The role of regulated secretion in neurite outgrowth, synapse formation and neuronal survival

Munc18-1 as a spider in the web

Joost Heeroma

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Chapter 1

Introduction

Reading maketh a full man, conference a ready man,
and writing an exact man.

Francis Bacon, Essays: Of Studies (1601)

General introduction

During brain development neuronal precursor cells divide until there are billions of neurons. After being born (defined as having stopped dividing) neurons migrate to their final position in the brain, either by manoeuvring through layers of neurons that arrived previously, or by following the physical lead of radial glial cells that project to the desired region of the brain. Finally, the neurons project a number of neurites to other cells and make synapses, resulting in a network consisting of hundreds of billions of connections. Actually some neurons begin projecting neurites while migrating to their final position and these neurons probably use their neurites either to gain environmental information about where to grow, to or as physical anchors to which they can pull themselves.

One of the main questions in neurobiology is how the wiring scheme of the brain is determined and very much related to this, how the brain is changed during life by experience and learning processes. One option is that the genetic code somehow encrypts the entire initial wiring scheme. According to recent estimates the human genome contains roughly 30.000 genes, 10.000 of which are thought to be expressed in the brain. As many of these genes are involved in metabolic pathways etc. it is difficult to imagine how a few thousand genes could code for the exact wiring scheme of up to a million billion connections between a thousand billion neurons. Furthermore, this option does not offer a solution to the molecular basis of the neuronal plasticity underlying learning processes. A more economic solution (and therefore more biologic) would be that the genetic code provides a number of ground rules for the basic layout of the brain and that neuronal communication fills in the details of how the how the neurons in the brain are interconnected and how these connections are modified by experience.

In this introduction, I will briefly describe some aspects of neuronal migration and neurite projection and a selection of genes that are thought to direct this process. The next section will deal with the formation of basic synapses. Then, the different forms of communication between neurons are discussed. The rest of the introduction will be focussed on regulated secretion, its known role in synaptic and network plasticity and its functions that are still under dispute. Finally, some current models to resolve the function of regulated secretion in neuronal plasticity are discussed and the aim and outline of this thesis are described.

Mapping of the brain by guidance cues and adhesion molecules

In order for the brain to function properly, the right connections need to be formed. Neurons must thus migrate to the appropriate brain region and project neurites to the correct area. Guidance cues and adhesion molecules are major determinants of neuronal migration and projection. In general, guidance cues and adhesion molecules can be secreted and received by neurons, but glial cells can also participate. Here, I will define guidance cues as molecules in the extracellular matrix that direct neuronal migration and outgrowth and adhesion molecules as membrane attached molecules with the same function.

In the developing cerebral cortex neurons originate from the ventricular zone or the subventricular zone, and then accumulate under the pia mater to form the preplate. Neurons that are subsequently born migrate to the preplate and form the cortical plate inside out (with new cells migrating past earlier formed cells to form a new layer on top of the old layers). In the layering process, some cells of the preplate are pushed up to form the marginal zone and some cells are pushed down to form the subplate. Integrins and Reelin are involved in this layering process as deletion of these proteins results in disruption of cortical layering (Anton *et al*., 1999; Graus-Porta *et al*., 2001; Forster *et al*., 2002). Layering is sharpened further as cells, destined for different layers, express different Ephrins and Ephrin receptors. Ephrins and their receptors regulate the expression of other adhesion molecules and thereby attract or repel other cells (Xu *et al*., 2000; Mellitzer *et al*., 1999).

Ephrins and other regulatory molecules also direct projections of neurons to the correct region. For instance, molecules like limbic associated membrane proteins (LAMPs), chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) and cellular adherins (Cadherins) 6, 8 and 11 are expressed in specific brain areas and guide thalamic neurons to their appropriate cortical partners (Lopez-Bendito & Molnar, 2003; Gao *et al*., 2000). Other molecules like netrin-1 do not specifically guide axons to a particular region but rather serve to promote axonal outgrowth towards the proper region as specified by other guidance molecules (Braisted et al., 2000).

Once a projection is in the proper brain region it must arborize and form synapses in the right place, for instance the correct cortical layer. Ephrins, again are involved in this process. Together with molecules like semaphorins these proteins repel and attract neuronal projections until these are in the right position and synapses can be formed (Gao *et al*., 1998; Mackarehtschian

et al., 1999; Mann *et al*., 2002; Uziel *et al*., 2002; Messersmith *et al*., 1995; Bagnard *et al*., 1998).

Synapse formation

One of the most studied synapses in the central nervous system is the glutamatergic synapse between pyramidal cells in the hippocampus and neocortex. Another well-characterised synapse is the neuromuscular junction in the peripheral nervous system. Therefore these synapses will be used here as models to describe synapse formation in general. Before a synapse can be formed, the future pre- and postsynaptic partners need to be in close vicinity of each other; neurons must migrate to the proper region and project neurites towards each other before a synapse can be formed. When the axonal and dendritic compartments meet, synaptogenesis can begin.

Induction of synapse formation

It is not known which compartment initiates synapse formation. Observations of synaptogenesis at the neuromuscular junction, where motor neurons are motile, project to a static muscle and form synapses suggested that the presynaptic nerve determines the place of future synapses. However, as will be discussed below, postsynaptic specializations are already present in the future endplate region of the muscle before the nerve arrives, suggesting the muscle is instructive in initial contact formation (Yang et al., 2001). In the central nervous system, axonal projections cover large distances growing towards their targets, and therefore are intuitively considered as the instigators of synapse formation. However, dendrites also actively grow out to possible presynaptic partners, and dendritic growthcones/filopodia even appear to draw axonal protrusions towards the dendritic shaft (Wong & Wong, 2000; Smith, 1999; Jontes & Smith, 2000; Ziv & Smith, 1996), making the postsynaptic compartment equally suitable in determining where a synapse will be formed. From a physiological point of view it also makes sense that the postsynaptic compartment determines the site of future synapses; when a presynaptic neuron fires an action potential, the same signal reaches every presynaptic terminal and, regardless of their position, enables them to release neurotransmitter. On a dendrite, on the other hand, each synaptic event elicits a graded potential and the position

of a synapse is the main determinant of how each signal will be integrated (whether or not an action potential will be generated) and therefore what the effect of this synaptic event will be. It seems theoretically more likely that the postsynaptic compartment instructs the presynaptic compartment where a synapse will be formed, but direct experimental evidence remains to be found.

Adhesion molecules

Axons and dendrites that are in close proximity need to recognise each other as suitable synaptic partners. This is probably mediated by adhesion molecules. Many adhesion molecules have been identified and shown to function in synapse formation. For one couple, neurexin and neuroligin, it has even been shown that expression of neuroligin in heterologous cells induces neurons to form synapses upon them through neurexin (Scheiffele et al., 2000). Since thousands of splice variants can be formed of both neurexin and neuroligin, these molecules also make interesting candidates for "handshake" molecules, ensuring that synapses are not made in a random fashion but only between suitable partners expressing the right isoform of neurexin or neuroligin (Missler & Sudhof, 1998). Apart from determining synapse specificity, adhesion molecules are also involved in synaptic plasticity (Dityatev *et al.*, 2000; Muller *et al.*, 1996; Cremer *et al.*, 2000). Besides establishing physical contact through adhesion molecules the synaptic components must be equipped with the right molecular machinery; the presynaptic compartment must have the machinery to release neurotransmitters and neuromodulatory substances in a regulated fashion and the postsynaptic compartment must have the proper receptors.

Presynaptic development

Since synapses can be functional within hours after initial contact (Friedman *et al.*, 2000; Ahmari *et al.*, 2000) it is not likely that synapses are assembled from randomly diffusing components but rather are transported as pre-assembled functional units. In axons an 80 nm vesicle was discovered that contains many of the essential presynaptic components (Ahmari et al., 2000). These vesicles contain the adhesion molecule N-cadherin, structural active zone proteins like piccolo and bassoon, tSNARE (soluble N-ethylmaleimide-sensitive-fusion-protein (NSF) attachment-protein (SNAP) receptor (SNARE) of the target membrane) proteins syntaxin and SNAP-25 (synaptosome associated protein of 25 kD), priming factor munc13

(mammalian uncoordinated 13), vesicle cycling factor Rab3a/c, Rab effector RIM (rab3 interacting molecule) and fusion regulator munc18-1 (mammalian uncoordinated 18-1) and N-type calcium channels (Shapira *et al.*, 2003; Zhai *et al.*, 2001). Synaptic vesicles or known synaptic vesicle proteins like synaptophysin or synaptobrevin are not found in these 80 nm vesicles (Shapira *et al.*, 2003; Zhai *et al.*, 2001). Analysis of hippocampal cultures, transfected with green fluorescent protein (GFP)-tagged bassoon, showed the presence of fluorescent puncta at axonal growth cones and along the axon. A distinction could be made between highly mobile puncta, moving at speeds up to 0.35 μm/s in both directions, and static puncta. Most of the static puncta co-localized with FM 4-64, an endocytosis marker suggesting the presence of a functional synaptic terminal. Also, the static puncta were two to three times more fluorescent than the mobile puncta, suggesting that two or three 80 nm vesicles are sufficient to create a functional synaptic terminal.

Postsynaptic development

Similar to the presynaptic compartment, there is evidence that postsynaptic elements are also delivered in prefabricated clusters. Postsynaptic density protein of 95 kD (PSD95) is one of the first elements that is localized to nascent synapses. PSD95 is a multidomain protein that can function as a structural organizer of the postsynaptic density. In addition, PSD95 is involved in the recruitment of AMPA (α-amino-3hydroxy-5-methylisoxazole) receptors (AMPArs) to the postsynaptic density and making these functional (Beique & Andrade, 2003). In the first three weeks in culture (rat hippocampal neurons) most PSD95 clusters shift from extrasynaptic to synaptic location (Rao et al., 1998). In the same period dendritic filopodia gradually become less motile both *in vitro* and *in vivo* (Ziv & Smith, 1996; Dailey & Smith, 1996; Dunaevsky *et al.*, 1999; Lendvai *et al.*, 2000). In fact, the stabilization of dendritic filopodia and their transition into spines is both spatially and temporally correlated with the synaptic localization of PSD95 (Jontes & Smith, 2000; Okabe *et al.*, 2001). As was shown for the active zone precursor vesicles, PSD95 moves along the dendritic shaft in clustered form and becomes associated with stabilizing filopodia. However PSD95 is probably not the stabilizing factor of filopodia, as transfection of a mutant form of PSD95 that does not cluster or target to synapses has no effect on the formation of stable synapses (Prange & Murphy, 2001).
Both AMPArs and NMDArs (N-methyl-D-aspartate receptors) are transported along dendrites in packets (prefabricated clusters) (Washbourne et al., 2002a).

