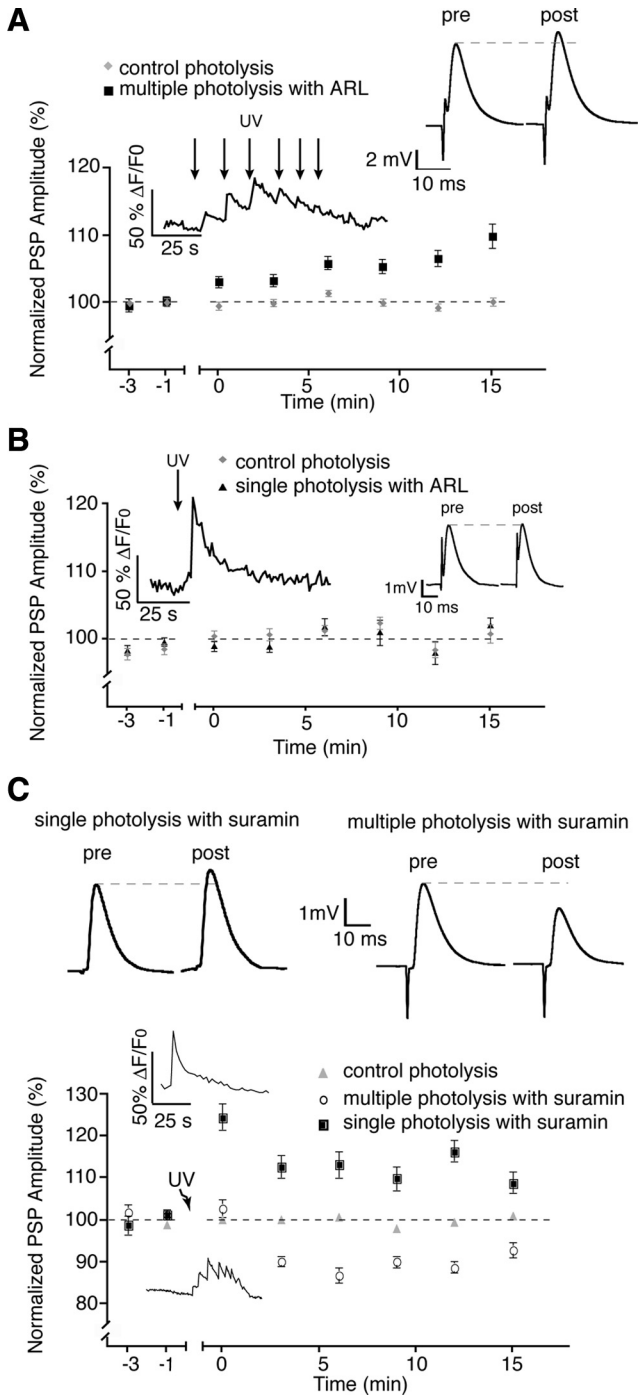

Figure 6. Purines but not ATP are implicated in plasticity. ***A***, Blockade of ATP hydrolysis with ARL-67156 prevented post-tetanic depression normally observed when activating PSCs with the multiple photolysis protocol. ***B***, The presence of ARL-67156 prevented post-tetanic potentiation normally induced by single photolysis of NP-EGTA. ***C***, Addition of 100 μM suramin had no effect on potentiation or depression induced by the two photo-activation protocols. Insets in the graphs illustrate typical PSC Ca^{2+} responses elicited by photo-activation of NP-EGTA. Insets on the right show representative PSPs (averages of 30 events) taken from a single recording at the 15 min time point. pre, Before; post, after.

mulation of ATP acting directly on P2 receptors since the presence of the nonselective P2 receptor antagonist suramin (100 μM) (Cunha et al., 1998) did not affect the outcome of the photolysis induced potentiation (105.9 ± 3.5%, $p = 0.87$) or depression (92.9 ± 1.8%, $p = 0.13$) (Fig. 6*C*). The apparent lack of ATP receptor involvement is further supported by these results, con-

sidering that this antagonist is notorious for its weak specificity and is therefore likely to have inhibited any form of ATP receptor present. Hence, this suggests that the endogenous modulation of synaptic plasticity is due to the degradation of glial ATP into adenosine.

Adenosine A_1 receptor mediate post-tetanic depression

We next tested the possibility that the blockade of ATP hydrolysis resulted in absence of adenosine, and an imbalance in A_1 and A_{2A} receptor regulation. Throughout the nervous system, A_1 and A_{2A} adenosine receptors mediate depression and potentiation, respectively (Correia-de-Sá et al., 1996; Lopes et al., 2002; Pousinha et al., 2010). Hence, one would predict that blocking A_1 receptors would alter PSC-mediated depression, whereas blocking A_{2A} receptors would alter PSC-mediated potentiation. Consistent with this possibility, bath application of an A_1 receptor agonist (CPA, 30 nm) (O'Neill et al., 2007; Serpa et al., 2009) reduced PSP amplitude to 81.8 ± 5.7%% of control ($p < 0.05$), whereas an A_{2A} agonist (CGS21680, 7.5 nM; Loram et al., 2009; D'Alimonte et al., 2009) increased PSP amplitude to 108.2 ± 2.2% relative to control ($p < 0.05$) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Consistent with the known role of A_1 receptors at the neuromuscular synapse (Redman and Silinsky, 1994), addition of the A_1 adenosine receptor antagonist PSB-36 (5 nM) (Weyler et al., 2006) did not affect base line synaptic transmission but when applied before photolysis-mediated activation of PSCs resulted in a post-photolysis potentiation of PSP amplitude rather than a post-tetanic depression (105.8 ± 1.3%, $n = 8$; $p = 0.002$, two-tailed t test) (Fig. 7*A*). The involvement of A_1 receptors in post-tetanic depression was further confirmed through the use of A_1 receptor knock-out mice. We observed no depression in A_1 −/− (98.7 ± 1.7%, $n = 8$) NMJ preparations (Fig. 7*B*), whereas depression was normal in +/+ controls (88.6 ± 1.6%) after the multiple photolysis protocol ($p < 0.0001$). Furthermore, the PSC-induced depression was occluded by prior activation of A_1 receptors with the selective (Lohse et al., 1988) A_1 agonist CCPA (0.1 μM; 104.2 ± 1.5%; $p = 0.05$; two-tailed t test; $n = 6$) (Fig. 7*C*).

Finally, depression was not significantly altered when performing the multiple photolysis protocol in the presence of SCH-58261 (50 nM) (Rebola et al., 2008), an A_{2A} receptor antagonist (depression of 94.4 ± 1.3%, from control) (Fig. 7*D*) ($p < 0.0001$, $n = 6$) or when tested at NMJs from A_{2A} −/− mice compared with +/+ controls (Fig. 7*E*) (−/−, 91.6 ± 1.2%; +/+, 91.0 ± 1.7%; $p > 0.05$, $n = 4$). As a whole, these experiments indicate that the endogenous pattern of activity leading to glial-mediated post-tetanic depression involves A_1 receptor activation after ATP degradation.

Adenosine A2 receptors mediate post-tetanic potentiation

Since our hypothesis is based on the different forms of short-term plasticity generated by a balanced A_1 and A_{2A} receptor activation, we next tested the involvement of A_{2A} receptor activation in the modulation of glial-mediated potentiation. We used the single photolysis protocol to test for the involvement of A_{2A} receptors in the glial-mediated post-tetanic potentiation. The presence of an A_{2A} antagonist SCH-58261 (50 nM) had no effect on basal synaptic transmission but blocked the expression of the glial-induced post-tetanic potentiation, and resulted in depression (Fig. 8*A*) (93.0 ± 1.5%; $n = 5$; $p < 0.0001$, two-tailed t test, $n = 7$). In addition, post-tetanic potentiation was absent at NMJs of A_{2A} −/− mice (99.6 ± 1.3%) (Fig. 8*B*) but not in the +/+ littermates

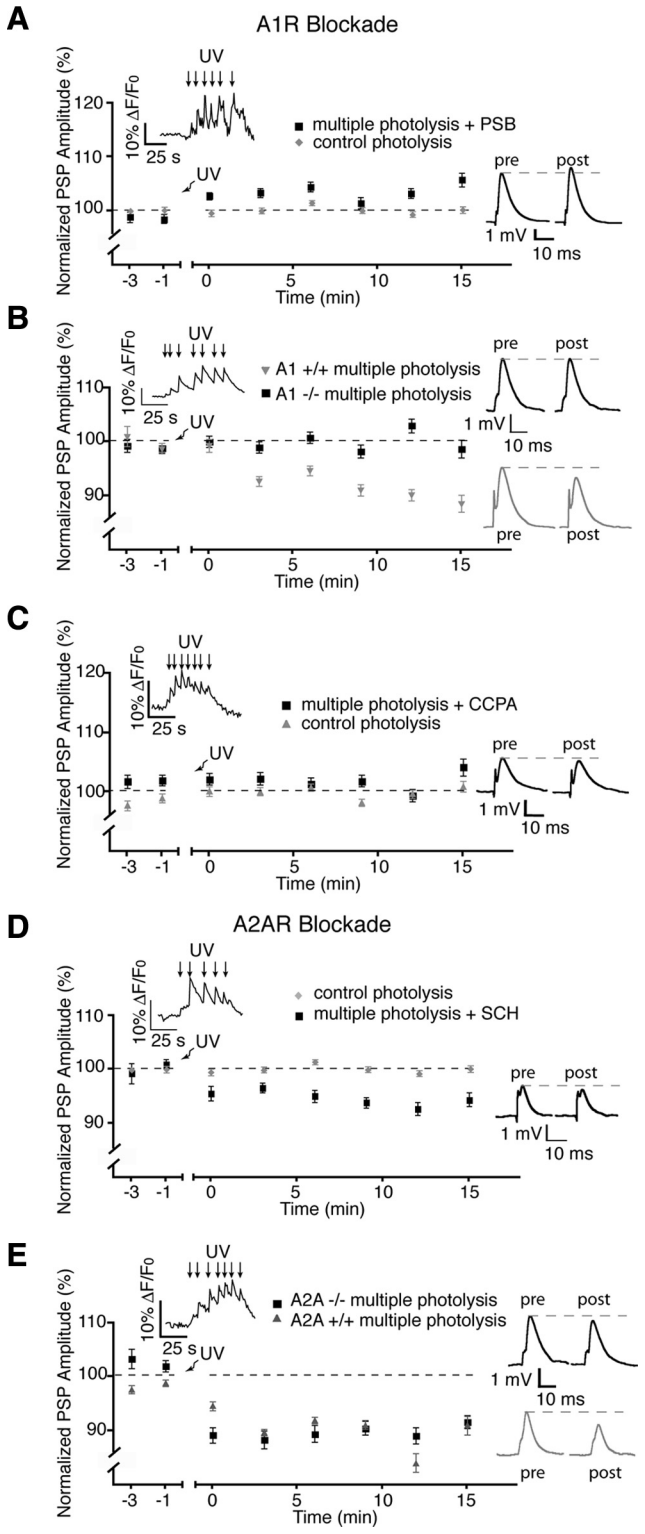

Figure 7. Post-tetanic depression is regulated by A1 adenosine receptors. ***A***, Post-tetanic depression induced by multiple photolysis of NP-EGTA in glial cells was blocked by the A_1 receptor antagonist PSB-36. ***B***, No post-tetanic depression was elicited by multiple photolysis at NMJs of A_1 −/− animals. ***C***, Post-tetanic depression was occluded by prior addition of the A_1 agonist CCPA. ***D***, Presence of the A_{2A} receptor antagonist SCH has no effect on post-tetanic depression. ***E***, Post-tetanic depression was evoked by the multiple photolysis protocol at NMJs from A_{2A} −/− mice. Insets in the graphs illustrate typical PSC Ca^{2+} responses elicited by photo-activation of NP-EGTA. Insets on the right show representative PSPs (averages of 30 events) taken from a single recording at the 15 min time point. pre, Before; post, after.

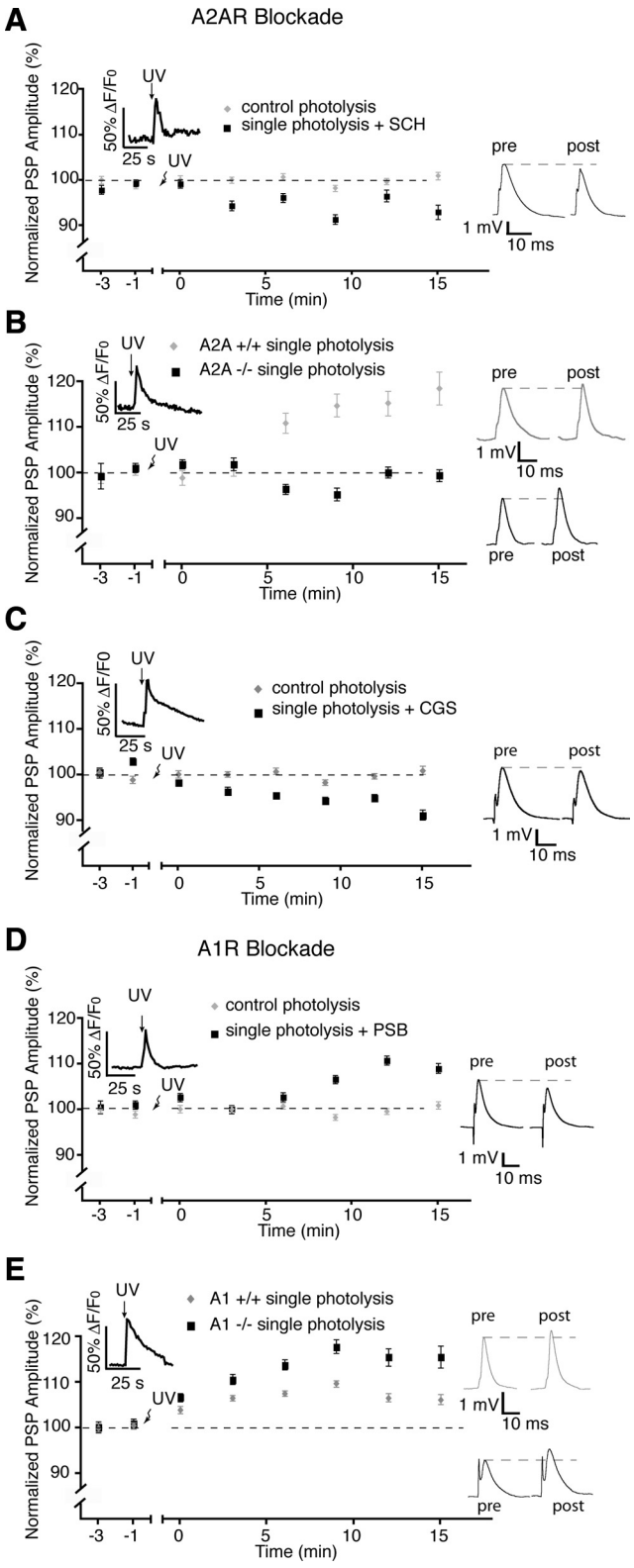

Figure 8. A_{2A} receptors regulate post-tetanic potentiation. ***A***, The presence of the A_{2A} receptor antagonist, SCH, prevented post-tetanic potentiation induced by single photolysis of NP-EGTA in PSCs. ***B***, No post-tetanic potentiation was elicited with the single photolysis protocol at NMJs of A_{2A} −/− animals. ***C***, Post-tetanic potentiation was occluded by prior addition of the A_{2A} agonist CGS21680. ***D***, Addition of the A_1 receptor antagonist PSB-36 had no effect on post-tetanic potentiation. ***E***, Post-tetanic potentiation was induced by the single photolysis protocol at NMJs from A_1 −/− mice. pre, Before; post, after.

(118.6 ± 3.6%; $p < 0.0001$, two-tailed t test, $n = 4$). Prior application of CGS-21680 (30 nM) (Rebola et al., 2008), an A_{2A} agonist, occluded the PSC-induced potentiation and resulted in a depression (91.2 ± 1.2%) that was significantly reduced from controls ($p < 0.0001$, two-tailed t test, $n = 6$), further confirming the involvement of A_{2A} receptors in potentiation (Fig. 8*C*). Finally, use of the single photolysis protocol in the presence of the A_1 receptor antagonist PSB-36 (5 nM) resulted in potentiation of PSP amplitude (109.1 ± 1.1%; $p < 0.0001$; $n = 6$) (Fig. 8*D*), as did the use NMJs from A1 −/− mice (see Fig. 10*E*) (115.6 ± 2.4%, $n = 6$), which was greater than +/+ littermates (106.3 ± 1.2%; $p = 0.0001$, $n = 6$). Together, the results strongly implicate a glial-regulated activation of A_1 and A_{2A} receptors in the generation of pattern-dependent induction of depression and potentiation at the NMJ.

Adenosine receptors mediate the plasticity induced by motor nerve stimulation

Knowing that the glial mechanisms regulating the sustained potentiation and depression involved a balance of antagonistic adenosine effects, we next tested whether these receptors were involved in the plasticity events induced by motor nerve stimulation and endogenous release of neurotransmitters. As shown in Figure 9, *A* and *B*, bath application of the A_1 antagonist PSB-36 (5 nM) had no effect on basal level of neurotransmission but completely prevented the expression of the synaptic depression that is normally observed when using the bursting protocol, and a small potentiation was even observed (103% ± 1.4%, $p < 0.05$). Conversely, rather than the potentiation, a small depression (92.1 ± 1.9%, $p < 0.05$) was observed in the presence of the A_{2A} receptor antagonist (SCH-58261, 50 nM) when stimulating the motor nerve with the continuous paradigm. Hence, these data suggest that the endogenous plasticity events are also mediated by a balanced A_1/A_{2A} regulation.

Adenosine receptors do not mediate the sustained plasticity after blockade of PSC activity

The observations that the polarity of post-tetanic plasticity events was reversed after the blockade of PSCs suggest that different mechanisms are recruited when glial regulation is perturbed. A possibility might be that the reversal is due to the activation of the antagonistic adenosine receptor, A_1 rather than A_{2A} and vice versa. This possibility would be consistent with the involvement of these receptors in the endogenous plasticity events elicited by motor nerve stimulation (Fig. 9*A*,*B*). This possibility was examined by testing the effects of A_1 and A_{2A} receptor antagonists on the reversal of the plasticity events observed after Ca^{2+} chelation in PSCs.

As shown above, blockade of PSC activity by photo-activation of diazo-2 resulted in a depression of 65 ± 11% with continuous motor nerve stimulation (rather than potentiation) and a potentiation of 103% ± 1.1% with the bursting protocol (rather than depression) (Fig. 9*C*,*D*). However, a depression was still observed in the presence of the A_1 antagonist (PSB-36, 5 nM; 60 ± 11%) while PSP amplitude remained potentiated even when the A_{2A} antagonist (SCH-58261, 50 nM) was present (106.1 ± 2.4%). Hence, the reversal observed in the post-tetanic plasticity cannot be explained by a switch in the adenosine receptor involved.

Discussion

Glial cells display finely tuned responsiveness to neuronal activity. They detect subtle changes in the frequency of activity (Pasti et al., 1997) and discriminate between different synaptic inputs

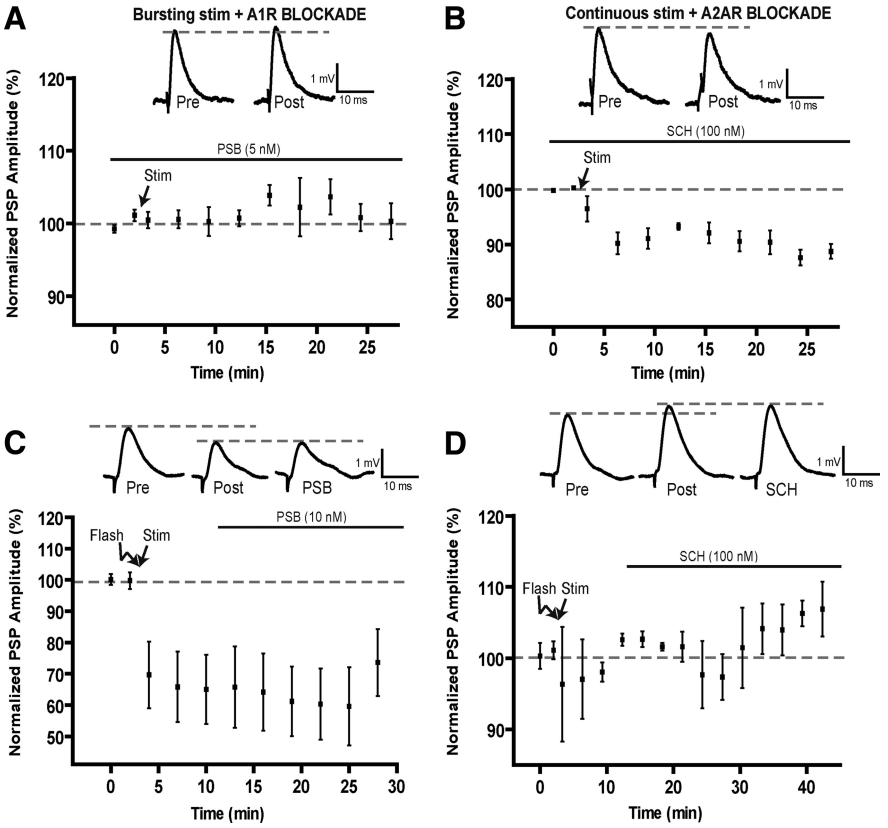

Figure 9. Role of adenosine receptors in post-tetanic plasticity induced by motor nerve stimulation. ***A***, ***B***, Changes in PSP amplitude before and after bursting stimulation in the presence of the A_1 antagonists PSB-36 (***A***) and before and after continuous stimulation in the presence of the A_{2A} antagonist SCH-58261 (***B***). Note that the A_1 antagonist prevented the production of post-tetanic depression, whereas the A_{2A} antagonist prevented the potentiation, as a depression was even observed. Insets represent representative PSPs before (Pre) and after (Post) the motor nerve stimulation. ***C***, Changes in PSP amplitude before and after photo-activation of diazo-2 in PSCs (Flash) and the stimulation of the motor nerve using the continuous paradigm (Stim). Bath application of the A_1 antagonist (PSB-36, black bar) was started 10 min after the end of the continuous stimulation period. Insets show a typical PSP before (Pre), after the diazo-2 photo-activation and motor nerve stimulation (Post) and during bath application of the A_1 antagonist (PSB). Note that the A_1 antagonist did not affect post-tetanic depression. ***D***, Changes in PSP amplitude before and after photo-activation of diazo-2 in PSCs (Flash) and the stimulation of the motor nerve using the bursting paradigm (Stim). Bath application of the A_{2A} antagonist (SCH-58261, black bar) was started 10 min after the end of the bursting stimulation paradigm. Insets indicate a typical PSP before (Pre), after the diazo-2 photo-activation and motor nerve stimulation (Post), and during bath application of the A_{2A} antagonist (SCH). Note that the potentiation was not altered by the presence of the A_{2A} antagonist.

(Perea and Araque, 2005). Here, we show that glia differentiate patterns of synaptic activity, an important element regulating synaptic plasticity. They integrate and decode the pattern of neuronal and synaptic activity to, in turn, influence synaptic transmission. Our data indicate that this regulation likely occurs by processing incoming information that alters and tunes subsequent feedback to a given synapse.

Neuronal communication depends on interplay between glial cells and neurons

Our data show that PSCs were differentially activated by different patterns of neuronal activity whereas blockade of their Ca^{2+}-dependent activity perturbed the outcome of synaptic plasticity. This indicates that neurons are no longer the only cells that decode information from patterns of neuronal activity and process it to influence the outcome of synaptic communication. Owing to this decoding capability, glial cell regulation of short-term and long-term synaptic plasticity should no longer be seen as an all-or-none event, but rather as an adaptable regulation dependent on the context of previous neuronal activity. This additional level of detection would allow glial cells to mediate and regulate a large array of synaptic events. Also, our results are consistent with the notion presented earlier (Robitaille, 1998; Castonguay and Robitaille, 2001) that glial cells may not produce plasticity events themselves, but rather modulate and control presynaptic and postsynaptic mechanisms. Indeed, the persistence of some forms of plasticity after Ca^{2+} chelation in PSCs argues in favor of this, whereas the lack of purinergic sensitivity of the residual plasticity suggests that other receptor systems also regulate synaptic efficacy at the NMJ. Hence, glial cells add another level of regulation to neuronal synaptic plasticity.

Impacts on CNS synapses

The data obtained here using a PNS synapse are likely applicable to synapses throughout the nervous system. Indeed, it has been demonstrated previously that direct activation of retinal glial cells can induce both positive and negative changes in neuronal activity (Newman and Zahs, 1998), suggesting similar roles to those described here. Furthermore, Panatier et al. (2006) recently described a phenomenon similar to ours, occurring in the hypothalamus. They showed that glial cells determined the outcome of synaptic plasticity (potentiation or depression) as a result of changes in the glial synaptic coverage and release of D-serine. These are only some examples while more are continually added, suggesting the involvement of glial cells at synapses throughout the nervous system (Serrano et al., 2006; Perea and Araque, 2007; Fellin et al., 2009; Gordon et al., 2009; Henneberger et al., 2010). Here, we have built on the current knowledge of glial involvement in synaptic function by providing evidence that glial cells, acutely responding to neuronal activity, can supply context-dependent feedback to synapses by decoding the pattern of neuronal activity. Hence, our data indicate that glial regulation of neuronal plasticity is not only a matter of slow long-term modification to the glial environment, but that it occurs over a matter of minutes. Morphological synaptic changes are an interesting avenue to explore since they can occur within a similar time window (Matsuzaki et al., 2004).