NMDA receptor containing packets move relatively fast, at ~4 μm/min both antero- and retrogradely along microtubules. AMPA receptor packets move relatively slow at ~2 μm/min. NMDArs appear very quickly after synapse formation, together or within minutes after arrival of PSD95. AMPArs do not arrive within an hour after synapse formation. Interestingly, synapses that are formed between axonal and dendritic shafts become functional (defined by presynaptic vesicle/FM dye cycling) within minutes while synapses formed at axonal growth cones may take up to four hours before they are functional. Apart from the delivery of receptors in prefabricated clusters there are numerous reports of individual receptors diffusing laterally in and out of synaptic regions and being shuttled between plasma membrane and cytosole (as will be discussed in the section about synaptic plasticity). There are also reports that PSD95 is not transported in packets but becomes synaptically clustered from a soluble cytoplasmic pool (Bresler *et al*., 2001; Marrs *et al*., 2001). Moreover, PSD95, NMDArs and AMPArs are delivered to synapses in different packages and little is known about the recruitment of other postsynaptic proteins. It also makes sense that at least part of the postsynaptic machinery is not delivered in prefabricated packages because dendrites are relatively short and have their own protein synthesising machinery (Black & Baas, 1989). Nonetheless, the evidence above suggests that in principle all components of basic synapses can be rapidly delivered in prefabricated packages, which is compatible with the observed velocity of functional synapse formation in cultures.

Box 1: Basic morphology and physiology of a neuron

A neuron, as a rule of thumb, has an input-receiving compartment consisting of a number of dendrites, a cell body and an output-generating compartment consisting of a single axon. Since there are many variations in the morphology of neurons, the rest of this description will concern an extensively studied model neuron: the pyramidal cell.

A pyramidal cell has a single apical dendrite receiving input from projection areas elsewhere in the brain and several basal dendrites receiving input from their local environment. Each of these primary dendrites branches several times, giving rise to secondary and tertiary

Box 1continued

dendrites. The dendritic surface is covered with spiny protrusions/ dendritic spines. Signal transduction in dendrites is passive; when an ion channel is opened, for instance by the binding of neurotransmitter to its receptor, a number of ions flow across the membrane and change the membrane potential. These ions diffuse laterally until the difference in membrane potential is dissipated and equilibrium is reached with the former state of the dendrite. The smaller the diameter of a dendrite the higher the resistance to spread of electrical signals and the more local a signal remains. A second effect of the high input resistance of lower order dendrites and spines is that relatively small ion fluxes result in large potential differences.

The majority of synapses on pyramidal cells are on the heads of dendritic spines and are excitatory in nature but excitatory synapses are also formed on dendritic shafts. Vice versa, most inhibitory synapses are formed on dendritic shafts but inhibitory synapses are also formed on the shafts of dendritic spines.

Signals that are generated in synapses can integrate, both spatially and temporally, and in this way a local signal can expand to other regions of a dendrite and eventually to the cell body.

Apart from the ligand-gated ion channels that are responsible for the generation of graded synaptic potentials the neuronal membrane contains voltage-gated ion channels that are responsible for the generation and maintenance of action potentials. The axon hillock contains the highest concentration of voltage gated (sodium) channels and generates the vast majority of action potentials. The rest of the neuronal membrane contains a lower density of voltage-gated channels, high enough to actively propagate the action potential, but generally too low to generate action potentials. Once generated, the action potential propagates both antero- and retrogradely. In forward direction the action potential mainly serves to evoke neurotransmitter release, which, dependent on the probability of release can occur at each individual nerve terminal. In backward direction the action potential “informs” the entire postsynaptic compartment of the neuron at which time the cell was activated, information that is crucial for synaptic plasticity as will be discussed below.

Forms of communication in the brain

Signalling in the brain is not uniform; various ways of communication between neurons (and other cells) exist. Here, the most important forms of communication in the brain, and their function in brain plasticity, are discussed. The first distinction can be made between direct physical/electrical coupling and chemical coupling.

Electrical coupling

Neurons can be physically coupled through gap junctions enabling them to directly relay electrical signals. Electrical synapses only make up a fraction of the total amount of synapses in the brain. Electrical interneuronal communication is basically the same as intraneuronal (passive) electrical signalling. One advantage of electrical coupling is that it is fast. In addition it is simple; no extra machinery is required for this kind of signal transmission. There are a number of disadvantages of electrical coupling. First, electrical synapses can only transmit depolarizing signals; probably hyperpolarizing signals are too weak in amplitude, as they cannot be converged into action potentials. Second, electrical synapses only function in direct/fast signalling; as no receptor-ligand binding is involved, no long lasting effect can be obtained. Third, the postsynaptic effect depends directly on the size of the presynaptic cell; the larger the presynaptic cell, the more ions are involved in its depolarisation, the larger the depolarisation of the postsynaptic cell. Fourth, there is no evidence that electrical synapses can alter their strength or reliability and therefore the direct contribution of an electrical coupling in synaptic plasticity is limited.
Although direct electrical coupling is not involved in synaptic plasticity, it is implicated in plasticity on a network level. Throughout life, but especially during the “critical” period of network formation (see below) neurons are reported to form transient gap-junctions. This enables neurons to develop a synchronous firing pattern, which, according to Hebbian rules (discussed below), strengthens chemical synapses and serves to consolidate networks (Schmitz *et al.*, 2001; Bem *et al.*, 2002; Peinado *et al.*, 1993).

Chemical coupling

Another form of communication between neurons is through receptors and ligands; one neuron presents a ligand and when this ligand binds

to its receptor a signal is transmitted. This ligand can be membrane bound as is the case with adhesion molecules, or can be secreted as is observed with neurotransmitters and neuromodulatory substances. Since adhesion molecules (and other guidance cues) are already discussed in the previous sections, the rest of this section will deal with the secretion of neurotransmitters and neuromodulatory substances.

Non-vesicular neurotransmitter release

The most prevalent form of neuronal communication is through the secretion and binding of neurotransmitter molecules. The simplest form of this neurotransmission is leakage (or non-vesicular release) of neurotransmitter molecules from the presynaptic compartment to postsynaptic receptors. Leakage can occur through channels that are specific for a particular kind of neurotransmitter or by diffusion across the plasma membrane. Although the exact mechanism by which leakage of neurotransmitter occurs has not been determined, this form of neurotransmission could potentially influence synapse formation and synaptic function. For instance in *Drosophila* mutants, in which vesicular secretion was abolished, the amount of presynaptically available neurotransmitter (which was manipulated by mutations of glutamate synthesizing and degrading enzymes) had a strong effect on the postsynaptic receptor field size (Featherstone et al., 2002). Since there is no known machinery for the regulation of non-vesicular neurotransmitter release, its presumed information content is low. Therefore, as synaptic plasticity is generally assumed to underlie information storage in the brain, non-vesicular release is not likely to direct synaptic plasticity.

Regulated neurotransmitter secretion

Contrary to non-vesicular release, vesicular release of neurotransmitter does offer the possibility of transneuronal communication with high information content. First, because vesicular release enables instant presentation of high concentrations of neurotransmitter to postsynaptic receptors (resembling binary “all or none” signaling). Second, because dozens of proteins have been identified that regulate synaptic vesicular release and hereby automatically change the information flow between neurons.

SNAREs are thought to be essential for vesicle fusion events throughout the cell. Synaptobrevin 1 is a transmembrane protein on synaptic vesicles. Synaptobrevin can form a ternary complex with the transmembrane protein syntaxin-1 and the membrane attached protein SNAP-25. The SNARE complex can undergo a highly exothermic conformational change, which can catalyze the energetically unfavorable fusion of two membranes (Jahn

& Sudhof, 1999). Although SNAREs are believed to be essential for vesicle fusion, synaptic vesicle fusion is not completely blocked by deletion of neuron specific SNAREs SNAP-25 or synaptobrevin (Washbourne *et al*., 2002b; Schoch *et al*., 2001). It is possible that membrane fusion is somehow possible without SNAREs, but it is more likely that related SNAREs can partially take over the function of the cognate SNAREs, especially because most SNAREs are quite promiscuous in their interactions (Toonen & Verhage, 2003).

A number of proteins function in the regulation of SNARE mediated membrane fusion; whether or not fusion occurs and perhaps to which exent fusion takes place (discussed below). Elevated presynaptic calcium levels are essential for evoked secretion and a molecular calcium sensor is likely to regulate SNARE mediated fusion. The prime candidate for this calcium sensor is synaptotagmin 1, as a point mutation in its calcium-sensing domain strongly reduces evoked neurotransmitter secretion without affecting parameters like spontaneous secretion(Fernandez-Chacon et al., 2001). Other calcium sensors that might be involved in the regulation of neurotransmitter release are Doc2 (double C2 domain), munc13, rabphilin, RIM and picollo (Dresbach *et al*., 2001; Garner *et al*., 2000; Gerber *et al*., 2001).

Fusion of synaptic vesicles is thought to exclusively take place at active zones (Zenisek et al., 2000). Active zones are electron dense structures aligned along the presynaptic membrane and consist of SNAREs to accomplish fusion, modulatory proteins to regulate fusion and structural proteins. A selection of these proteins that have a marked effect on neurotransmitter release is listed below.

Spectrin is a synapsin (synaptic vesicle protein) binding protein that is believed to form a filamentous network that holds together and spaces the presynaptic particle web in the active zone (Phillips et al., 2001). Although its exact function is not known, injection of peptide fragments of spectrin blocks neurotransmission (Zimmer *et al*., 2000; Sikorski *et al*., 2000). Bassoon is a large (420 kD) protein with multiple protein interaction domains, allowing it to structurally organize the active zone (Gundelfinger et al., 2003). The exact function of bassoon is not known but its deletion in mice completely blocks neurotransmitter secretion in ~10% of all synapses while leaving other synapses unaffected (Altrock et al., 2003). Munc13 is a protein that is involved in priming/making synaptic vesicles ready for fusion. In the cerebrum two isoforms exist; munc13-1 primarilly drives excitatory synapses and munc13-2 primarilly drives inhibitory synapses. Both isoforms can function in any synape, as is observed in culture (Rosenmund et al., 2002). When both isoforms are deleted all neurotransmitter secretion is

blocked apart from a sporadic fusion event after application of α-latrotoxin (Varoqueaux *et al.*, 2002a). RIM binds to munc13 and interacts with rab3A. Deletion of RIM1α does not have a strong effect on basic neurotransmitter release but showed defects in synaptic plasticity that resemble the deletion of rab3A (Schoch *et al.*, 2002; Castillo *et al.*, 2002). Therefore, either RIM has a modulatory role in synaptic vesicle fusion rather than an essential one, or RIM2α can compensate for the loss of RIM1α. Munc18-1 is a syntaxin binding protein that regulates the amount of vesicles that are docked at the membrane/ready for priming. Deletion of munc18-1 results in the most dramatic block of neuronal vesicular fusion observed in any mutant (Verhage *et al.*, 2000; Voets *et al.*, 2001). Mints are munc18-1 interacting proteins that also bind to CASK (calmodulin dependent serine kinase), amyloid precursor protein, presenillin and kinesin Kif17. Deletion of mint 1 has no effect on basal synaptic transmission and a minor effect on short term plasticity resembling the deletion of munc13-2 (Rosenmund *et al.*, 2002; Ho *et al.*, 2003). Therefore mints are either not essential for synaptic vesicle secretion, or mint 2 can compensate for the loss of mint 1. Finally, piccolo is a very large (530 kD) protein that has multiple domains allowing it to interact with multiple proteins (Fenster et al., 2000). Even though it's function is not known, its multiple interactions with other active zone proteins suggest it is a key player in the regulation of synaptic function. One proposed function of piccolo is to regulate endocytosis of synaptic vesicles at the active zone (Fenster et al., 2003).