Unlike the work discussed above, Agulhon et al. (2010) reported that Ca^{2+} elevation in astrocytes did not alter short-term and long-term plasticity. Interestingly, they evoked sustained and massive agonist-induced activation of a foreign receptor overexpressed in astrocytes, whereas Henneberger et al. (2010) and ourselves used more subtle glial regulation and stimulation paradigms. An interesting possibility might be that intense stimulation of glial cells puts them in a less responsive mode that depresses their normal Ca^{2+}-dependent mechanisms. However, experiments directly addressing this issue need to be performed to solve this conundrum.

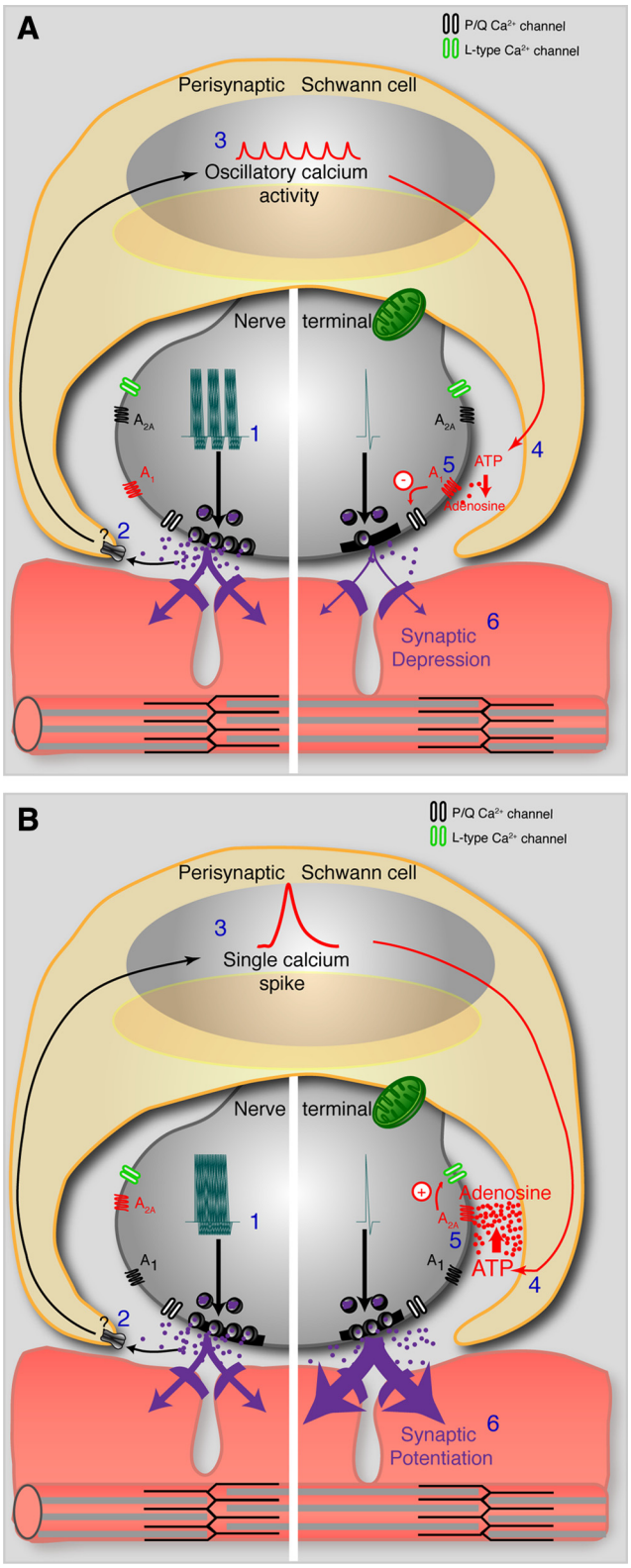

Figure 10. Model of glial-mediated bidirectional modulation of synaptic plasticity. ***A***, Bursts of activity (1) induce the release of neurotransmitter to activate perisynaptic Schwann cells (2) and the postsynaptic terminal. Receptor activation on PSCs leads to oscillatory calcium elevations (3) and the release of a lesser amount of glial-derived ATP (4) that is degraded to adenosine. Relatively low levels of synaptic adenosine lead to synaptic depression through activation of A_1 receptors and a decrease in presynaptic calcium entry through P/Q-type calcium channels (5 and 6). ***B***, Continuous presynaptic activity (1) induces the release of neurotransmitter to activate perisynaptic Schwann cells (2) and the postsynaptic terminal. Receptor activation on PSCs leads to a single calcium elevation (3) and the release of a larger amount of glial-derived ATP (4) that is degraded to adenosine. Relatively high levels of synaptic adenosine lead to activation of A_{2A} receptors, activation of L-type calcium channels (5) and synaptic potentiation (6).

Glial cells decode neuronal signaling through calcium dynamics

We found that PSCs decode the pattern of activity by producing selective signature of global Ca^{2+} elevation. Notwithstanding spatial and temporal limitations inherent to the Ca^{2+} imaging technique, our data suggest that specific glial responses have direct consequences on the output of synaptic plasticity. Indeed, small and brief responses repeated over a longer period induced depression, whereas responses with larger amplitude and duration caused potentiation. Interestingly, this situation is reminiscent of the calcium hypothesis of the switch between long-term potentiation and long-term depression production in hippocampus where a rapid and short-lasting Ca^{2+} elevation leads to a potentiation, whereas smaller and repeated elevations lead to depression (Yang et al., 1999).

This observation suggests that the excitability of neurons and glial cells share similar biochemical mechanisms since both amplitude and duration of an intracellular chemical signal such as Ca^{2+} determine the outcome of the biological process. Also, considering Ca^{2+}-related regulation, these data suggest that glial cells and neurons regulate neuronal communication within the same time domain. This strengthens the idea that glial and neuronal elements need to be in tune with each other for proper neuronal communication to occur (Serrano et al., 2006).

Glial regulation occurs through a balanced A_1/A_{2A} receptor activation

Activation of A_1 and A_{2A} receptors results in inhibition and activation of different types of presynaptic calcium channels to regulate transmitter release at synapses in general and at the mammalian NMJ in particular (Correia-de-Sá et al., 1996; De Lorenzo et al., 2004; Oliveira et al., 2004; Silinsky, 2005; Cunha, 2008). Moreover, an adenosine-dependent long-term depression has been reported (Redman and Silinsky, 1994) that involves an inhibition of presynaptic calcium currents (Silinsky, 2004, 2005). Our data indicate that the balanced A_1-A_{2A} regulation can be controlled, not only by the presynaptic pool itself in an autocrine manner, but also by perisynaptic glial cells. In addition, adenosine-dependent glial regulation appears both necessary and sufficient, as indicated by the blockade of the potentiation and depression induced by direct glial activation or by motor nerve stimulation.

Our data are consistent with the general perspective in the field of purinergic research where the synaptic regulation by adenosine depends on a balanced activation of inhibitory A_1 and facilitatory A_{2A} receptors. Importantly, this balanced regulation depends not only on the level of extracellular adenosine but also on direct interactions between A_1 and A_{2A} receptors through cross-regulatory actions (Cunha, 2001). This tight autoregulated interaction is believed to be the basis for the unbalanced effects that are observed when one receptor type is specifically blocked. Indeed, similar to what we observed in this work, blocking a receptor type does not necessarily results in a simple blockade but rather to a reversed effect. Hence, the governance by adenosine receptors cannot be interpreted on the basis of individual receptor regulation but on combined and interactive functions of all receptors.

Model of bidirectional glial regulation

We propose a model (Fig. 10) based on the properties of A_1-A_{2A} receptor interactions as discussed above (see also Johansson et al., 2001), the ability of glial cells to release ATP (Fields and Burnstock, 2006), and the balanced regulation of cell activity during spatiotemporal changes in intracellular Ca^{2+} levels. Smaller but sustained accumulation of glial calcium elicited by bursting synaptic activity would induce the release of a smaller amount of ATP at any given time, leading to a lower concentration of adenosine in the synaptic cleft, the activation of A_1 receptors, and post-tetanic depression. Conversely, post-tetanic potentiation would be induced by larger and more transient glial calcium responses elicited by sustained synaptic activity causing the release of a greater quantity of ATP, therefore leading to more adenosine and the activation of A_{2A} receptors. Furthermore, our data and model are consistent with adenosine receptor pharmacology and knock-out manipulations reported previously (Fredholm et al., 2005) where the outcome could not be accounted for simply by the regulation of a single type of receptor. Importantly, possible involvement of other adenosine receptors such as A_{2B} and A_3 receptors cannot be ruled out completely.

Potential involvement in diseases

Our data show that PSC regulation leads to changes in synaptic potency that would facilitate or reduce motoneuronal control of the NMJ, which is based on the pattern of neuronal activity itself. Interestingly, the PSC-mediated adenosine regulation could be a target for possible treatments of muscular diseases (e.g., myasthenia gravis) or conditions that lead to weakened synapses (e.g., aging) whereby patterns of activity could be made to favor PSC-dependent potentiating pathway, thereby strengthening the efficacy of the NMJ. Albeit small, these changes in plasticity could influence the level of excitability when repeated over the course of the daily neuromuscular activity.

In addition, these principles of neuron–glial interactions may apply to the basic function of CNS synapses, and their perturbation may contribute to a number of malfunctions. For instance, there is evidence suggesting that inadequate interactions between A_{2A} and D2 receptors may be in part responsible for certain aspects of the pathophysiology observed in Parkinson's disease and certain forms of drug addictions (Ferré et al., 2008). These interactions may not be solely neuronal in nature but may involve glial cells owing to their role in the regulation of adenosine receptors and their functions.

We demonstrate that glial cells can govern the outcome of synaptic plasticity based on their ability to decode the patterns of neuronal communication. These results indicate that glial cells can act as pattern detectors, a role that could influence many CNS functions.

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Annexe II

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SYNAPSE–GLIA INTERACTIONS ARE GOVERNED BY SYNAPTIC AND INTRINSIC GLIAL PROPERTIES

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Abstract—It is believed that glial cell activation and their interactions with synapses are predominantly dependent upon the characteristics of synaptic activity and the level of transmitter release. Because synaptic properties vary from one type of synapse to another, synapse-glia interactions should differ accordingly. The goal of this work was to examine how glial cell activation is dependent upon the properties of their respective synapses as well as the level of synaptic activity. We contrasted Ca^{2+} responses of perisynaptic Schwann cells (PSCs) at neuromuscular junctions (NMJs) with different synaptic properties; the slow-twitch *soleus* (SOL) and the fast-twitch levator auris longus (LAL) muscles. Amplitude of PSC Ca^{2+} responses elicited by repeated motor nerve stimulation at 40, 50 and 100 Hz were larger and their kinetics faster at LAL NMJs and this, at all frequencies examined. In addition, a greater number of PSCs per NMJ was activated by sustained synaptic transmission at NMJs of LAL in comparison to SOL. Differences in PSC activation could not be explained solely by differences in levels of transmitter release but also by intrinsic PSC properties since increasing transmitter release with tetraethylammonium chloride (TEA) did not increase their responsiveness. As a whole, these results indicate that PSC responsiveness at NMJs of slow- and fast-twitch muscles differ not only according to the level of activity of their synaptic partner but also in accordance with inherent glial properties.

Key words: perisynaptic Schwann cells, synaptic strength, synaptic plasticity, synapse–glia interactions, neuromuscular junction, neuromuscular properties.

In recent years, the traditional view that glial cells of central (CNS) and peripheral nervous systems (PNS) mainly provide support to neurons and synapses has been challenged. Indeed, glial cells are now considered to be active members of their associated synapses as they have been shown to be activated by synaptic transmission and, in turn, to modulate synaptic activity (Auld and Robitaille, 2003; Halassa et al., 2007). Glial cell activation as a function of synaptic activity is therefore a key feature of these bi-directional interactions. Knowing that synapses differ in terms of probability of release, quantal content, level of activity and plasticity, one would predict that glial properties should be adapted with their environment and tuned with their respective synaptic partners. However, it is unclear whether the properties of perisynaptic glial cells differ at synapses with different properties. Therefore, the aim of the present study was to examine the properties of glial cells at differing synapses and to study their activation as a function of the type of synapses they are associated with.