Box 2: Exocytosis, endocytosis and cycling

Once a vesicle fuses with the plasma membrane the vesicle must be retrieved. If not, the synaptic membrane will expand and change synaptic morphology. The most common mechanism of vesicle retrieval is by endocytosis. In short, adapter protein 2 recruits clathrin to the plasma/vesicle membrane, where a vesicle shaped lattice is built that invaginates the membrane. Interaction with adapter protein 180 determines the size of the lattice and therefore the amount of retrieved membrane. Once the clathrin coat is finished, dynamin forms a collar around the neck of the invagination and pinches off the vesicle (Gundelfinger et al., 2003). The endocytosed vesicle can now be incorporated into an endosome where its protein content can be

Box 2 continued

changed or it can be directly refilled with neurotransmitter and returned to the releasable pool (Buckley et al., 2000).

A number of variants on this scheme of membrane retrieval have been described, among which a mechanism named "kiss and run" (Aravanis *et al*., 2003;Gandhi & Stevens, 2003). Here, the vesicle does not fully merge, but apart from the fusion pore, remains separated from the target membrane, and is retrieved on the spot. This has the advantage that the lipids and proteins of the vesicle and active zone are not mingled and thus enables faster vesicle cycling. An even faster way of vesicle recycling has been proposed, named "kiss and stay" (Sudhof, 2000). In this case, the vesicle does not leave the active zone, keeps its SNAREs and is fusion ready again within an instant. Experimental evidence for a "kiss and stay" mechanism was found in large dense core vesicle (LDCV) secreting chromaffin cells (Tabares et al., 2001). The authors found that a fraction of LDCVs did not secrete catecholamines, suggesting that these vesicles were instantaneously fusion ready again *i.e.* before refilling could occur (the use of these fusion events is questionable of course). Another interesting finding was that the fusion pore of these vesicles expanded significantly slower that that of other, catecholamine filled, vesicles, implying that the release kinetics during secretion events can be regulated as well.

The three described ways of exocytosis, full fusion, "kiss and run" and "kiss and stay", offer interesting possibilities in terms of information content of vesicular release. First, because the different forms of fusion could be associated with different release kinetics. Second, because these fusion types could participate in synaptic plasticity; "kiss and stay" fusion could enable extremely rapid firing of a presynaptic terminal. Full fusion and endocytosis, on the other hand, enable a change in vesicle membrane protein composition or even content and therefore the formation of distinct populations of synaptic vesicles within a synaptic terminal. Evidence for the existence of different populations of vesicles that respond to different stimuli was found in LDCV secreting chromaffin cells (Duncan *et al*., 2003; Sudlow *et al*., 1994).

Regulated secretion of neuromodulatory substances

Apart from the small clear cored vesicles which function in neurotransmitter secretion, LDCV secretion is also tightly regulated in neurons. LDCVs are not secreted at the active zone; in chromaffin cells LDCVs can fuse at random locations. Whether synaptic LDCVs can fuse at random sites or need specific location bound machinery is not known. It is known that many proteins that regulate synaptic vesicle secretion, like munc13-1 and munc18-1 also participate in LDCV secretion (Voets *et al*., 2001; Ashery *et al*., 2000). However, there are also proteins that function specifically in LDCV fusion, for instance CAPS, a protein that shows functional resemblance with munc13-1 and is essential for LDCV secretion in synaptosomes and *Drosophila* NMJs (Tandon *et al*., 1998; Renden *et al*., 2001). In contrast to synaptic vesicle secretion, one action potential is usually not sufficient to trigger LDCV exocytosis at synaptic terminals. Rather, LDCVs are secreted after a train of action potentials (Cowan et al., 2000). As a consequence, LDCVs are thought not to function in fast neurotransmission, but in long lasting modulation of neuronal function.

Glial cells

Even though glial cells are not neurons, and therefore by definition cannot contribute to neuronal communication, these cells deserve mentioning. The general concept is that neurons form the primary communicating organ while glial cells serve merely to support neurons, by physical support, electrical isolation, nutrition and waste disposal, balancing environmental osmotic values etc. However, in recent years, glial cells have become increasingly involved in brain communication. Like neurons, glial cells have been shown to form networks through which calcium waves are transmitted. Also, since glia actively regulate the amount of neurotransmitter (metabolites) in the neuron's environment it affects the neurons ability to transmit signals. Furthermore, glia have been shown to possess the SNARE machinery required for fast synaptic transmission and are able to perform SNARE mediated secretion. Finally, glia have been implicated in the secretion of neuromodulatory substances (Smit et al., 2003). This together with the estimation that there are approximately 10 times more glial cells in the brain than neurons, make glial cells an important factor in neuronal communication/brain function (Fields & Stevens-Graham, 2002; Araque *et al*., 1999; Bezzi & Volterra, 2001).

Examples of activity based plasticity

Thus far, the general formation of the brain, the generation of basic synapses and the potential forms of communication between neurons have been discussed. This section will focus on the role that neuronal activity, in the form of neurotransmitter secretion, has in the rewiring of the initial neuronal/synaptic networks. First two well-studied models for developmental plasticity are described. Then, ongoing plasticity in living neuronal networks is discussed.

Ocular dominance column formation

In cats, and later in other mammals, it was found that the primary visual cortex is grouped in alternating columns of neurons that preferentially respond to one eye (Hubel & Wiesel, 1970). This columnar organisation already exists before birth and is therefore innate (Des Rosiers *et al*., 1978; Rakic, 1976). However, during the so called critical period, these ocular dominance columns must be consolidated by visually driven activity; when new born animals are dark-reared or when visual circuit activity is suppressed by blocking action potentials, blocking NMDArs or stimulating GABAergic input (Berardi et al., 2000), columnar organisation and eye preference disappear. Furthermore, ocular dominance is driven by differential input as strobe rearing, thus equalizing visual input for both eyes also dissipated columnar organisation. Moreover, monocular sensory deprivation dramatically reduces columnar width of the corresponding eye at the expense of the columns responding to the other eye (Katz & Crowley, 2002).
Interestingly, when both eyes are removed upon birth, not only excluding visually driven activity but also spontaneous neurotransmitter signalling of the retina towards the lateral geniculate nucleus and cortex, the existing columnar organisation of the visual cortex remains intact (Crowley & Katz, 1999). Thus, at least in the visual system, evoked neuronal activity serves to confirm an already existing neuronal network instead of creating it. In addition, spontaneous neurotransmitter release appears to play an important role in the dissipation of “unused” neuronal circuits, and is probably also instructive in the initial formation of these circuits.

Neuromuscular junction elimination

During embryonic development each muscle fiber is innervated by multiple

motor axons and each motor axon innervates multiple muscle fibers. Therefore, when any given motor neuron fires an action potential, muscle fibers at different locations in the muscle contract simultaneously, resulting in sub-optimal muscle contraction. In order for a muscle to contract smoothly and generate maximal power the stimulation of individual muscle fibers by various axons must be well controlled both spatially and temporally. Adjacent muscle fibers must be recruited/contracted in an ordered fashion (Stotz & Bawa, 2001). Nerve terminals that have such a firing pattern that, together, they elicit a smooth muscle contraction eventually survive. Nerve terminals that fire out of synchrony with the optimal pattern are retracted (Lichtman & Colman, 2000). Since before the onset of NMJ elimination all nerve terminals cover AChR patches of a similar size, the absolute postsynaptic response that a nerve terminal elicits is not likely to be the sole determinant of which terminal survives. Rather, the terminals that participate in the largest overall postsynaptic responses survive.

The exact molecular mechanism is underlying NMJ elimination is unknown. However, a number of components have been identified. There must be a signal that maintains the AChR clusters underneath the surviving nerve terminals. This positive/aggregating signal is agrin; in mice deficient for agrin, the prepatterned AChR clusters that the muscle autonomously forms before the arrival of nerve terminals gradually disappear during embryonic development (Yang *et al.*, 2001; Lin *et al.*, 2001). Many components that function downstream of agrin, such as muscle specific kinase (MuSK) and rapsyn have been identified (Gautam et al., 1999). It has also been proposed that there must be a negative component that disperses the AChR clusters underneath the nerve terminals that are to be eliminated. The most likely candidate for this is the muscle activity that is elicited by motor neurons. The rationale behind this concept is that nerve evoked muscle activity elicits a general negative signal, dispersing all AChR clusters, exept those that are sufficiently supplied with agrin by the "winning" nerve terminals. In chapter two the role of nerve evoked muscle activity in NMJ development will be discussed in more detail.

The exact mechanism by which the muscle "keeps track" of which nerve terminals participated in the generation of optimal muscle contraction, and therefore should be excluded from the elimination process, is still unknown. It is assumed that retrograde messengers, such as GDNF (Nguyen & Lichtman, 1996), are involved. For instance, it is possible that an optimal muscle contraction elicits postsynaptic retrograde messenger release, but only at those sites that were involved in the generation of muscle depolarization. In this way only the nerve terminals that acted in controlled muscle contraction

are “rewarded” by retrograde messengers and stimulated to secrete agrin that can serve to maintain the underlying receptor patch from degradation.

General plasticity

The presence of ocular dominance columns and the elimination of NMJs are both examples of activity based synaptic competition during development. However, activity based plasticity of synaptic connections is a process that is thought to rewire the brain continuously during learning and memory formation (Cajal, 1893). The most dramatic forms of synaptic plasticity are long term potentiation and depression where synapses actually can be added or deleted, but synaptic activity also underlies minor/short lasting modifications of synaptic efficacy.

Short term presynaptic plasticity

The shortest form of presynaptic plasticity is facilitation; when two action potentials arrive shortly after each other, the second action potential has a higher probability of evoking neurotransmitter release. The most probable cause for facilitation is the accumulation of residual calcium from the first action potential and calcium influx after the second action potential at the active zone. The increased calcium concentration increases the probability of release and thus facilitates neurotransmitter secretion after the second action potential. The facilitation effect is maximal when the initial probability of release is low and vice versa.

Augmentation requires a short train of action potentials and lasts for 8-20 seconds. Post tetanic potentiation requires more action potentials and lasts for minutes. Similar to facilitation, raised calcium levels are likely to be responsible for augmentation and post tetanic potentiation, as artificial elevation of internal calcium levels are sufficient to induce these types of plasticity (Zengel *et al.*, 1994; Brain & Bennett, 1995; Delaney & Tank, 1994). Exactly how calcium regulates these forms of potentiation is unknown, but calcium binding proteins such as munc13, DOC2, rabphilin, RIM, picollo and synaptotagmin (mentioned above) are interesting candidates.

Short-term postsynaptic plasticity

In addition to these presynaptic forms of short-term plasticity, synaptic strength can also be regulated postsynaptically. The fastest form of postsynaptic plasticity is desensitization; binding of a ligand to a receptor can cause the receptor to be unresponsive to ligands that are presented shortly after (milliseconds to minutes, reviewed in (Jones & Westbrook,

1996). For AMPA and NMDA it was shown that these receptors can laterally diffuse in and out of the synapse and thereby influence the postsynaptic response (Borgdorff & Choquet, 2002; Tovar & Westbrook, 2002). AMPA receptors are also shuttled between the cytosol and plasma membrane to regulate synaptic strength, a process which is mediated by NMDA receptor activation (Lu et al., 2001). Finally, the subunit composition of a receptor can be changed thereby changing the properties of the receptor (Passafaro et al., 2001).

Long-term potentiation and depression

Like short-term plasticity, long term plasticity is dependent on raised calcium levels but in addition it requires protein synthesis and structural changes; the number of docked and primed vesicles, the number of receptors, the respective size of active zone and postsynaptic density, the number of synapses etc.. One of the characteristics of long term plasticity is that it generally requires activation of NMDArs. Hereto, the synapse must have been activated very recently (to release the voltage dependent magnesium block). In addition, timing of depolarization is important; when the presynaptic compartment is depolarized before the postsynaptic compartment, the synapse is potentiated. When the postsynaptic compartment is activated before the presynaptic compartment, the synapse is depressed. Furthermore, the time difference between pre- and postsynaptic activation is important; the closer the time window, the stronger the effect.

As in normal plasticity, long term plasticity partially relies on lateral diffusion and insertion and excision of receptors/subunits. In addition, more drastic effects can occur; extreme potentiation can result in the duplication of a synapse, just as extreme depression can result in the silencing or deletion of a synapse.

Long term plasticity can be modulated by other neurotransmitters like serotonin, dopamine, acetylcholine and noradrenalin (Buzsaki & Gage, 1988). For example, acetylcholine decreases potassium channel permeability, increasing the excitation level of a neuron, which influences plasticity (Nakajima et al., 1986).

The location of a synapse is also of importance for plasticity. As mentioned before, whether a synapse is potentiated or depressed depends on the relative timing of pre- and postsynaptic activity and the amount of plasticity is determined by the absolute time difference between pre- and postsynaptic activity. Postsynaptic activity is mostly determined by action potentials that propagate back into the dendritic tree. As the signal propagates further into a dendrite, the diameter of the dendrite decreases and the resistance

increases. As a result the signal propagates slower, which in turn gives a larger time window for plasticity to occur (Rao & Sejnowski, 2001). Since a higher input resistance also increases the degree of depolarization, this might also have an effect on the degree of plasticity. Therefore, the location of a synapse on the dendritic tree determines whether or not, and to which extent, potentiation and depression occur.

Spontaneous fusion; by product of regulated secretion or a functional event?

In the sections above, a multitude of experiments was discussed, which together built a strong case for the importance of neurotransmitter-mediated neuronal activity in neurite outgrowth and synaptic plasticity. Neurotransmitter mediated neuronal activity in general is viewed as an action potential evoking the release of a number of transmitter-filled synaptic vesicles which, in turn, evoke postsynaptic potentials. When enough vesicles fuse simultaneously, these postsynaptic potentials can in trigger an action potential in the postsynaptic neuron.
However, not every fusion event is triggered by an action potential. As these events are not synchronised, and by themselves only elicit a small postsynaptic effect, they have very little chance of eliciting a postsynaptic action potential. For this reason these fusion events are often nicknamed miniature or “spontaneous” and discarded as noise in the system. The finding that innate ocular dominance columns disappear when evoked activity is inhibited, unless the entire eye is removed upon birth, suggests that spontaneous neurotransmitter secretion has a function in synapse formation (Katz & Shatz, 1996). In hippocampal slice cultures, dendritic spines could be maintained when evoked neurotransmitter release was inhibited with tetrodotoxin. When spontaneous release was impaired with botulinum toxins, spines could not be maintained, unless simultaneously treated with AMPA agonists, suggesting spontaneous release is functional in dendritic spine/synaptic maintenance (McKinney et al., 1999). In acute rat hippocampal slices miniature/spontaneous release events, in principle, are sufficient to induce action potentials in pyramidal cells (Sharma & Vijayaraghavan, 2003). Finally, it was recently shown in developing synapses in island culture that asynchronous release can compensate for depressed synchronous release and maintain secretion at near maximal levels (Otsu et al., 2004). Therefore, asynchronous release can indeed be functional, and arguably should not be designated spontaneous.

In addition to experimental findings suggesting a function for spontaneous neurotransmitter secretion, there is also a theoretical reason to assume this; action potential-evoked, synchronised fusion is a highly effective form of regulated secretion that is useful to simultaneously inform many postsynaptic partners that a given neuron is active. However, this simultaneous activation of all presynaptic terminals also imposes a major restriction on the potential information processing content of a neuronal network; probably not every signal needs to be transmitted in 1000-fold in order for the information to be reliably received.
Non-synchronised release is not necessarily driven by action potentials, but presumably still regulated and therefore offers the possibility for a neuron to communicate at a single synapse. The simplistic view of neurons only receiving input at their dendrites and only generating output at their axonal terminal has gained complexity over the years; axo-axonal synapses have been described and also dendritic retrograde signalling was found in the last few years (Ludwig & Pittman, 2003). In addition, mossy fiber terminals for instance are sites where synapses of various modalities converge in an area of only a few micrometers (thus enabling a single neurotransmitter quantum to locally depolarise above firing treshold). These are therefore sites where single vesicles could theoretically be functional and effective in highly localized feed-forward and feedback excitation and inhibition, without involving the entire neuritic trees of the corresponding neurons.

Unevoked synaptic vesicle fusion may be involved in clustering and organisation of postsynaptic receptor clusters as was observed *Drosophila* NMJs; in flies that lack syntaxin or dynamin (a protein involved in endocytosis at the synapse) all synaptic vesicle fusion is abolished and postsynaptic glutamate receptor clusters fail to develop at the NMJ (these mutants and a number other regulated secretion defects that influence neurite outgrowth or synapse formation are listed in table 1). In flies that lack synaptobrevin or cysteine string protein, evoked release is blocked but spontaneous fusion can still occur. Flies carrying these mutations have normally developed glutamate receptor clusters. This suggests that spontaneous fusion is critical for the development of receptor clusters (Saitoe et al., 2001). Interestingly, the authors found that injection of agriotoxin (which blocks glutamate receptors) has no effect on glutamate receptor clustering, suggesting that not the binding of neurotransmitters to receptors, but some other secreted agent is crucial for glutamate receptor clustering.
Another group, using the same mutants, came to different conclusions; neither evoked, nor spontaneous fusion of neurotransmitter vesicles is

required for glutamate receptor clustering at the fly NMJ (Featherstone et al., 2002). The authors did mention that the amount of presynaptic glutamate determined the size of the postsynaptic receptor field; loss of function of the glutamate producing enzyme glutamate oxaloacetate transaminase increased the postsynaptic receptor field. Loss of function of the glutamate degrading enzyme glutamate decarboxylase decreased the receptor field. The size of the receptor field in the latter mutant was not affected by overexpression of (synaptobrevin cleaving) tetanus neurotoxin. These data suggest that, even though synaptic vesicle fusion is not involved, presynaptic glutamate can negatively regulate the size of postsynaptic receptor field, again contradicting the data by Saitoe *et al.*. Thus, some presynaptic factor organizes the postsynaptic receptor field and whether this is spontaneous neurotransmitter secretion is still under debate.

Regulated secretion and neurite outgrowth

Regulated secretion of neurotransmitters plays a critical role in synaptic plasticity, as was described above. Plasticity often involves neurite remodeling or outgrowth and thus vesicular fusion to accommodate incorporation of new membrane. This offers the interesting possibility that the synaptic vesicles, SNAREs and regulatory proteins that are instrumental in delivering the neurotransmitters that induce plastic events are also directly involved in delivering the building blocks for these changes.

In PC12 cells it was found that newly synthesized synaptophysin was transported to the plasma membrane in constitutive exocytosis vesicles and subsequently endocytosed to mature synaptic vesicles (Regnier-Vigouroux et al., 1991). In cultured hippocampal neurons, SV2, synaptobrevin and synaptophysin, all present on mature synaptic vesicles are transported differentially and co-localize at synaptic terminals, suggesting that these proteins are joined in synaptic vesicles on site (Mundigl et al., 1993). Moreover, different synaptic vesicle proteins are transported to synaptic terminals by different motor proteins (Okada et al., 1995). These findings suggest that synaptic vesicles are formed by endocytosis and thus reduce the likelihood that fusion of synaptic vesicles contributes to membrane expansion or the first signalling events between synaptic partners. Apart from experimental evidence there is also a theoretical constraint against synaptic vesicles subserving growth cone expansion. Given that an axon of 1μm diameter can grow up to 40 μm per hour this would take 100.000 synaptic vesicles of 20 nm diameter (per hour) when these vesicles were the

sole source of membrane at the growth cone. Fusion of 100.000 vesicles per hour, or ~25 per second would be pushing the limits of the fastest mature synapses and are therefore extremely unlikely to occur at a growth cone that lacks fully differentiated synaptic vesicle fusion machinery. Thus, synaptic vesicles are unlikely to contribute to membrane expansion in neurite outgrowth.