The neuromuscular junction (NMJ) is a valuable model to test this possibility. Indeed, studies of synapse-glia interactions at the NMJ have provided amongst the strongest evidence for the involvement of glial cells in the regulation of synaptic functions as part of a tripartite synapse (Robitaille, 1998; Castonguay and Robitaille, 2001; Todd and Robitaille, 2006; Todd et al., 2007; Feng and Ko, 2007, 2008). Furthermore, the properties of NMJs of various nerve-muscle preparations have been well characterized, in particular, NMJs of fast- and slow-twitch muscles (Gertler and Robbins, 1978; Wood and Slater, 2001; Slater, 2008). These synapses are both cholinergic in nature but differ in terms of their electrophysiological properties and functions. For instance, studies conducted in freely moving rats demonstrated that motor neurons innervating the fast-twitch *extensor digitorum longus* (EDL) muscle preferentially discharge at high frequencies (≈80 Hz) for short periods, therefore behaving in a phasic fashion. On the other hand, motor neurons innervating the slow-twitch soleus (SOL) muscle fire at lower frequencies (20 to 40 Hz) for longer periods of time, exhibiting a tonic behaviour (Henning and Lømo, 1985). Importantly, it has been demonstrated that synapses of fast- and slow-twitch muscles of the rat can also be distinguished by their neurotransmitter release properties where quantal content at NMJs of fast-twitch muscles was shown to be greater than that of NMJs from slow-twitch muscles (Reid et al., 1999). Interestingly, differences in neurotransmitter release properties between EDL and SOL NMJs of the rat have been shown to influence the outcome of post-tetanic plasticity (Lev-Tov, 1987).

Considering that motoneurons at NMJs of phasic and tonic muscles have different patterns of activity that are suited to their normal functioning, we compared the properties of perisynaptic Schwann cell (PSC) activation by monitoring Ca^{2+} responses at NMJs of the predominantly fast-twitch *Levator auris longus* (LAL) muscle (Erzen et al., 2000) and NMJs of the predominantly slow-twitch SOL

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Abbreviations: ACh, acetylcholine; EDL, *extensor digitorum longus* muscle; Em, membrane potential; EPPs, end plate potentials; HEPES, N′-2-Hydroxyethylpiperazine-N′-2 ethanesulphonic acid; LAL, *levator auris longus* muscle; NMJ, neuromuscular junction; PNS, peripheral nervous system; PSCs, perisynaptic Schwann cells; PTP, post-tetanic potentiation; SOL, *soleus* muscle; TEA, tetraethylammonium chloride; TPEN, tetrakis-(2-pyridylmethyl) ethylenediamine.

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muscle (Bishop and Milton, 1997) at different levels of synaptic activity. We observed that PSC Ca^{2+} responses elicited at all frequencies tested were larger and displayed faster kinetics at NMJs of the fast-twitch LAL in comparison to NMJs of the slow-twitch SOL. Additionally, a greater number of PSCs was activated by synaptic activity at NMJs of the fast-twitch LAL. Increasing synaptic activity at NMJs of the slow-twitch SOL muscle did not enhance the ability of PSCs to respond to synaptic activity.

EXPERIMENTAL PROCEDURES

Nerve–muscle preparations

LAL and SOL muscles, with their respective motor nerve, were dissected from CD-1 mice (males, 22–24 g; Charles River Laboratories, St.-Constant, QC, Canada) under deep anaesthesia (0.1 ml g^{-1} midozolam and hypnorm dissolved in distilled water, administered i.p.). This procedure was performed in accordance with the regulations of the Canadian organization for animal welfare and the Animal care committee of the Université de Montréal such that number of animals as well as their suffering were minimized. Nerve-muscle preparations were pinned in an experimental Sylgard-coated recording chamber filled with normal Ringer's solution containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 1$NaHCO_3$, 2 $CaCl_2$, 25 HEPES and 10 glucose, oxygenated with 100% O_2. In some experiments, a solution with no Ca^{2+} added (low Ca^{2+}/high Mg^{2+}) was used. It contained: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 5 $MgCl_2$, 1 $NaHCO_3$, 25 HEPES and 10 glucose. All experiments were performed within the same regions of the muscles to minimize variability between experiments.

Calcium imaging of PSCs

For Ca^{2+} imaging of PSCs, both types of nerve-muscle preparations described above were incubated for 90 min in an oxygenated Ringer's solution containing 10 or 20 μM fluo-4 AM (Invitrogen Canada Inc., Burlington, ON, Canada), 0.02% pluronic acid (Invitrogen Canada Inc., Burlington, ON, Canada), and 1% DMSO (Sigma, St.-Louis, MO, USA), at room temperature (21 °C). Following the loading period and prior to the experiment, nerve-muscle preparations were perfused for 20 min with normal Ringer's solution containing 20 μM TPEN (tetrakis-(2-pyridylmethyl) ethylenediamine; Invitrogen) to buffer heavy metals which diminish binding of fluo-4 to Ca^{2+} ions. Experiments were performed with Ringer's solution heated to 30 °C with an automatic temperature controller (Warner Instrument; Hamben, CT, USA). Excitation of fluo-4 was provided by the 488 nm line of the argon ion laser of a Bio-Rad MRC 600 laser-scanning confocal microscope attenuated to 1% of its maximal intensity. Emitted fluorescence was detected using a long-pass filter with a cut-off at 515 nm. Localization of NMJs was achieved using transmitted light microscopy with a 40× water immersion objective (0.75 NA: Olympus, Tokyo, Japan). Changes in fluorescence were measured over PSC somata and expressed as:

$$\% \Delta F/F = (F - F_{rest})/F_{rest} \times 100.$$

The level of resting fluorescence was always within the same range for all experiments and all preparations. These values range between 20 and 30 pixel intensity values and experiments were deemed successful only when this basal value remained constant within each experiment. Importantly, the hardware adjustments were also standardized from experiment to experiment where the offset (black level) was between 4.9 and 5.1 and the gain was between 8 and 9. No other hardware amplifications and filtering were performed. A cell was considered responsive when the amplitude of the response was more than 5 pixel intensity above the resting level.

The different parameters of Ca^{2+} responses were defined as follows: latency is the time interval between the start of the motor nerve stimulation and the start of the Ca^{2+} response determined when $\Delta F/F$ is larger than 5% above baseline; time-to-peak is the time between the beginning of the Ca^{2+} response and its maximal amplitude; maximal amplitude is the maximal value of the Ca^{2+} response obtained at its peak; duration corresponds to the time interval measured at 10–90% of responses amplitudes.

Motor nerve stimulation for Ca^{2+} imaging of PSCs

The cut end of the nerve, for both nerve-muscle preparations, was stimulated with a suction electrode at twice the threshold level for muscle contraction (Grass medical instrument; Quincy, MA, USA) at various frequencies (40, 50 and 100 Hz) while maintaining the duration of the stimulus train constant (30 s). A lower frequency (e.g. 20–30 Hz) that would be more in tune with the properties of the SOL muscle was not used because PSCs of LAL muscle were quite unresponsive at this frequency. Muscle contractions generated by neurotransmitter release were prevented by blocking nicotinic receptors with α-bungarotoxin (20 μM for the LAL and 60 μM for the SOL; Invitrogen Canada Inc., Burlington, ON, Canada). Synaptically evoked PSC Ca^{2+} responses show a rundown whereby the amplitude of Ca^{2+} responses are reduced when elicited at 20 min intervals (Jahromi et al., 1992; Rochon et al., 2001). To avoid rundowns of PSC Ca^{2+} responses elicited by repeated nerve stimulations, experiments performed in the presence of tetraethylammonium chloride (TEA) were compared to a different set of experiments performed in control conditions.

Electrophysiological recordings of synaptic transmission

The distal end of the motor nerve was stimulated using a suction electrode at intensity twice above the threshold for muscle contractions. Muscle contractions were prevented by adding D-tubocurarine chloride (1.0 to 1.5 μM; Sigma) to the perfusion solution. Intracellular recordings of end plate potentials (EPPs) were performed with glass microelectrodes (10–15 MΩ) filled with KCl (2 M). Synaptic signals were amplified 100 to 500 times and filtered at 2 kHz with a Warner DC amplifier. Evoked synaptic responses at various frequencies were digitized at 10 kHz and recorded with Strathclyde electrophysiology software (J. Dempster, University of Strathclyde, Scotland). All experiments were performed on muscle fibres having a membrane potential (Em) more negative than −65 mV. Experiments were discarded if Em was found to be depolarized by more then 10 mV. Experiments were performed with continuous perfusion of normal Ringer solution heated to 30 °C. EPPs were recorded at 0.2 Hz for 15 min to establish the basal level of synaptic transmission.

Synaptic plasticity of the SOL and LAL muscles was studied as follows. After the recording of a control period with stimulation at 0.2 Hz, frequency of motor nerve stimulation was increased to one of the following: 10, 20 or 40 Hz for 30 s or 50 Hz for 60 s. Data for EPP amplitude was smoothed by using a moving bin averaging of three consecutive events. Synaptic depression following high frequency stimulation was defined by the reduction in amplitude measured for the last five EPPs of the train of stimuli and expressed as percentage of the averaged amplitude of the EPPs during the control period. Post-tetanic potentiation (PTP) was determined by calculating the ratio of the averaged amplitude of the 10 EPPs that immediately followed the end of the high frequency stimulation over the averaged amplitude of EPPs during the control period.

Drugs

Acetylcholine (ACh), adenosine, ATP and muscarine (20 μM) were dissolved in the same Ringer's solution used for the perfu-

sion of the preparation during the experiment. Drugs were locally applied onto selected PSC somata by micropressure (5–10 psi; pulse duration 200 ms) with a glass micropipette (tip diameter 2–4 μm), using a Picospritzer II (General Valve, Fairfield, NJ, USA). TEA (1 to 2.5 mM; Sigma) and atropine (20 μM) were prepared daily in physiological solution and continuously applied by bath perfusion of the heated Ringer's solution.

Statistical analysis

Results for Ca^{2+} imaging experiments are expressed as mean$\pm$ SEM. Data were compared using two-way ANOVA with NMJ type (SOL vs. LAL) as one factor and synaptic activity level (40, 50 and 100 Hz) as the other. When significant interactions were obtained, one-way ANOVA for each level of the NMJ type factor and Student *t* test for each frequency of the synaptic activity level factor were used to determine the main effects. If significance was achieved for a one-way ANOVA or for the factor synaptic activity level without a significant interaction, Tukey post hoc comparisons were carried out. If group variances were heterogeneous, *P*-values were calculated on transformed data (log). Statistical analysis was performed at a confidence interval of 95% with α=0.05. *N* indicates the number of nerve-muscle preparations in one condition and *n* refers to the number of PSCs.

RESULTS

Before studying synapse-glia interactions of the different NMJs, we needed to further characterize synaptic plasticity events at LAL NMJs as these were not well defined and compared them to plasticity events observed at SOL NMJs. Conversely, we further characterize synapse-glia interactions at the slow-twitch SOL NMJs as they had not been systematically studied yet.

Synaptic plasticity at LAL and SOL NMJs

NMJs of fast and slow twitch muscles exhibit different electrophysiological properties that are in part influenced by the difference in synaptic transmission at these types of synapses (Bradacs et al., 1997; Nguyen et al., 1997).

Synaptic plasticity of LAL NMJs was first examined at different frequencies of stimulation. Synaptic depression was observed at all frequencies tested while potentiation was only evident at higher frequencies (Fig. 1). As expected, synaptic depression induced by repetitive motor nerve stimulation increased with the frequency of stimulation (Fig. 1A–D) (10 Hz, 29.7$\pm$3.0%; 20 Hz, 34.1$\pm$6.7%; 40 Hz, 40.8$\pm$9.3% and 50 Hz, 64.3$\pm$8.0%; n=5, N=5 for each frequency tested).

Synaptic depression induced by a 50 Hz train of stimulation was significantly greater than that observed following 10 Hz or 20 Hz stimulation (Fig. 1E, $P<0.05$). A potentiation of 73.7$\pm$9.6% of EPP amplitude was observed with a 40 Hz train of stimulation (Fig. 1C, F) while a potentiation of 90.1$\pm$22.9% was observed when stimulating at 50 Hz (Fig. 1D, F). These are not statistically different from one another ($P>0.05$).

Unlike LAL, synaptic plasticity events recorded at NMJs from the central portion of the SOL muscles were characterized by stable synaptic depression at all frequencies (Fig. 2A–E). However, the small difference in synaptic depression induced by 10 Hz and 20 Hz stimulation reached significance with the one observed at 50 Hz ($P<0.05$). Synaptic potentiation was also quite different at SOL NMJs since it was observed at the frequency of 20 Hz and increased in size at 40 and 50 Hz (Fig. 2A–D, F).

As a whole, these data indicate that both types of synapses have unique synaptic properties that result in the expression of distinct plasticity events during sustained nerve activity. These results allowed us to next compare the glial properties and the communication that take place between the neuromuscular and glial elements of the synapses of SOL and LAL muscles.

Neurotransmitter-induced Ca^{2+} responses in PSCs of SOL NMJs

The properties of PSC activation at NMJs of amphibian and mammalian fast-twitch muscles have been studied and were shown to mainly depend on Ca^{2+} released from internal stores (Jahromi et al., 1992; Robitaille, 1995; Robitaille et al., 1997; Rochon et al., 2001). However, the properties of activation of PSCs at NMJs of slow-twitch muscles are not as well characterized. Hence, we studied PSC activation by locally applying onto their somata the main neurotransmitters found at the NMJs of the slow-twitch SOL muscles.

First, we locally applied ACh (20 μM; 200 ms, 8–10 psi) on PSCs at SOL NMJs. As shown in Fig. 3A, local applications of ACh induced Ca^{2+} elevations of 194.9$\pm$ 31.9% in the presence of external Ca^{2+} (n=11, N=6; Fig. 3A). Also, similarly to PSCs at amphibian and mammalian NMJs of fast-twitch muscles (Jahromi et al., 1992; Robitaille et al., 1997; Rochon et al., 2001), local applications of the muscarinic agonist muscarine (20 μM) caused a Ca^{2+} increase of 218.8$\pm$28.5% in PSCs at SOL NMJs (n=17, N=5; Fig. 3B). This increase is mainly attributable to Ca^{2+} being released from internal stores since applications of muscarine induced Ca^{2+} elevations of 162.6$\pm$22.3% when no external Ca^{2+} was added (n=8, N=4; Fig. 3C). Interestingly, however, unlike PSCs of NMJs at fast-twitch muscles, Ca^{2+} responses elicited in absence of external Ca^{2+} were significantly smaller than those elicited in the presence of external Ca^{2+} ($P<0.001$). This indicates that there is an important contribution of Ca^{2+} entry at PSCs of slow-twitch SOL NMJs but not at NMJs of amphibian and mammalian fast-twitch muscles (Jahromi et al., 1992; Rochon et al., 2001).

We then tested whether the co-transmitter ATP caused Ca^{2+} increases in PSCs at NMJs of SOL muscles knowing that it is a potent activator of PSCs at NMJs of amphibian and mammalian fast-twitch muscles (Jahromi et al., 1992; Robitaille, 1995; Rochon et al., 2001). As shown in Fig. 3D, local applications of ATP (20 μM) induced Ca^{2+} responses of 195.1$\pm$12.0% (n=28, N=4). These Ca^{2+} responses were caused by the release of Ca^{2+} from internal stores since ATP applications in the absence of extracellular Ca^{2+} still induced Ca^{2+} increases in PSCs. Also, similarly to what was observed in PSCs of NMJs of fast-twitch muscles, ATP-induced Ca^{2+} responses were significantly

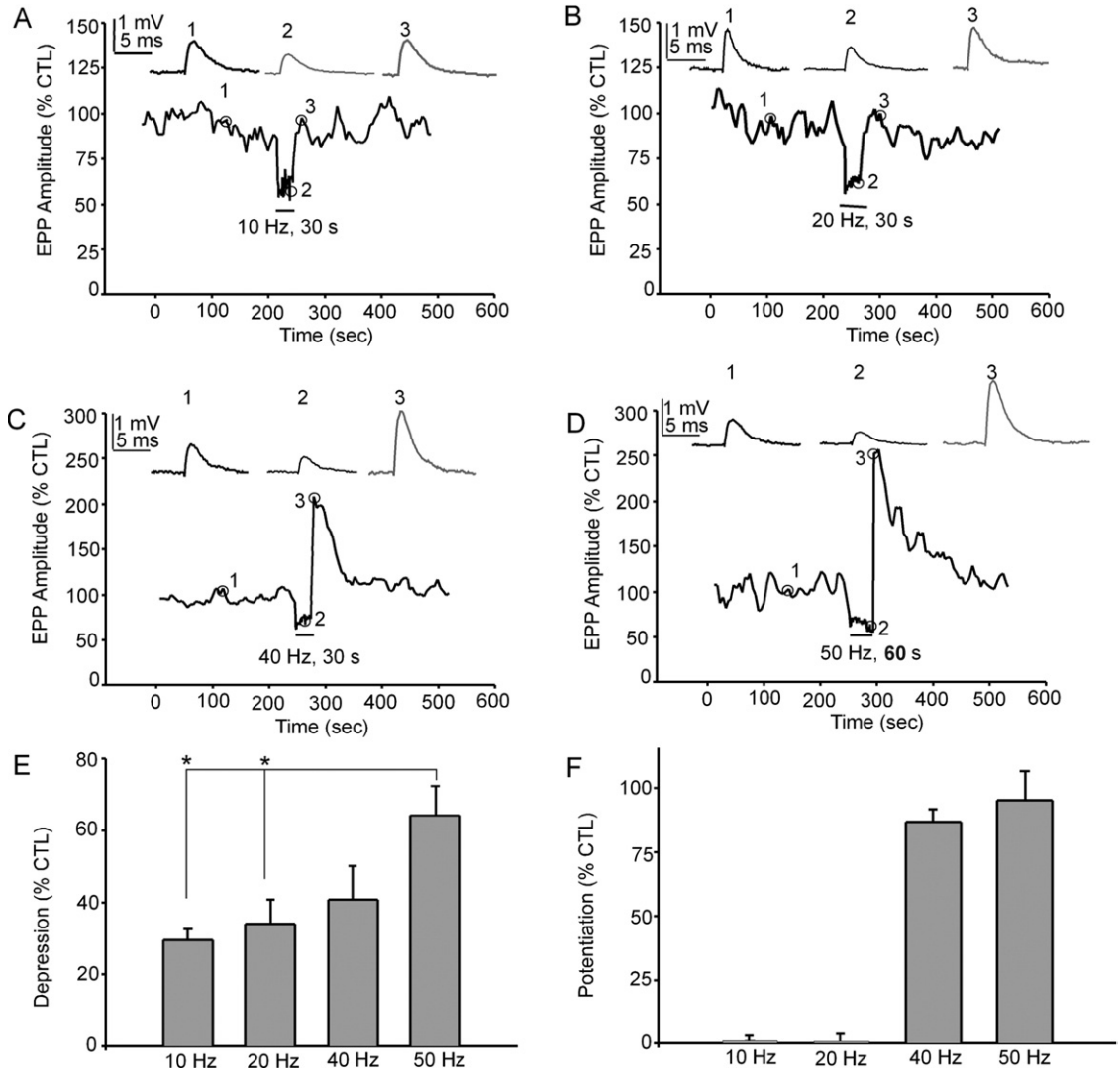


Fig. 1. Synaptic plasticity at NMJs of LAL muscles. Relative EPP amplitude (% of control) before during and after repetitive motor nerve stimulation at 10 Hz, 30 s (A), 20 Hz, 30 s (B), 40 Hz, 30 s(C) and 50 Hz, 60 s (D). Insets (upper panels) show representative EPPs obtained before (black), during (light grey) and after (dark grey) high frequency stimulation. (E) Histograms depicting the mean±SEM of synaptic depression (% of control) induced by motor nerve stimulation at 10 Hz, 30 s, 20 Hz, 30 s, 40 Hz, 30 s and 50 Hz, 60 s (n=5). (F) Histograms depicting the mean±SEM of synaptic potentiation (% of control) induced by motor nerve stimulation at 10 Hz, 30 s, 20 Hz, 30 s, 40 Hz, 30 s and 50 Hz, 60 s (n=5, same as in Fig. E). * indicates significance at $P<0.05$.

larger in absence of external Ca^{2+} (396.9±33.6%; n 48 cells in four muscles Fig. 3E) ($P<0.001$).

In addition to ATP, adenosine is also an important neurotransmitter involved in synapse-glia interactions at NMJs of fast-twitch muscles (Rochon et al., 2001). We therefore locally applied adenosine (20 μM) onto PSC somata at NMJs of SOL muscles. As for PSCs at NMJs of fast-twitch muscles, local applications of adenosine resulted in Ca^{2+} increases of 258.0±24.1% (n=28, N=4) in PSCs at NMJs of slow-twitch SOL muscles (Fig. 3F). As a whole, these results indicate that functional differences exist between PSCs at NMJs of slow- and fast-twitch muscles regarding cholinergic excitation, in particular, with regards to the role of Ca^{2+} entry. However, the mechanism of purinergic signalling appears to be maintained at NMJs of the two muscle types.

Nerve-evoked glial Ca^{2+} responses in PSCs of SOL NMJs

After characterizing the mechanisms of PSC activation at slow-twitch SOL synapses we further characterized their activation by neurotransmitters released during synaptic activity (see also Todd and Robitaille, 2006).

As was previously shown by Rochon et al. (2001), repeated motor nerve stimulation resulted in an increase in fluo-4 fluorescence of 102.7±14.9% (n=17, N=8) indicating a Ca^{2+} elevation in PSCs at LAL NMJs (Fig. 4A). Knowing that transmitter release properties differ at NMJs of slow-twitch and fast-twitch muscles, we next wondered whether the level of transmitter release at SOL NMJs was sufficient to activate PSCs. Changes in intracellular Ca^{2+} were monitored before, during and after repeated motor

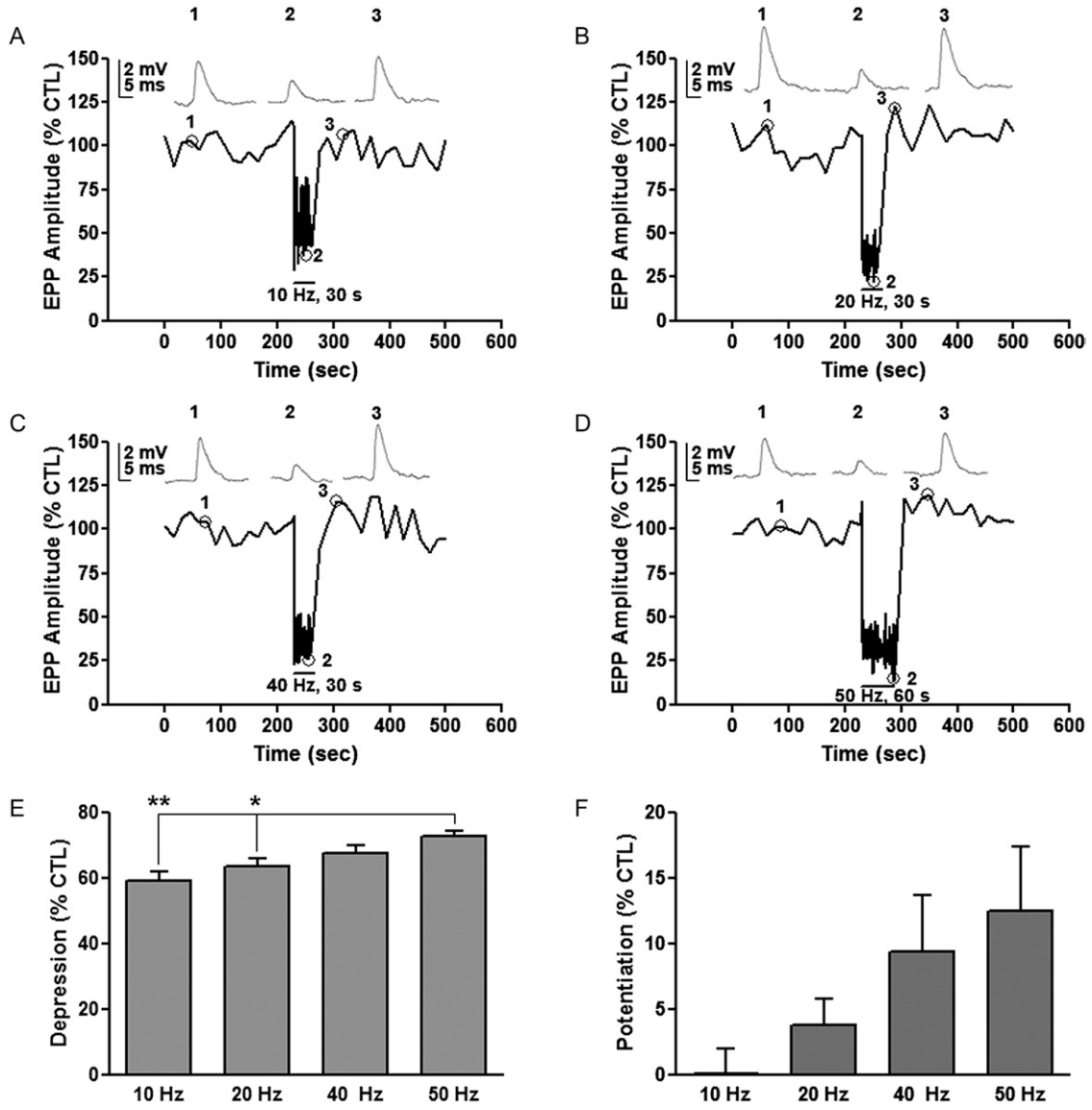


Fig. 2. Synaptic plasticity at NMJs of SOL muscles. Relative EPP amplitude (% of control) before during and after repetitive motor nerve stimulation at 10 Hz, 30 s (A), 20 Hz, 30 s (B), 40 Hz, 30 s(C) and 50 Hz, 60 s (D). Insets (upper panels) show representative EPPs obtained before (black), during (light grey) and after (dark grey) high frequency stimulation. (E) Histograms depicting the mean±SEM of synaptic depression (% of control) induced by motor nerve stimulation at 10 Hz, 30 s (n=4), 20 Hz, 30 s (n=5), 40 Hz, 30 s and 50 Hz, 60 s (n=6). (F) Histograms depicting the mean±SEM of synaptic potentiation (% of control) induced by motor nerve stimulation at 10 Hz, 30 s, 20 Hz, 30 s, 40 Hz, 30 s and 50 Hz, 60 s (same experiments as in Fig. E). * indicates significant differences at $P<0.05$ and ** at $P<0.01$.

nerve stimulation (50 Hz, 30 s). Interestingly, the same stimulation protocol used to induce Ca^{2+} responses in PSCs at LAL NMJs also caused Ca^{2+} responses in PSCs at SOL NMJs. However, the peak amplitude of the Ca^{2+} responses was on average 75.7±15.6% (n=19, N=11) (Fig. 4B) which is significantly smaller than the peak amplitude elicited in PSCs at LAL NMJs ($P<0.001$). Hence, this observation indicates that responsiveness to synaptic activity of PSCs is maintained at synapses with different transmitter release properties, although their level of activation appears to be lower.