The core components of the SNARE complex (syntaxin, synaptobrevin and SNAP-25) have been proposed to function in membrane expansion even before the complex has matured enough to be functional in regulated secretion (Igarashi et al., 1997). Syntaxin-1 has been proposed to be involved in membrane expansion at the growth cone of growing axons (Ahnert-Hilger et al., 1996)(not experimentally shown). Cleaving of syntaxin-1 with BoNT-C (which also cleaves SNAP-25) results in growth cone collapse and inhibits neurite outgrowth (Igarashi et al., 1996), but also causes neuronal death, which obscures the effect of BoNT-C on neurite outgrowth (Osen-Sand et al., 1996).
Studies with anti-sense RNA against SNAP-25 and SNAP-25-cleaving BoNT-A suggested that SNARE proteins are required for neurite outgrowth, both *in vivo* and *in vivo* (Osen-Sand *et al*., 1993; Osen-Sand *et al*., 1996). However, another study suggests that BoNT-A has no detectable effect on neurite outgrowth, synapse formation or neuronal viability (Williamson & Neale, 1998). In addition, mice with a null mutation for SNAP-25 show no impairment of neurite outgrowth or synapse formation (Washbourne *et al*., 2002b). It is possible that partial redundancy with comparable SNARES, like SNAP-23, can compensate for the loss of SNAP-25.
Cleaving synaptobrevin with TeNT or BoNT-B did not prevent neurite outgrowth or synapse formation suggesting that in contrast to SNAP-25 (and syntaxin), synaptobrevin is dispensable for neurite outgrowth and that evoked neurotransmitter secretion is dispensable for synapse formation (Osen-Sand et al., 1996). This can be explained by the presence of a TeNT insensitive form of synaptobrevin (Ti-VAMP) that could function in neurite extension (Galli et al., 1998). In agreement with these data, *Drosophila* and mouse null mutations of n-syb/synaptobrevin 1 did not show any impairment in neurite outgrowth or synapse formation. Again, other isoforms of synaptobrevin could compensate for the loss of n-syb/synaptobrevin 1.
Apparently the SNAREs that are required for regulated secretion are not required for neurite outgrowth or are redundant with other SNAREs. Syntaxin-13 for example is implicated in neurite outgrowth. When syntaxin-13 is overexpressed in PC12 cells neurite outgrowth is accelerated (Hirling

et al., 2000). For comparison, overexpression of syntaxin-1 has no effect on neurite outgrowth. Both in PC12 cells and in dissociated neurons, syntaxin-13 is present in punctate and tubular patterns in perinuclear regions and in neurites and co-localizes with SNAP25. Syntaxin-13 was first described to function early in the endocytotic cycle where it is involved in separating endocytosed components (receptors etc.) that are destined to be returned to the plasma membrane from the bulk endocytotic mass (Prekeris et al., 1998). Syntaxin-13 is predominantly expressed in brain, by neurons (Advani et al., 1998). Brain expression levels of syntaxin-13 peak specific during neurite outgrowth, but are moderate both before and after (Sarria et al., 2002). This could imply that syntaxin-13 both has a role in the separation of endocytosed components, for which low levels are sufficient, and in neurite elongation, for which higher levels are required. Thus, the SNAREs that are required for synaptic vesicle secretion are not essential or at least redundant to SNAREs that have a clear function in neurite outgrowth.

One regulatory/non-SNARE protein complex that is involved in neurite outgrowth was first described in yeast; a complex of proteins was discovered to be essential for constitutive exocytosis and the budding of new yeast cells and was conveniently named the exocyst complex (Hazuka et al., 1997). In rat hippocampal neurons the exocyst complex is localized at growth cones and in synaptic regions (Hazuka et al., 1999), and therefore a good candidate to function in neurite outgrowth and synapse formation. In *Drosophila*, NMJ formation was analysed in a sec5 null mutant (Murthy et al., 2003). In the first stages of larval development neurite outgrowth and synapse formation were normal. This was probably due to maternal sec5 because, as the maternal contribution diminished, neurite outgrowth arrested and no more synaptic boutons were added to the existing NMJs. Even though vesicles with synaptic proteins were still transported to synaptic regions and accumulated there, these vesicles could no longer fuse. Sec5, however, does not appear to function in neurotransmitter secretion.
Still, several proteins that regulate SNARE mediated membrane fusion have been implicated in neurite outgrowth. Rab3 is a protein is a protein with mild regulatory function in secretion, but its deletion in *C. elegans* results in aberrant sprouting of 3% of motor neurons (Zhao & Nonet, 2000). Synaptotagmin is a calcium binding protein that presumably triggers evoked secretion(Fernandez-Chacon et al., 2001). In *C. elegans* deletion of this gene, *snt1*, results in aberrant sprouting of 25% of motor axons. Similarly, deletion of *unc13* results in aberrant sprouting of 50% of motor axons (Zhao & Nonet, 2000). Interestingly, deletion of *Drosophila* and mammalian orthologues

drosophila unc-13 and *munc13-1* and *munc13-2* gave no defect in neurite outgrowth or synapse formation (Aravamudan *et al.*, 1999;Varoqueaux *et al.*, 2002b), but this might be explained by partial redundancy with CAPS (Renden et al., 2001).
Mutation of *munc18-1* has the largest effect on regulated secretion and is discussed below. The yeast ortholog of *munc18-1* is sec1, and when it is deleted membrane expansion and budding stop (Novick et al., 1980). Although any relationship between neuronal outgrowth and budding yeast is far fetched, this suggests that munc18-1-like proteins are critically involved in regulated secretion events. In *C. elegans* the deletion of *unc18* has less clear effects; one mutation (*e81*) does not prevent neurite outgrowth, but gives massive aberrant sprouting of cholinergic SAB axons and consequently, mismatched synapses (Zhao & Nonet, 2000). Another mutation (*e234*), however, shows no effect on GABAergic neurons/synapses (Weimer et al., 2003). The reason for this discrepancy is unknown. However, with respect to the proposed role of neuronal activity in neurite outgrowth and synapse formation, it is conceivable that a secretion defect in hyperpolarising/ GABAergic synapses would be less conspicuous than a defect in excitatory/ cholinergic synapses. In *Drosophila*, the deletion of *munc18* ortholog *rop* has no effect on neurite outgrowth or synapse formation (Harrison et al., 1994). However, it is anticipated that maternal rop obscures the phenotype. In rat PC12 cells, overexpression of *munc18-1* reduced total neurite length, an effect that was enhanced by a mutant form of *munc18-1* that could no longer be phosphorylated by Cdk5 and has increased affinity for syntaxin (Steiner et al., 2002). Interestingly, the same group found that in hippocampal neurons, overexpression of *munc18-1* increased total neurite length by increasing the amount of branches, not individual neurite length. In conclusion, analysis of genetically or otherwise manipulated yeast, worms, flies, mice or cultured neurons did not elucidate the role of regulated secretion in neurite outgrowth and synapse formation but do underline its significance in these processes.

The munc18-1 knockout mouse

In *C.elegans*, synaptic vesicle fusion in not completely blocked by deletion of *unc18* (Zhao & Nonet, 2000;Weimer *et al.*, 2003). In contrast, mice deficient for mammalian *unc18* (*munc18-1*) appear to completely lack neurotransmitter secretion and as a result are paralysed and stillborn (Verhage et al., 2000). The assumption that *munc18-1* deficient neurons

are devoid of both spontaneous and evoked neurotransmitter secretion was based on two observations. First, field stimulation in acute neocortical preparations did not elicit GABA receptor stimulation in the mutant. Second, application of α-latrotoxin did not elicit a postsynaptic response in mutant diaphragm muscle (Verhage et al., 2000). It is theoretically possible that *munc18-1* deficient neurons are to some degree capable of neurotransmitter secretion. Also, it is not known whether neuromuscular synapses are actually present in mutant mice, thus obscuring the α-latrotoxin data.

Apart from neurotransmitter secretion LDCV fusion in neurosecretory cells is affected in the absence of *munc18-1*. In *munc18-1* null adrenal chromaffin cells the secretion of catecholamines is reduced to ~10% after stimulation with calcium (Voets et al., 2001). Basal secretion of growth hormone (GH) and adrenocorticotropic hormone (ACTH) by neurosecretory cells in the pituitary was reduced to less than 2% and 50% respectively (Korteweg *et al*., unpublished data). Excessive stimulation of secretion with calcium raised GH and ACTH ~2-fold in both mutant and wildtype cells. The total content of GH and ACTH was not lower in mutant cells. This suggests that the capacity to secrete hormones, not the activity level of the pituitary cells (which are not activated in mutant brains) or the level of available hormone is limiting hormone secretion in *munc18-1* deficient cells. It is not known whether the mechanisms of pituitary hormone granule secretion or chromaffin cells LDCV secretion are identical to the mechanism of LDCV secretion in neurons, but it can be assumed that neuronal LDCV secretion is affected to some extend in *munc18-1* null neurons.

The exact function of munc18-1 in regulated secretion is not known, but in chromaffin cells munc18-1 has a clear effect on vesicle docking to the membrane; normally ~50% of the vesicles are docked (i.e. less than one vesicle radius removed from the plasma membrane). When *(m)unc18-1* is absent, only a few vesicles are docked to the membrane and the rest are dispersed over the cytoplasm (Voets *et al*., 2001; Weimer *et al*., 2003). In the *munc18-1* null pituitary a similar phenotype is observed; the mean granule distance from the plasma membrane has nearly tripled and the amount of morphologically docked granules is reduced (Korteweg *et al*., unpublished data). Also in the neocortex, *munc18-1* appears to have a positive effect on both synaptic vesicle and LDCV docking (Bouwman *et al*., 2004). However, the amount of vesicles in the developing neocortex is very low, which makes analysis of docking less clear-cut. Regardless of the exact role of *munc18-1* in regulated secretion, its deletion results in the most severe neurosecretory phenotype described to date.

Despite the (presumed) absence of regulated secretion, gross morphological

development of the brain is normal. Synaptic vesicle markers synapsin and synaptobrevin are enriched in layers 1 and 4 of the cortex and electron microscopical analysis confirmed the presence of synapses in layer 1 (the marginal zone) of the neocortex. This suggests that brain development, up to the level of synapse formation, can occur normally in the absence of *munc18-1* (-dependent secretion). It is not known however, if the final stages of neurite outgrowth and the bulk formation of synapses, which occur in the peri-natal period are also unaffected in the absence of *munc18-1*.
After initial development the brain of *munc18-1* deficient embryos degenerates. This degeneration occurs in developmental order with the first formed brain areas degenerating first (Verhage et al., 2000). It is not known what causes this degeneration but it is tempting to speculate that this is related to the observed defect in regulated secretion.

Aim and outline of this thesis

The aim of this thesis is to elucidate the role of regulated secretion in neuronal viability, neurite outgrowth and synapse formation. The most profound neurosecretory phenotype known to date was obtained by genetic deletion of *munc18-1*. In mutant chromaffin cells, docking and secretion of LDCVs is reduced by 90% (Voets *et al.*, 2001). Synaptic vesicle secretion is completely absent in the mutant (Verhage *et al.*, 2000). For this reason, the *munc18-1* null mutant mouse was used as a model system.
The aim of chapter 2 is to describe neurite outgrowth, synapse formation and neuronal survival in the peripheral nervous system of developing *munc18-1* deficient embryos. Neurotransmitter secretion is tested by application of α-latrotoxin and direct stimulation of the phrenic nerve. As NMJs are extremely large synapses and much is known about their development, this is the ideal preparation to identify the role of spontaneous vesicular fusion in postsynaptic AChR receptor cluster organisation. The characteristic innervation pattern enables analysis of the role of regulated secretion on neurite outgrowth and synapse formation. The potential link between presynaptic secretion and neuronal survival is analysed by comparison of sensory and motor neuronal survival. The results suggest that neurotransmitter secretion is required for proper terminal, not initial, neurite outgrowth, AChR cluster refinement and motor neuronal survival.
Chapter 3 further addresses the potential relationship between presynaptic secretion and neuronal survival. Different components of regulated presynaptic secretion are mimicked by culturing *munc18-1* deficient neurons

in the presence of (combinations of) glial cells, insulin, BDNF, dissociated wildtype neurons or wildtype organotypic hippocampal slices. To minimize potential developmental defects and and optimize trophic support, mutant neuronal survival is tested in a cerebellar Purkinje-cell-specific *munc18-1* deficient mouse. We conclude that trophic support, not neurotransmitter secretion delays neuronal degeneration but *munc18-1* is ultimately required for long-term neuronal survival.