Since PSC activation is dependent upon muscarinic and purinergic signalling (Robitaille, 1995; Robitaille et al., 1997; Rochon et al., 2001) and since the level of ACh release is smaller at NMJs of slow-twitch muscles (Reid et al., 1999), it is possible that the contribution of the muscarinic components to PSC activation at NMJs of slow-twitch muscles differs from the one observed at NMJs of fast-twitch muscles. To test this possibility, we studied Ca^{2+} responses elicited by synaptic activity in the presence of the general muscarinic receptor blocker atropine (20 μM) (Rochon et al., 2001). As shown in Fig. 4C, PSC Ca^{2+} responses evoked by repeated motor nerve stimulation in the presence of atropine (20 μM) were significantly reduced (29.4±11.4%, n=6, N=3; $P<0.001$; Fig. 4C), indicating that, similarly to NMJs at fast-twitch muscles, re-

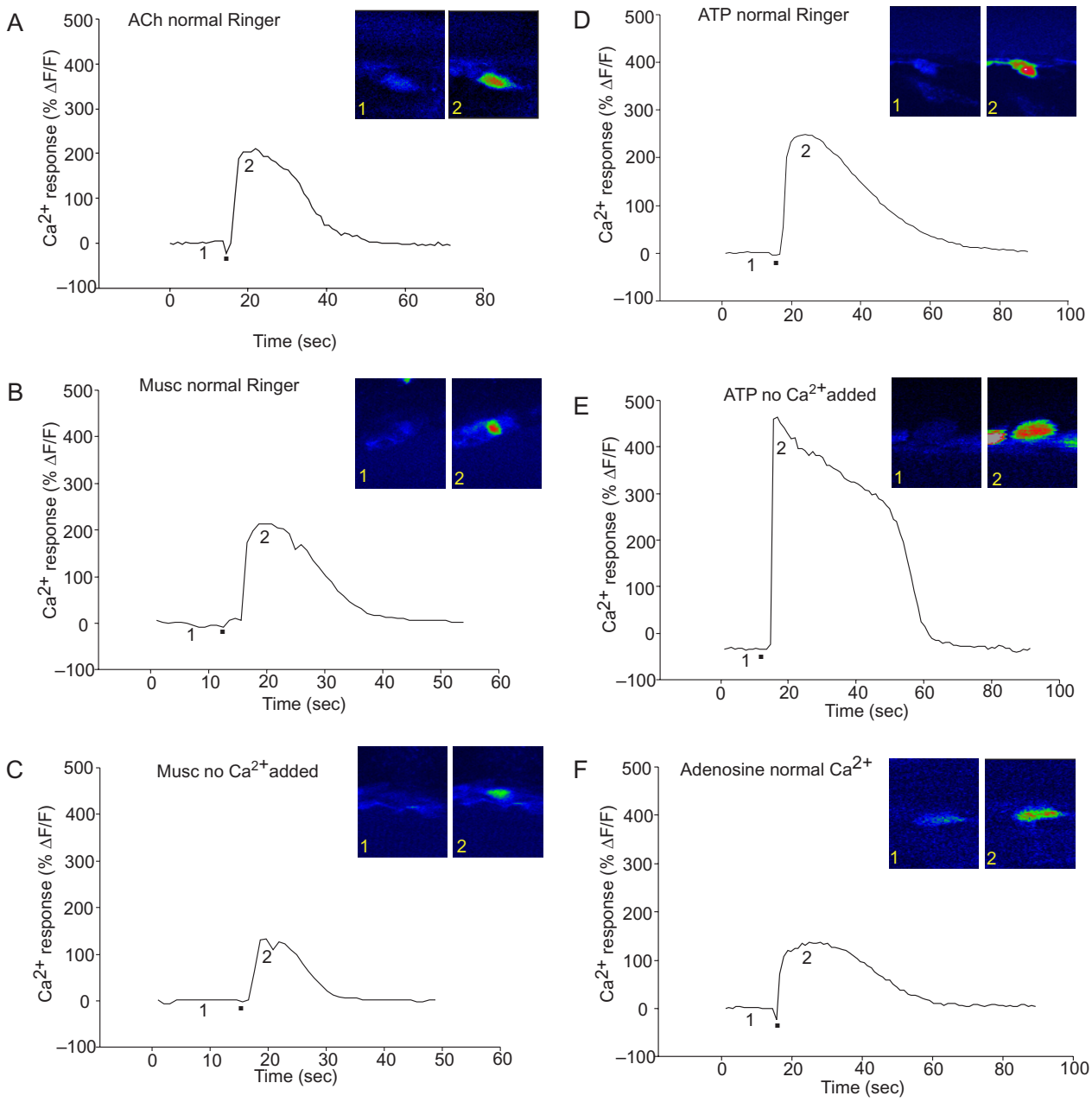


Fig. 3. PSC Ca^{2+} responses induced by local neurotransmitter applications at SOL NMJs. (A) Time course of fluorescence changes in PSC induced by the local application of ACh (20 μM). (B) Time course of fluorescence changes in PSC following the local application of muscarine (20 μM) in the presence of external Ca^{2+} and (C), when no external Ca^{2+} was added. (D) Time course of fluorescence changes in PSCs before and after local application of ATP (20 μM) in the presence of external Ca^{2+} and (E), in absence of external Ca^{2+}. (F) Time course of fluorescence changes in PSC following the local application of adenosine (20 μM) in normal physiological solution. Insets show false color confocal of PSCs loaded with fluo-4 AM at rest (1) and at the peak (2) of the Ca^{2+} response for each of the conditions tested. Figs. (A–F) were obtained from different preparations. The time of local agonist application is indicated by a shaded square under each trace of Ca^{2+} response. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

leased ACh by the presynaptic nerve terminal during synaptic activity at SOL NMJs is also an important activator of PSCs through muscarinic receptors.

Synapse-glia interactions at SOL and LAL NMJs

Having better characterized the synaptic and glial properties of the different NMJs and knowing that PSCs at SOL NMJs are also activated by synaptic transmission we next examined the characteristics of synapse-glia interactions at SOL and LAL NMJs. To achieve this goal, considering that transmitter release and synaptic plasticity differ at NMJs of slow- and fast-twitch muscles, we compared SOL and LAL PSC Ca^{2+} responses at different levels of synaptic activity by varying the frequency of motor nerve stim-

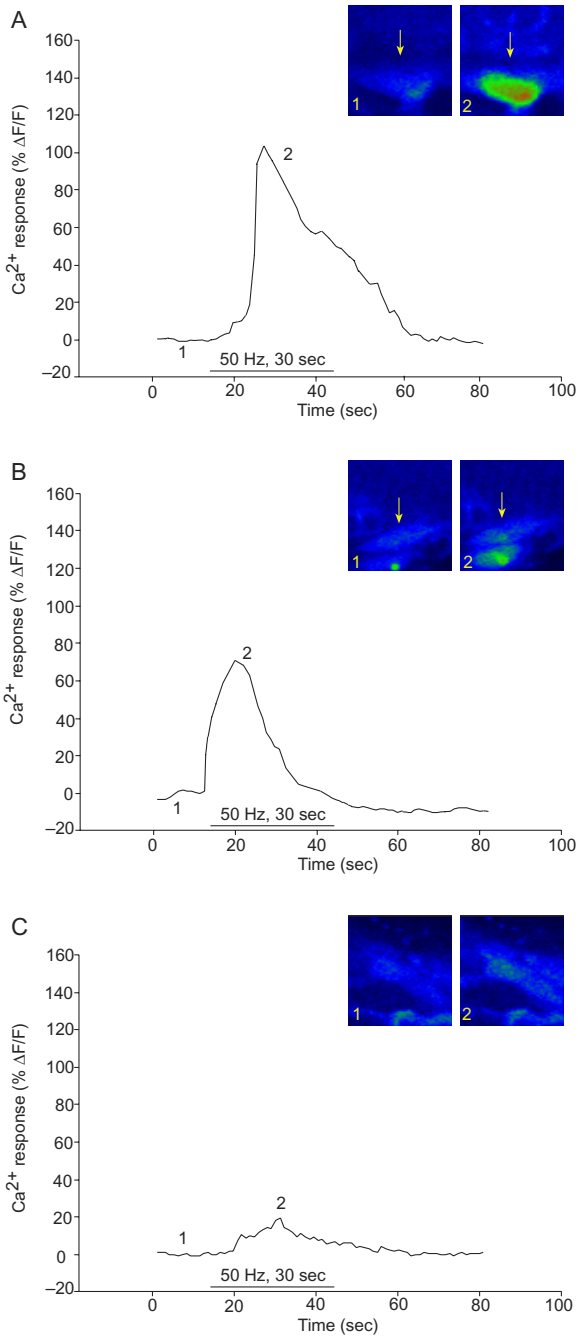


Fig. 4. Synaptically-induced Ca^{2+} responses in PSCs. (A) Time course of fluorescence changes of a PSC (yellow arrow) at a fast-twitched NMJ of the LAL, before, during and following repeated motor nerve stimulation (50 Hz, 30 s). (B) Time course of fluorescence changes of a PSC (yellow arrow) at a slow-twitched NMJ of the SOL, before, during and following repeated motor nerve stimulation (50 Hz, 30 s). (C) Time course of fluorescence changes of a PSC at a slow-twitched NMJ of the SOL, before, during and following repeated motor nerve stimulation (50 Hz, 30 s) in the presence of the muscarinic antagonist atropine (20 μM). Insets show false color confocal images of a PSC loaded with fluo-4 AM at rest (1) and at the peak (2) of the Ca^{2+} response in the different conditions depicted. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Table 1. Properties of PSC Ca^{2+} responses at weak (SOL) and strong (LAL) NMJs

	40 Hz	50 Hz	100 Hz
Latency (s)			
SOL NMJ	2.6±0.6*	2.3±0.5**,*	1.9±0.5**,*
LAL NMJ	3.6±0.6*	5.3±0.5*	2.4±0.5*
Time-to-peak (s)			
SOL NMJ	11.8±1.9***	11.2±1.8	8.4±1.8***
LAL NMJ	16.4±1.9***	13.1±1.9	8.1±1.7***
Peak response (% Δ F/F)			
SOL NMJ	59.1±13.8*	75.7±15.6*	81.1±13.7*
LAL NMJ	83.8±10.7*	102.7±14.9*	111±14.6*
Duration (s)			
SOL NMJ	46.5±4.6	45.7±4.2	61.7±4.2
LAL NMJ	49.3±4.6	44.1±4.5	48.5±3.9
Responding PSC/NMJ (%)			
SOL NMJ	54.5±8.1§	50.6±7.8§	52.3±8.5§
LAL NMJ	82.0±12.0§	72.5±9.5§	95.2±10.2§

* Indicates significant differences between PSCs at SOL NMJs and PSCs at LAL NMJs within one frequency.

** Indicates significant differences between frequencies of stimulation within one NMJ type.

*** Indicates significant differences between frequencies for both NMJ types.

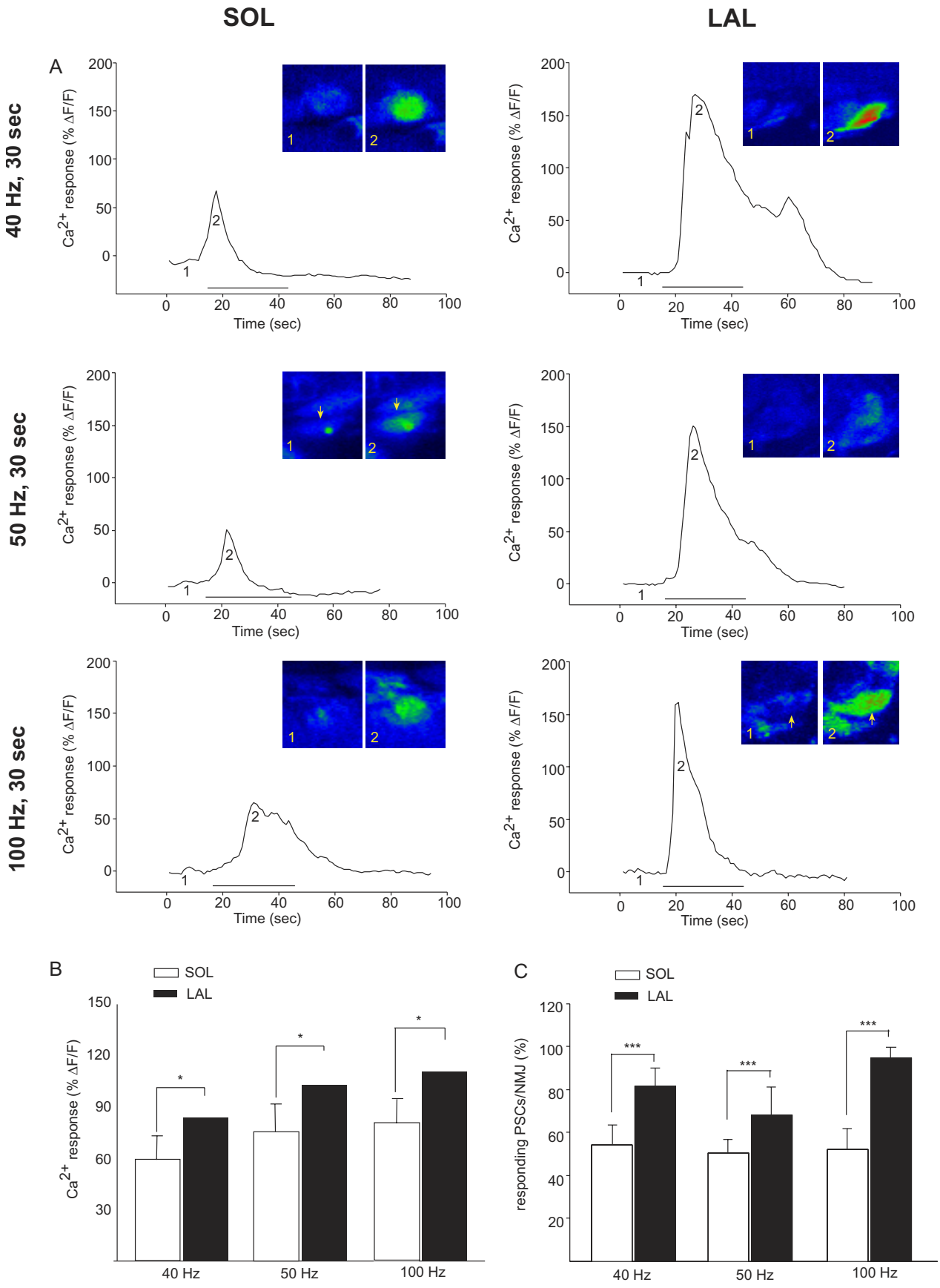
§ Indicates significant differences between frequencies percentage of responding cells in both NMJ types.

* $P<0.05$, § $P<0.001$, ** $P<0.05$, *** $P<0.05$. Number of observations for SOL NMJs: 40 Hz: 16 cells in 10 muscles, 50 Hz: 19 cells in 11 muscles and 100 Hz: 19 cells in 13 muscles. Number of observations for LAL NMJs, 40 Hz: 16 cells in six muscles, 50 Hz: 17 cells in eight muscles and 100 Hz: 22 cells in seven muscles.

ulation while maintaining the duration of the train of stimuli constant.

Differences in Ca^{2+} responses were analyzed in relation to the peak of the responses, the time-to-peak, the decay time as well as the delay-to-onset. Results revealed a significant difference between SOL and LAL NMJs as the peak amplitude of PSC Ca^{2+} responses was consistently greater at LAL NMJs than at SOL NMJs for all frequencies tested ($P<0.05$; Fig. 5A, B; summarized in Table 1). Furthermore, a greater number of PSCs per NMJ were activated by transmitter release following repeated motor nerve stimulation at LAL NMJs in comparison to SOL NMJs at all frequencies evaluated ($P<0.001$; Fig. 5C, summarized in Table 1). These results suggest that the responsiveness of PSCs at SOL NMJs is reduced in comparison to PSCs at LAL NMJs.

The time-to-peak of PSC Ca^{2+} responses was not significantly different between SOL and LAL NMJs at the different frequencies tested. However, the rise time of Ca^{2+} responses was significantly faster at both types of NMJs when induced by a 100 Hz stimulation in comparison to a 40 Hz stimulation (SOL: 11.8±1.9 s at 40 Hz vs. 8.4±1.8 s at 100 Hz; LAL: 16.4±1.9 s at 40 Hz vs. 8.1±1.7 s at 100 Hz; $P<0.05$) (Fig. 6A, B). This indicates that the kinetic of PSCs Ca^{2+} responses is dependent on the pattern of activity of the synapse regardless of the synaptic strength of the synapse.


SOL
LAL
A
40 Hz, 30 sec
50 Hz, 30 sec
100 Hz, 30 sec
Ca2+ response (% ΔF/F)
Time (sec)
B
SOL
LAL
Ca2+ response (% ΔF/F)
40 Hz
50 Hz
100 Hz
C
SOL
LAL
responding PSCs/NMJ (%)
40 Hz
50 Hz
100 Hz

The latency of PSC Ca^{2+} responses at LAL NMJs generated by 100 Hz stimulations was significantly shorter than those induced by 50 Hz stimulations (100 Hz: 2.4±0.5 s vs. 50 Hz: 5.3±0.5 s; $P<0.05$; Fig. 7) while the same tendency was also observed at PSCs of the SOL NMJs. A higher efficacy of activation of PSCs at LAL NMJs seems to occur when considering the differences of the aforementioned parameters of the Ca^{2+} responses. Hence, one would expect that the delay-to-onset of Ca^{2+} responses would be faster for PSCs at LAL NMJs. However, surprisingly, the delay-to-onset was significantly longer for PSCs at LAL NMJs for all frequencies examined ($P<0.05$; Table 1).

Responsiveness of PSCs at SOL NMJs upon increase of transmitter release

The results presented above suggest that the responsiveness of PSCs at SOL NMJs was lower in comparison to that observed at LAL NMJs. One possible explanation for this difference may be attributable to purported lower level of transmitter release at NMJs at slow-twitch muscles in comparison to NMJs at fast-twitch muscles. Hence, if this is the case, increasing the level of transmitter release at SOL synapses should result in larger Ca^{2+} responses in PSCs. This was tested by monitoring Ca^{2+} responses in PSCs elicited by motor nerve stimulation at different frequencies of the SOL NMJs in the presence of TEA (1 to 2.5 mM). This concentration of TEA tripled the amount of transmitter release at SOL NMJs (Fig. 8A) and does not affect PSCs excitability by local application of agonists (data not shown). Interestingly, as shown in Fig. 8B–D, the maximal amplitude of PSC Ca^{2+} responses induced by stimulation at 40 Hz (45±15.5%, $N=7$, $n=9$), 50 Hz (88.6±19.5%, $N=4$, $n=5$) and 100 Hz (99±27%, $N=4$, $n=7$) of SOL synapses was not significantly increased in the presence of TEA in comparison to control (see Table 1; $P=0.468$). In addition, the percentage of responsive cells remained unchanged at all frequencies tested. Importantly, this cannot be explained by a saturation of the Ca^{2+} indicator or the inability of PSCs to produce larger Ca^{2+} responses since the larger Ca^{2+} responses were elicited by local ATP in the same cells (144±13.9% ΔF/F). These responses are larger than those evoked by nerve stimulation in presence of TEA at all frequencies tested.

DISCUSSION

The present work aimed at determining whether synapse-glia interactions are simply governed by synaptic properties or whether PSC properties are also involved. By comparing PSC Ca^{2+} responses induced by repeated motor nerve stimulation at SOL synapses to those of PSCs at LAL synapses we found that the responsiveness of PSCs at SOL synapses is lower in comparison to those at LAL synapses. Importantly, this phenomenon cannot be accounted solely by differences in neuronal synaptic properties since intrinsic PSC properties also appear to contribute to the differential responses at the two types of NMJs.

Receptors of PSCs at different NMJs

PSCs of the two types of NMJs do not differ in terms of the types of functional receptors expressed at their membrane. Indeed, as was previously observed for PSCs at NMJs of amphibian and mammalian fast-twitch muscles (Robitaille et al., 1997; Rochon et al., 2001), PSCs at NMJs of slow-twitch muscles also express muscarinic receptors as indicated by glial Ca^{2+} responses elicited by the application of muscarine as well as ACh. In addition, synaptically-induced PSC Ca^{2+} responses were still observed in the presence of the nicotinic antagonist α-bungarotoxin but significantly reduced by the muscarinic antagonist atropine. This provides further evidence that, similar to PSCs at NMJs of fast-twitch muscles, released ACh at SOL NMJs activates PSC cholinergic receptors of the muscarinic sub-type. However, it appears that the mechanisms governing the Ca^{2+} dynamics in PSCs of the SOL NMJs are different since muscarinic-induced responses involved Ca^{2+} entry, an element that was not observed at LAL NMJs where only internal stores were at play. Hence, this suggests that PSC properties governing their responsiveness to synaptic activity are fundamentally similar at different synapses although functional differences exist. The impact that such differences in the mechanism of PSC activation may have on the NMJ function remains to be determined.

Furthermore, it is established that ATP is co-released with ACh (Dowdall et al., 1974) and that PSCs at NMJs of fast-twitch muscles express P1 and P2 purinergic receptors (Robitaille, 1995; Rochon et al., 2001). Local applications of ATP and adenosine resulted in Ca^{2+} elevations in PSCs at SOL NMJs, indicating that P1 and P2 receptors are also expressed by PSCs at these synapses. Taken together, these results indicate that PSCs at SOL NMJs express the appropriate receptors for the main neurotransmitters being released and that PSC Ca^{2+} signalling mechanisms are preserved across NMJs with different synaptic properties. Furthermore, differences observed in the responsiveness of PSCs at LAL and SOL NMJs cannot be explained by the absence of one of the main receptor systems used by PSCs to detect synaptic activity.