In chapter 4 the aim is to analyze the role of regulated secretion in neurite outgrowth and branching in cultured hippocampal and neocortical neurons. Neurotransmitter secretion is tested by application of sucrose and α-latrotoxin. Control and mutant neurons are compared to analyze the role of regulated secretion in neurite outgrowth, branching, and growth cone dynamics. We conclude that *munc18-1* is initially dispensable for initial neurite outgrowth but later is required for normal dendrite outgrowth and branching. In the absence of *munc18-1* net growth cone elongation is reduced while more and longer growth cone filopodia are formed.

The aim of chapter 5 is to rescue cultured *munc18-1* deficient from degeneration and to create a system to analyse mutant forms of munc18-1 and their effect on neuronal function. Hereto, neuronal viability, neurite outgrowth, synapse formation and neurotransmitter secretion are tested in late embryonic munc18-1 deficient neurons that are transduced with *munc18-1*. In addition, several mutant forms of *munc18-1* are expressed on a null background to test whether the affinity of munc18-1 for syntaxin-1 and mint-1 affects these parameters. We find that introduction of *munc18-1* in late embryonic null mutant neurons are restores neuronal viability, morphology and neurotransmitter secretion and conclude that *munc18-1* deficient neurons are healthy at the time of plating. Furthermore, preliminary results suggest that the affinity of munc18-1 for syntaxin-1 and mint-1 is not a critical determinant of dendrite outgrowth, branching and synaptic vesicle fusion.

Finally, in chapter 6, the results are summarized and the relationship between regulated secretion, *munc18-1* and neuronal survival, neurite outgrowth and synapse formation is discussed.

Reference List

Advani, R. J., Bae, H. R., Bock, J. B., Chao, D. S., Doung, Y. C., Prekeris, R., Yoo, J. S., & Scheller, R. H. (1998). Seven novel mammalian SNARE proteins localize to distinct membrane compartments. *J Biol Chem* 273, 10317-24.

Ahmari, S. E., Buchanan, J., & Smith, S. J. (2000). Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci* 3, 445-51.

Ahnert-Hilger, G., Kutay, U., Chahoud, I., Rapoport, T., & Wiedenmann, B. (1996). Synaptobrevin is essential for secretion but not for the development of synaptic processes. *Eur J Cell Biol* 70, 1-11.

Altrock, W. D., tom Dieck, S., Sokolov, M., Meyer, A. C., Sigler, A., Brakebusch, C., Fassler, R., Richter, K., Boeckers, T. M., Potschka, H., Brandt, C., Loscher, W., Grimberg, D., Dresbach, T., Hempelmann, A., Hassan, H., Balschun, D., Frey, J. U., Brandstatter, J. H., Garner, C. C., Rosenmund, C., & Gundelfinger, E. D. (2003). Functional inactivation of a fraction of excitatory synapses in mice deficient for the active zone protein bassoon. *Neuron* 37, 787-800.

Anton, E. S., Kreidberg, J. A., & Rakic, P. (1999). Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. *Neuron* 22, 277-89.

Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci* 22, 208-15.

Aravamudan, B., Fergestad, T., Davis, W. S., Rodesch, C. K., & Broadie, K. (1999). Drosophila UNC-13 is essential for synaptic transmission. *Nat Neurosci* 2, 965-71.

Aravanis, A. M., Pyle, J. L., & Tsien, R. W. (2003). Single synaptic vesicles fusing transiently and successively without loss of identity. *Nature* 423, 643-7.

Ashery, U., Varoqueaux, F., Voets, T., Betz, A., Thakur, P., Koch, H., Neher, E., Brose, N., & Rettig, J. (2000). Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. *Embo J* 19, 3586-96.

Bagnard, D., Lohrum, M., Uziel, D., Puschel, A. W., & Bolz, J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Development* 125, 5043-53.

Beique, J. C. & Andrade, R. (2003). PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. *J Physiol* 546, 859-67.

Bem, T., Le Feuvre, Y., Simmers, J., & Meyrand, P. (2002). Electrical coupling can prevent expression of adult-like properties in an embryonic neural circuit. *J Neurophysiol* 87, 538-47.

Berardi, N., Pizzorusso, T., & Maffei, L. (2000). Critical periods during sensory development. *Curr Opin Neurobiol* 10, 138-45.

Bezzi, P. & Volterra, A. (2001). A neuron-glia signalling network in the active brain. *Curr Opin Neurobiol* 11, 387-94.

Black, M. M. & Baas, P. W. (1989). The basis of polarity in neurons. *Trends Neurosci.* 12, 211-214.

Borgdorff, A. J. & Choquet, D. (2002). Regulation of AMPA receptor lateral movements. *Nature* 417, 649-53.

Bouwman, J., Maia, A. S., Camoletto, P. G., Posthuma, G., Roubos, E. W., Oorschot, V. M. J., Klumperman, J., & Verhage, M. (2004). Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. *Neuroscience* 126, 115-126.

Brain, K. L. & Bennett, M. R. (1995). Calcium in the nerve terminals of chick ciliary ganglia during facilitation, augmentation and potentiation. *J Physiol* 489, 637-48.

Braisted, J. E., Catalano, S. M., Stimac, R., Kennedy, T. E., Tessier-Lavigne, M., Shatz, C. J., & O'Leary, D. D. (2000). Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. *J Neurosci* 20, 5792-801.

Bresler, T., Ramati, Y., Zamorano, P. L., Zhai, R., Garner, C. C., & Ziv, N. E. (2001). The dynamics of SAP90/PSD-95 recruitment to new synaptic junctions. *Mol Cell Neurosci* 18, 149-67.

Buckley, K. M., Melikian, H. E., Provoda, C. J., & Waring, M. T. (2000). Regulation of neuronal function by protein trafficking: a role for the endosomal pathway. *J Physiol* 525 Pt 1, 11-9.

Buzsaki, G. & Gage, F. H. (1988). Mechanisms of action of neural grafts in the limbic system. *Can J Neurol Sci* 15, 99-105.

Cajal, R. y. (1893). Neue Darstellung vom histologischen Bau des Centralnervensystems. *Arch.Anat.Physiol. Anat.Abt.Suppl.* 319-428.

Castillo, P. E., Schoch, S., Schmitz, F., Sudhof, T. C., & Malenka, R. C. (2002). RIM1alpha is required for presynaptic long-term potentiation. *Nature* 415, 327-30.

Cowan, W. M., Sudhof, T. C., & Stevens, C. F. (2000). *Synapses* Baltimore: John Hopkins University Press.

Cremer, H., Chazal, G., Lledo, P. M., Rougon, G., Montaron, M. F., Mayo, W., Le Moal, M., & Abrous, D. N. (2000). PSA-NCAM: an important regulator of hippocampal plasticity. *Int J Dev Neurosci* 18, 213-20.

Crowley, J. C. & Katz, L. C. (1999). Development of ocular dominance columns in the absence of retinal input. *Nat Neurosci* 2, 1125-30.

Dailey, M. E. & Smith, S. J. (1996). The dynamics of dendritic structure in developing hippocampal slices. *J Neurosci* 16, 2983-94.

Delaney, K. R. & Tank, D. W. (1994). A quantitative measurement of the dependence of short-term synaptic enhancement on presynaptic residual calcium. *J Neurosci* 14, 5885-902.

Des Rosiers, M. H., Sakurada, O., Jehle, J., Shinohara, M., Kennedy, C., & Sokoloff, L. (1978). Functional plasticity in the immature striate cortex of the monkey shown by the [14C]deoxyglucose method. *Science* 200, 447-9.

Dityatev, A., Dityateva, G., & Schachner, M. (2000). Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM. *Neuron* 26, 207-17.

Dresbach, T., Qualmann, B., Kessels, M. M., Garner, C. C., & Gundelfinger, E. D. (2001). The presynaptic cytomatrix of brain synapses. *Cell Mol Life Sci* 58, 94-116.

Dunaevsky, A., Tashiro, A., Majewska, A., Mason, C., & Yuste, R. (1999). Developmental regulation of spine motility in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 96, 13438-43.

Duncan, R. R., Greaves, J., Wiegand, U. K., Matskevich, I., Bodammer, G., Apps, D. K., Shipston, M. J., & Chow, R. H. (2003). Functional and spatial segregation of secretory vesicle pools according to vesicle age.