Fig. 5. PSC Ca^{2+} responses at SOL and LAL NMJs induced by synaptic activity. (A) Typical traces showing time course of fluorescence changes of PSCs at slow-twitched SOL NMJs and fast-twitched LAL NMJs, before, during and following repeated motor nerve stimulation at 40, 50 Hz and 100 Hz for 30 s. Insets show false color confocal images of PSCs loaded with fluo-4 AM at rest (1) and at the peak (2) of the respective Ca^{2+} responses. (B) Histograms depicting mean±SEM of peak amplitude of Ca^{2+} responses of PSCs at low-twitched SOL NMJs (white bars) and fast-twitched LAL NMJs (black bars) following repeated motor nerve stimulation at 40, 50 Hz and 100 Hz for 30 s. (C) Histograms depicting mean±SEM of percentage of responding PSCs at low-twitched SOL NMJs (white bars) and fast-twitched LAL NMJs (black bars) following repeated motor nerve stimulation at 40, 50 Hz and 100 Hz for 30 s. * indicates significance of $P<0.05$ and *** indicates significance at $P<0.001$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

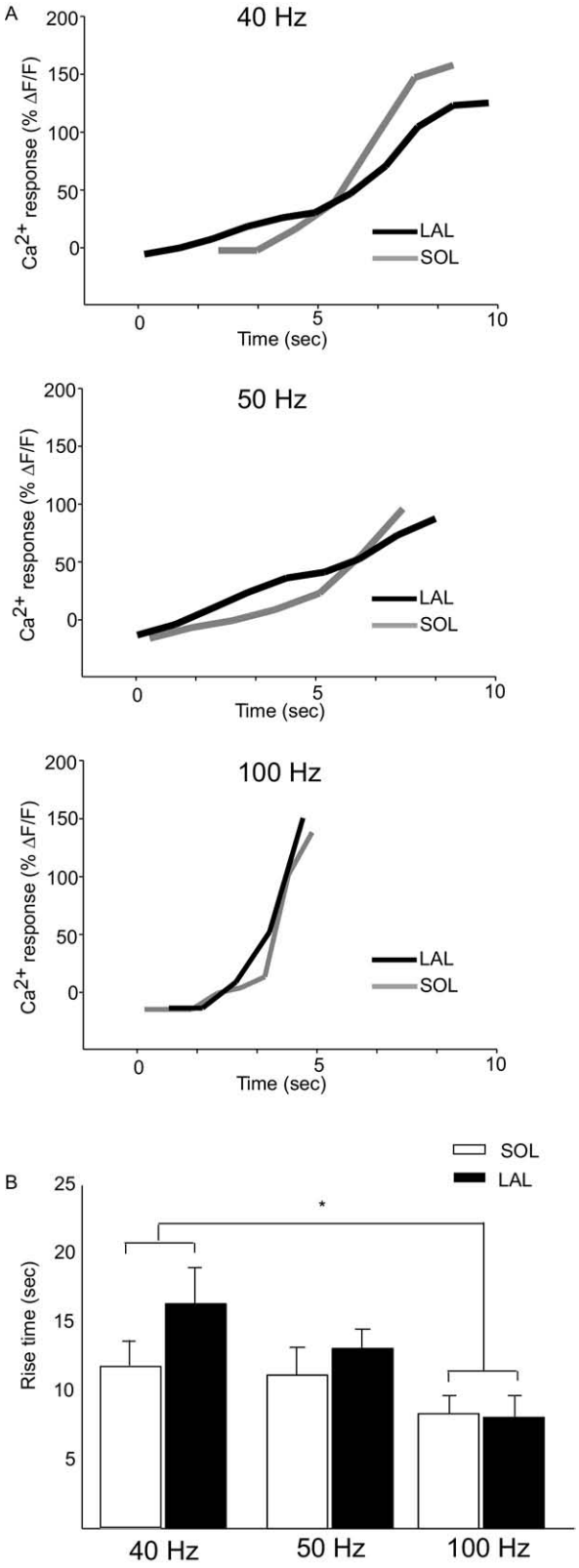


Fig. 6. Time-to-peak for PSC Ca^{2+} responses at SOL and LAL NMJs. (A) Typical traces showing time course of fluorescence changes during the time-to-peak phase of PSC Ca^{2+} responses of weak SOL NMJ (grey) and strong LAL NMJ (black) induced by a 40 Hz, 50 Hz and 100 Hz motor nerve stimulation. (B) Histogram comparing mean±SEM time-to-peak of PSCs Ca^{2+} responses at weak SOL NMJs (white bars) and strong LAL NMJs (black bars) following repeated motor nerve stimulation at 40, 50 Hz and 100 Hz for 30 s. * indicates significance of $P<0.05$.

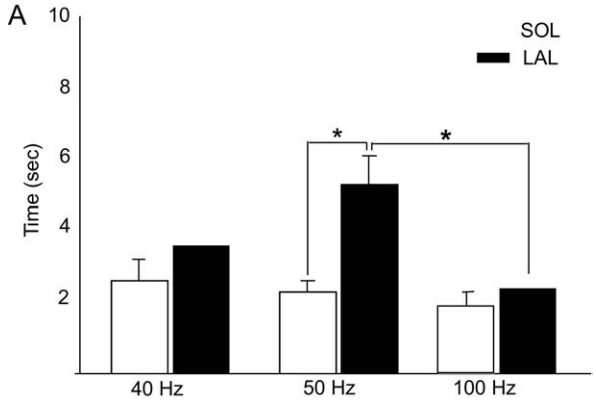


Fig. 7. Latency of PSC Ca^{2+} responses at SOL and LAL NMJs. (A) Histogram comparing mean±SEM latencies of PSCs at Ca^{2+} responses at weak SOL NMJs (white bars) and strong LAL NMJs (black bars) induced by repeated motor nerve stimulation at 40, 50 Hz and 100 Hz for 30 s. * indicates significance of $P<0.05$.

Synapse–glia interactions at different types of NMJs

Properties of NMJs of SOL and LAL muscles differ in terms of synaptic efficacy that is tuned with the properties of their respective muscle fibres (Wood and Slater, 2001). In line with these differences, the results of the present study clearly illustrate that acute synapse-glia interactions taking place at chemical synapses also vary at different synapses. The results presented here are the first evidence that glial Ca^{2+} responses induced at two synapses of the same nature (both cholinergic) but with varying synaptic properties differ in their characteristics. This suggests that glial activation is not restricted to synapses with higher levels of neurotransmission and that synapse-glia interactions are maintained at different synapses.

The kinetic of PSC responses is different at the two types of NMJs which implies that their activation is also different. In fact, as a whole, the characteristics of the PSC Ca^{2+} responses suggest a more efficient activation of PSCs at LAL NMJs. Indeed, the time course was faster even though the amplitude of the responses was larger in PSCs at LAL NMJs. However, in that respect, the longer delay of the Ca^{2+} responses at LAL NMJs elicited by synaptic activity is somewhat counterintuitive. Hence, although the overall data (amplitude, % of responsive cells, kinetics) argue that synapse-glia interactions at SOL synapses are inefficient, the fact that the delay is faster rather suggests that the properties of the interactions are simply adjusted to the synaptic properties and are neither better nor worse than the other. Interestingly, the properties of the NMJ and the muscle fibres are known to be plastic and undergo long-term changes in their properties (Bélair et al., 2005). We recently showed that PSCs also show long-term changes in their functional properties (Bélair et al., 2010).

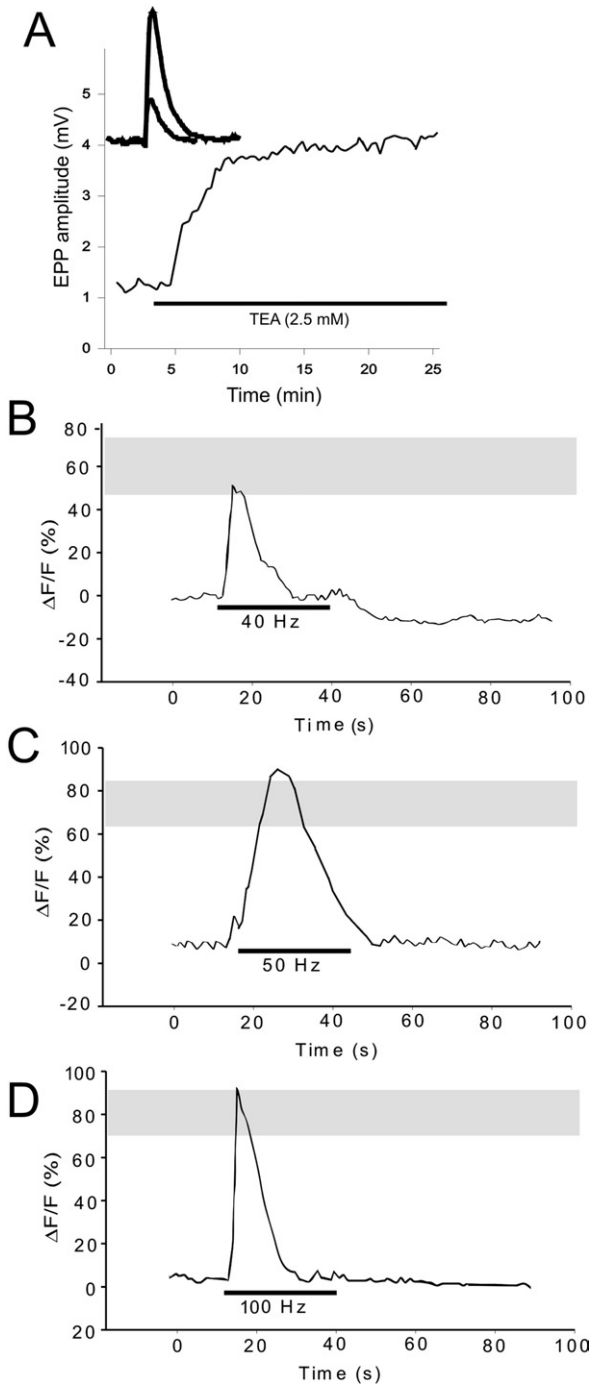


Fig. 8. Responsiveness of PSCs at SOL NMJs is unaffected by increases in transmitter release. (A) Changes in EPP amplitude induced by bath perfusion of TEA (2.5 mM). Insets show an EPP recorded before (ctrl) and at the plateau of the TEA effects (TEA). Changes of fluorescence in a PSC (% ΔF/F) before, during (black bar) and after motor nerve stimulation at 40 Hz (B), 50 Hz (C) and 100 Hz (D) in the presence of TEA (2.5 mM). The gray zone represents the mean±SEM of responses obtained in control conditions. Ca^{2+} responses obtained in the presence of TEA were not significantly different than controls (Student *t*-test, $P>0.05$). Figs. (A–D) were obtained from different experiments.

It is well established that glial responses are generated by synaptic transmission. Our results suggest that differences in synaptic properties influence the characteristics of PSC Ca^{2+} responses. Indeed, at all frequencies examined, PSC Ca^{2+} responses observed at LAL NMJs were larger in amplitude in comparison to those at SOL NMJs. These changes need to be taken as part of the whole kinetic of the complex temporal and spatial Ca^{2+} changes in PSCs. These results are in agreement with the fact that PSC Ca^{2+} responses are activated by neurotransmission and may reflect the greater quantal content that strong NMJs have, resulting in a larger amount of neurotransmitter molecules available for PSC activation. However, a direct link between the quantal content during sustained synaptic activity and PSC activation would need to be established to directly test that possibility. Interestingly, the level of neurotransmission at LAL NMJs may not only influence the peak amplitude of PSC Ca^{2+} responses but also influence the efficacy of PSC activation as indicated by the greater number of PSCs activated per NMJ.

PSCs of SOL and LAL NMJs differ

The properties of transmitter release at the two types of NMJs do not suffice to explain the observed differences in synapse-glia interactions. Indeed, our results showing that tripling transmitter release at SOL NMJs did not improve PSC responsiveness at these synapses strongly suggests that the excitability of PSCs is not a simple passive adjustment to the level of transmitter release. Hence, although the level of transmitter release undoubtedly governs the interactions that take place between nerve terminals and PSCs, the fundamental properties of PSCs also differ at different synapses. This observation has important implications for the understanding of the role of perisynaptic glia in modulating synaptic transmission and plasticity as it implies that the modulation of glial cells is not only dependent on the state of the synapse and its activity but that glial cells are fundamentally different and thus, are likely to play a different modulatory role. Indeed, the modulation of synaptic transmission and plasticity by glial cells is largely dependent on the level and dynamic of Ca^{2+} changes in these cells. Hence, since the properties of Ca^{2+} responses differ in PSCs of SOL and LAL NMJs, one would predict that this will result in a different modulatory role of PSCs at their respective synapses. Interestingly, these observed differences are in line with the concept of heterogeneity demonstrated in CNS glial cells. For instance, it was demonstrated that two functionally distinct populations of astrocytes can be found in the hippocampal CA1 region (Wallraff et al., 2004).

CONCLUSION

In conclusion, the results presented here provide evidence that the characteristics of glial cell activation are governed in part by the synaptic properties of their associated synapses and in part by the basic responsiveness of glial cells themselves. PSCs modulate neurotransmission in a positive and a negative fashion at NMJs of amphibian fast-twitch muscles (Robitaille, 1998; Castonguay et al., 2001). The fact that synapse-glia interactions are different at different synapses implies that the extent and the nature of such glial modulation

will be determined by the properties of glial cell activation which is based not only upon the level of transmitter release but also on their intrinsic properties. Interestingly, it was shown at the amphibian NMJ that part of the glial modulation was indirectly modulated by a feedback mechanism from the muscle fibre (Pinard and Robitaille, 2008). This would indicate that the three elements at the synapse are tuned to reflect the overall properties of the synaptic context.

Considering the extent of the diversity and plasticity of synaptic properties at CNS synapses, it is quite likely that glial modulation of synaptic activity in the CNS is governed by similar mechanisms to that observed in the PNS. In fact, this phenomenon may be even more pronounced in the CNS owing to the complexity of the astrocytic organization and the large diversity of synapses a given astrocyte is associated with (Auld and Robitaille, 2003). Astrocytes would then play an important integrative role by filtering and regulating the different properties of a large number of synapses interacting with them (Perea and Araque, 2005, 2007). Moreover, this diversity may even be regionalized within an astrocyte since glial processes may, in some conditions, act as isolated functional compartments (Grosche et al., 1999). Hence, based on our observations, glial modulation at CNS synapses is likely to be very diverse and, more importantly, be expressed as a function of the synaptic environment and the properties of the glial elements.

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