Nature 422, 176-80.
Featherstone, D. E., Rushton, E., & Broadie, K. (2002). Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. *Nat Neurosci* 5, 141-6.
Fenster, S. D., Chung, W. J., Zhai, R., Cases-Langhoff, C., Voss, B., Garner, A. M., Kaempf, U., Kindler, S., Gundelfinger, E. D., & Garner, C. C. (2000). Piccolo, a presynaptic zinc finger protein structurally related to bassoon. *Neuron* 25, 203-14.
Fenster, S. D., Kessels, M. M., Qualmann, B., Chung, W. J., Nash, J., Gundelfinger, E. D., & Garner, C. C. (2003). Interactions between Piccolo and the actin/dynamin-binding protein Abp1 link vesicle endocytosis to presynaptic active zones. *J Biol Chem* 28, 28.
Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C., & Sudhof, T. C. (2001). Synaptotagmin I functions as a calcium regulator of release probability. *Nature* 410, 41-9.
Fields, R. D. & Stevens-Graham, B. (2002). New insights into neuron-glia communication. *Science* 298, 556-62.
Forster, E., Tielsch, A., Saum, B., Weiss, K. H., Johanssen, C., Graus-Porta, D., Muller, U., & Frotscher, M. (2002). Reelin, Disabled 1, and beta 1 integrins are required for the formation of the radial glial scaffold in the hippocampus. *Proc Natl Acad Sci U S A* 99, 13178-83.
Friedman, H. V., Bresler, T., Garner, C. C., & Ziv, N. E. (2000). Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27, 57-69.
Galli, T., Zahraoui, A., Vaidyanathan, V. V., Raposo, G., Tian, J. M., Karin, M., Niemann, H., & Louvard, D. (1998). A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. *Mol Biol Cell* 9, 1437-48.
Gandhi, S. P. & Stevens, C. F. (2003). Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. *Nature* 423, 607-13.
Gao, P. P., Sun, C. H., Zhou, X. F., DiCicco-Bloom, E., & Zhou, R. (2000). Ephrins stimulate or inhibit neurite outgrowth and survival as a function of neuronal cell type. *J Neurosci Res* 60, 427-36.
Gao, P. P., Yue, Y., Zhang, J. H., Cerretti, D. P., Levitt, P., & Zhou, R. (1998). Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections. *Proc Natl Acad Sci U S A* 95, 5329-34.
Garner, C. C., Kindler, S., & Gundelfinger, E. D. (2000). Molecular determinants of presynaptic active zones. *Curr Opin Neurobiol* 10, 321-7.
Gautam, M., DeChiara, T. M., Glass, D. J., Yancopoulos, G. D., & Sanes, J. R. (1999). Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn. *Brain Res.Dev.Brain Res.* 114, 171-178.
Gerber, S. H., Garcia, J., Rizo, J., & Sudhof, T. C. (2001). An unusual C(2)-domain in the active-zone protein piccolo: implications for Ca(2+) regulation of neurotransmitter release. *Embo J* 20, 1605-19.
Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J. C., & Muller, U. (2001). Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. *Neuron* 31, 367-79.
Gundelfinger, E. D., Kessels, M. M., & Qualmann, B. (2003). Temporal and spatial coordination of exocytosis and endocytosis. *Nat Rev Mol Cell Biol* 4, 127-39.
Harrison, S. D., Broadie, K., van de Goor, J., & Rubin, G. M. (1994). Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission. *Neuron* 13, 555-66.
Hazuka, C. D., Foletti, D. L., Hsu, S. C., Kee, Y., Hopf, F. W., & Scheller, R. H. (1999). The sec6/8 complex is located at neurite outgrowth and axonal synapse- assembly domains. *J Neurosci* 19, 1324-34.
Hazuka, C. D., Hsu, S. C., & Scheller, R. H. (1997). Characterization of a cDNA encoding a subunit of the rat brain rsec6/8 complex. *Gene* 187, 67-73.
Hirling, H., Steiner, P., Chaperon, C., Marsault, R., Regazzi, R., & Catsicas, S. (2000). Syntaxin 13 is a developmentally regulated SNARE involved in neurite outgrowth and endosomal trafficking. *Eur J Neurosci* 12, 1913-23.
Ho, A., Morishita, W., Hammer, R. E., Malenka, R. C., & Sudhof, T. C. (2003). A role for Mints in transmitter release: Mint 1 knockout mice exhibit impaired GABAergic synaptic transmission. *Proc Natl Acad Sci U S A* 100, 1409-1414.
Hubel, D. H. & Wiesel, T. N. (1970). The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J Physiol* 206, 419-36.
Igarashi, M., Kozaki, S., Terakawa, S., Kawano, S., Ide, C., & Komiya, Y. (1996). Growth cone collapse and inhibition of neurite growth by Botulinum neurotoxin C1: a t-SNARE is involved in axonal growth. *J Cell Biol* 134, 205-15.
Igarashi, M., Tagaya, M., & Komiya, Y. (1997). The soluble N-ethylmaleimide-sensitive factor attached protein receptor complex in growth cones: molecular aspects of the axon terminal development. *J Neurosci* 17, 1460-70.
Jahn, R. & Sudhof, T. C. (1999). Membrane fusion and exocytosis. *Annu Rev Biochem* 68, 863-911.
Jones, M. V. & Westbrook, G. L. (1996). The impact of receptor desensitization on fast synaptic transmission. *Trends Neurosci* 19, 96-101.
Jontes, J. D. & Smith, S. J. (2000). Filopodia, spines, and the generation of synaptic diversity. *Neuron* 27, 11-4.
Katz, L. C. & Crowley, J. C. (2002). Development of cortical circuits: lessons from ocular dominance columns. *Nat Rev Neurosci* 3, 34-42.

Katz, L. C. & Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. *Science* 274, 1133-8.
Lendvai, B., Stern, E. A., Chen, B., & Svoboda, K. (2000). Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature* 404, 876-81.
Lichtman, J. W. & Colman, H. (2000). Synapse elimination and indelible memory. *Neuron* 25, 269-278.
Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R., & Lee, K. F. (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410, 1057-64.
Lopez-Bendito, G. & Molnar, Z. (2003). Thalamocortical development: how are we going to get there? *Nat Rev Neurosci* 4, 276-89.
Lu, W., Man, H., Ju, W., Trimble, W. S., MacDonald, J. F., & Wang, Y. T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* 29, 243-54.
Ludwig, M. & Pittman, Q. J. (2003). Talking back: dendritic neurotransmitter release. *Trends Neurosci* 26, 255-61.
Mackarehtschian, K., Lau, C. K., Caras, I., & McConnell, S. K. (1999). Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression. *Cereb Cortex* 9, 601-10.
Mann, F., Peuckert, C., Dehner, F., Zhou, R., & Bolz, J. (2002). Ephrins regulate the formation of terminal axonal arbors during the development of thalamocortical projections. *Development* 129, 3945-55.
Marrs, G. S., Green, S. H., & Dailey, M. E. (2001). Rapid formation and remodeling of postsynaptic densities in developing dendrites. *Nat Neurosci* 4, 1006-13.
McKinney, R. A., Capogna, M., Durr, R., Gahwiler, B. H., & Thompson, S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci* 2, 44-9.
Mellitzer, G., Xu, Q., & Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 400, 77-81.
Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S., & Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* 14, 949-59.
Missler, M. & Sudhof, T. C. (1998). Neurexins: three genes and 1001 products. *Trends Genet* 14, 20-6.
Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G., & Kiss, J. Z. (1996). PSA-NCAM is required for activity-induced synaptic plasticity. *Neuron* 17, 413-22.
Mundigl, O., Matteoli, M., Daniell, L., Thomas-Reetz, A., Metcalf, A., Jahn, R., & De Camilli, P. (1993). Synaptic vesicle proteins and early endosomes in cultured hippocampal neurons: differential effects of Brefeldin A in axon and dendrites. *J Cell Biol* 122, 1207-21.
Murthy, M., Garza, D., Scheller, R. H., & Schwarz, T. L. (2003). Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. *Neuron* 37, 433-47.
Nakajima, Y., Nakajima, S., Leonard, R. J., & Yamaguchi, K. (1986). Acetylcholine raises excitability by inhibiting the fast transient potassium current in cultured hippocampal neurons. *Proc Natl Acad Sci U S A* 83, 3022-6.
Nguyen, Q. T. & Lichtman, J. W. (1996). Mechanism of synapse disassembly at the developing neuromuscular junction. *Curr Opin Neurobiol* 6, 104-12.
Novick, P., Field, C., & Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205-15.
Okabe, S., Miwa, A., & Okado, H. (2001). Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J Neurosci* 21, 6105-14.
Okada, Y., Yamazaki, H., Sekine-Aizawa, Y., & Hirokawa, N. (1995). The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell* 81, 769-80.
Osen-Sand, A., Catsicas, M., Staple, J. K., Jones, K. A., Ayala, G., Knowles, J., Grenningloh, G., & Catsicas, S. (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. *Nature* 364, 445-448.
Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A., Montecucco, C., & Catsicas, S. (1996). Common and distinct fusion proteins in axonal growth and transmitter release. *J.Comp Neurol.* 367, 222-234.
Otsu, Y., Shahrezaei, V., Li, B., Raymond, L. A., Delaney, K. R., & Murphy, T. H. (2004). Competition between Phasic and Asynchronous Release for Recovered Synaptic Vesicles at Developing Hippocampal Autaptic Synapses. *Journal of Neuroscience* 24, 420-433.
Passafaro, M., Piech, V., & Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat Neurosci* 4, 917-26.
Peinado, A., Yuste, R., & Katz, L. C. (1993). Extensive dye coupling between rat neocortical neurons during the period of circuit formation. *Neuron* 10, 103-14.
Phillips, G. R., Huang, J. K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, W. S., Arndt, K., Frank, M., Gordon, R. E., Gawinowicz, M. A., Zhao, Y., & Colman, D. R. (2001). The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* 32, 63-77.
Prange, O. & Murphy, T. H. (2001). Modular transport of postsynaptic density-95 clusters and association with stable spine precursors during early development of cortical neurons. *J Neurosci* 21, 9325-33.
Prekeris, R., Klumperman, J., Chen, Y. A., & Scheller, R. H. (1998). Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. *J Cell Biol* 143, 957-71.

Rakic, P. (1976). Prenatal genesis of connections subserving ocular dominance in the rhesus monkey. *Nature* 261, 467-71.
Rao, A., Kim, E., Sheng, M., & Craig, A. M. (1998). Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *J Neurosci* 18, 1217-29.
Rao, R. P. & Sejnowski, T. J. (2001). Spike-timing-dependent Hebbian plasticity as temporal difference learning. *Neural Comput* 13, 2221-37.
Regnier-Vigouroux, A., Tooze, S. A., & Huttner, W. B. (1991). Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane. *Embo J* 10, 3589-601.
Renden, R., Berwin, B., Davis, W., Ann, K., Chin, C. T., Kreber, R., Ganetzky, B., Martin, T. F., & Broadie, K. (2001). Drosophila CAPS is an essential gene that regulates dense-core vesicle release and synaptic vesicle fusion. *Neuron* 31, 421-37.
Rosenmund, C., Sigler, A., Augustin, I., Reim, K., Brose, N., & Rhee, J. S. (2002). Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. *Neuron* 33, 411-24.
Saitoe, M., Schwarz, T. L., Umbach, J. A., Gundersen, C. B., & Kidokoro, Y. (2001). Absence of junctional glutamate receptor clusters in Drosophila mutants lacking spontaneous transmitter release. *Science* 293, 514-7.
Sarria, J. C., Catsicas, S., Hornung, J. P., & Hirling, H. (2002). Developmental and spatial expression pattern of syntaxin 13 in the mouse central nervous system. *Cell Tissue Res* 309, 209-18.
Scheiffele, P., Fan, J., Choih, J., Fetter, R., & Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101, 657-69.
Schmitz, D., Schuchmann, S., Fisahn, A., Draguhn, A., Buhl, E. H., Petrasch-Parwez, E., Dermietzel, R., Heinemann, U., & Traub, R. D. (2001). Axo-axonal coupling. a novel mechanism for ultrafast neuronal communication. *Neuron* 31, 831-40.
Schoch, S., Castillo, P. E., Jo, T., Mukherjee, K., Geppert, M., Wang, Y., Schmitz, F., Malenka, R. C., & Sudhof, T. C. (2002). RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* 415, 321-6.
Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Sudhof, T. C., & Kavalali, E. T. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science* 294, 1117-22.
Shapira, M., Zhai, R. G., Dresbach, T., Bresler, T., Torres, V. I., Gundelfinger, E. D., Ziv, N. E., & Garner, C. C. (2003). Unitary assembly of presynaptic active zones from piccolo-bassoon transport vesicles. *Neuron* 38, 237-52.
Sharma, G. & Vijayaraghavan, S. (2003). Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. *Neuron* 38, 929-939.
Sikorski, A. F., Sangerman, J., Goodman, S. R., & Critz, S. D. (2000). Spectrin (betaSpIIsigma1) is an essential component of synaptic transmission. *Brain Res* 852, 161-6.
Smit, A. B., Brejc, K., Syed, N., & Sixma, T. K. (2003). Structure and function of AChBP, homologue of the ligand-binding domain of the nicotinic acetylcholine receptor. *Ann.N.Y.Acad.Sci.* 998, 81-92.
Smith, S. J. (1999). Dissecting dendrite dynamics. *Science* 283, 1860-1.
Steiner, P., Sarria, J. C., Huni, B., Marsault, R., Catsicas, S., & Hirling, H. (2002). Overexpression of neuronal Sec1 enhances axonal branching in hippocampal neurons. *Neuroscience* 113, 893-905.
Stotz, P. J. & Bawa, P. (2001). Motor unit recruitment during lengthening contractions of human wrist flexors. *Muscle Nerve* 24, 1535-41.
Sudhof, T. C. (2000). The synaptic vesicle cycle revisited. *Neuron* 28, 317-20.
Sudlow, A. W., Wilde, C. J., & Burgoyne, R. D. (1994). Transforming growth factor-beta 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. *Biochem J* 304, 333-6.
Tabares, L., Ales, E., Lindau, M., & Alvarez de Toledo, G. (2001). Exocytosis of catecholamine (CA)-containing and CA-free granules in chromaffin cells. *J Biol Chem* 276, 39974-9.
Tandon, A., Bannykh, S., Kowalchyk, J. A., Banerjee, A., Martin, T. F., & Balch, W. E. (1998). Differential regulation of exocytosis by calcium and CAPS in semi- intact synaptosomes. *Neuron* 21, 147-54.
Toonen, R. F. & Verhage, M. (2003). Vesicle trafficking: pleasure and pain from SM genes. *Trends Cell Biol* 13, 177-86.
Tovar, K. R. & Westbrook, G. L. (2002). Mobile NMDA receptors at hippocampal synapses. *Neuron* 34, 255-64.
Uziel, D., Muhlfriedel, S., Zarbalis, K., Wurst, W., Levitt, P., & Bolz, J. (2002). Miswiring of limbic thalamocortical projections in the absence of ephrin-A5. *J Neurosci* 22, 9352-7.
Varoqueaux, F., Sigler, A., Rhee, J. S., Brose, N., Enk, C., Reim, K., & Rosenmund, C. (2002a). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc Natl Acad Sci U S A* 99, 9037-42.
Varoqueaux, F., Sigler, A., Rhee, J. S., Brose, N., Enk, C., Reim, K., & Rosenmund, C. (2002b). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc Natl Acad Sci U S A* 99, 9037-42.
Verhage, M., Maia, A. S., Plomp, J. J., Brussaard, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., van den Berg, T. K., Missler, M., Geuze, H. J., & Sudhof, T. C. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287, 864-869.

Voets, T., Toonen, R. F., Brian, E. C., de Wit, H., Moser, T., Rettig, J., Sudhof, T. C., Neher, E., & Verhage, M. (2001). Munc18-1 promotes large dense-core vesicle docking. *Neuron* 31, 581-91.
Washbourne, P., Bennett, J. E., & McAllister, A. K. (2002a). Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nat Neurosci* 5, 751-9.
Washbourne, P., Thompson, P. M., Carta, M., Costa, E. T., Mathews, J. R., Lopez-Bendito, G., Molnar, Z., Becher, M. W., Valenzuela, C. F., Partridge, L. D., & Wilson, M. C. (2002b). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci* 5, 19-26.
Weimer, R. M., Richmond, J. E., Davis, W. S., Hadwiger, G., Nonet, M. L., & Jorgensen, E. M. (2003). Defects in synaptic vesicle docking in unc-18 mutants. *Nat.Neurosci.* 6, 1023-1030.
Williamson, L. C. & Neale, E. A. (1998). Syntaxin and 25-kDa synaptosomal-associated protein: differential effects of botulinum neurotoxins C1 and A on neuronal survival. *J.Neurosci.Res.* 52, 569-583.
Wong, W. T. & Wong, R. O. (2000). Rapid dendritic movements during synapse formation and rearrangement. *Curr Opin Neurobiol* 10, 118-24.
Xu, Q., Mellitzer, G., & Wilkinson, D. G. (2000). Roles of Eph receptors and ephrins in segmental patterning. *Philos Trans R Soc Lond B Biol Sci* 355, 993-1002.
Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C., & Burden, S. J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* 30, 399-410.
Zengel, J. E., Sosa, M. A., Poage, R. E., & Mosier, D. R. (1994). Role of intracellular Ca2+ in stimulation-induced increases in transmitter release at the frog neuromuscular junction. *J Gen Physiol* 104, 337-55.
Zenisek, D., Steyer, J. A., & Almers, W. (2000). Transport, capture and exocytosis of single synaptic vesicles at active zones. *Nature* 406, 849-54.
Zhai, R. G., Vardinon-Friedman, H., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., Ziv, N. E., & Garner, C. C. (2001). Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. *Neuron* 29, 131-43.
Zhao, H. & Nonet, M. L. (2000). A retrograde signal is involved in activity-dependent remodeling at a C. elegans neuromuscular junction. *Development* 127, 1253-1266.
Zimmer, W. E., Zhao, Y., Sikorski, A. F., Critz, S. D., Sangerman, J., Elferink, L. A., Xu, X. S., & Goodman, S. R. (2000). The domain of brain beta-spectrin responsible for synaptic vesicle association is essential for synaptic transmission. *Brain Res.* 881, 18-27.
Ziv, N. E. & Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17, 91-102.

Chapter 2

Development of the mouse neuromuscular junction in the absence of regulated secretion

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Abstract

To investigate the role of neurotransmitter secretion in the development and stabilization of synapses, the innervation of the diaphragm and intercostal muscles was studied in *munc18-1* null mutant mice, which lack regulated secretion. We found that this mutant is completely devoid of both spontaneous and evoked neuromuscular transmission throughout embryonic development. At embryonic day (E) 14, axonal targeting and main branching of the phrenic nerve were normal in this mutant, but tertiary branches were elongated and no terminal branches were observed at this stage, in contrast to control littermates. Acetylcholinesterase staining was observed in the endplate region of mutant muscle from E14 onwards, but not as dense and confined to spots as in controls. Acetylcholine receptor staining was also present in the endplate region of the mutant muscle. In this case, the staining density and the concentration in spots (clusters) were similar to controls, but the distribution of these clusters was less organized. Starting at E15, some receptor clusters co-localized with nerve terminal staining, suggesting synapses, but most clusters remained a-neural. Electron microscopical analysis confirmed the presence of synaptic structures in the mutant. Between E14 and birth, the characteristic staining pattern of nerve

branches gradually disappeared in the mutant until, at E18, an elaborate meshwork of nerve fibers with no apparent organization remained. In the same period, most of the motor neuronal cell bodies in the spinal cord degenerated. In contrast, sensory ganglia in the dorsal root showed no obvious degeneration. These data suggest that regulated secretion is not essential for initial axon pathfinding, clustering of acetylcholine receptors, acetylcholinesterase or the formation of synapses. However, in the absence of regulated secretion, the maintenance of the motor neuronal system, organization of nerve terminal branches and stabilization of synapses is impaired and a-neural postsynaptic elements persist.

Introduction

Synapses are the main medium for information transfer among neurons and to their targets. Over the years interest has grown to understand the processes that govern the formation, maintenance and functioning of neuromuscular junctions (NMJs), as model synapses. Genetic approaches have elucidated several aspects of NMJ development and functioning. A striking finding was that pre- and postsynaptic elements do not need their counterparts for initial development. For instance, in the mouse diaphragm, postsynaptic acetylcholine receptors (AChRs) cluster in the normal endplate region, also when the phrenic nerve never arrives there (Yang et al., 2001). *Vice versa*, in zebrafish, postsynaptic AChRs are dispensable for axonal targeting and basic synaptogenesis on the muscle (Westerfield et al., 1990). Intriguingly, in mice deficient for rapsyn or MuSK, postsynaptic defects do appear to influence axonal targeting and synaptogenesis. Rapsyn and MuSK are proteins expressed in muscle and required for AChR clustering. In mice deficient for either of these proteins, axons branch and elongate excessively, leading to superfluous neuronal coverage of the muscle (Gautam et al., 1995; DeChiara et al., 1996; Banks et al., 2001). A similar phenotype is observed in mice deficient for agrin, a protein responsible for the induction of new AChRs and the reshaping of existing AChR clusters (Gautam et al., 1996). Another phenotype is observed in mice deficient for neuregulin-1 (Wolpowitz et al., 2000) or their receptors, erbs (Morris et al., 1999; Lin et al., 2000). In these mice, motor neurons do reach their target muscles, but instead of growing excessively, they degenerate and leave the muscles uninnervated, while AChR- and acetylcholinesterase (AChE) clusters persist in the normal endplate region (Morris et al., 1999; Lin et al., 2000; Wolpowitz et al., 2000).

From these and other studies, it was suggested that postsynaptic specializations are prepatterned and are subsequently refined by the nerve, through positively and negatively regulating signals. Finally, the muscle stabilizes certain nerve terminals, while eliminating others in an activity-dependent manner (Lichtman and Colman, 2000). Agrin is a good candidate for a positively regulating neuronal signal (Gautam et al., 1996; Bezakova et al., 2001). Electrical activity in the muscle, evoked by neurotransmitter secretion, has been proposed as a negatively regulating/destabilizing signal (Lin et al., 2001; Yang et al., 2001). This idea is consistent with earlier observations that both the total amount of AChR and the number of AChR clusters was increased in the presence of the action potential blocker tetrodotoxin *in utero* (Houenou et al., 1990). However, tetrodotoxin does not block spontaneous neurotransmitter secretion and the resulting miniature endplate potentials (MEPPs) may influence AChR clustering.
Genetic models have also been used to analyze the effect of neurotransmitter secretion on synaptic development in worms and flies. The genes *syntaxin* and *dynamin* are required for the synaptic vesicle secretion cycle. In *Drosophila*, the functionality of these genes was found to be essential for glutamate receptor localization at the NMJ (Saitoe et al., 2001). However, another study of the same mutants suggests that these genes are dispensable (Featherstone et al., 2002). In *Caenorhabditis elegans*, deletion of the syntaxin binding protein unc18 results in severe neurotransmitter secretion defects and aberrant sprouting of cholinergic SAB neurons (Zhao and Nonet, 2000).

The aim of this study was to analyze synaptic development in the mouse peripheral nervous system (PNS) in the *munc18-1* null mutant mouse. This mouse is the first mutant that is completely devoid of both spontaneous and evoked neurotransmitter secretion (Verhage et al., 2000). We analyzed neuromuscular transmission throughout prenatal development and the staining pattern of growth cone marker GAP-43, synaptic vesicle marker synapsin, synaptic cleft AChE and postsynaptic AChRs, at different embryonic stages, in diaphragm and intercostal muscles of control and null mutant littermates.

Experimental procedures

Animals

Munc18-1 deficient mice were generated as described before (Verhage et al., 2000). Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous matings. The day a vaginal plug was observed was considered to be embryonic day 0 (E0). Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines. All animal procedures were performed in accordance with the Ethical Committee on Animal Experiments of the University of Utrecht Medical Center. All efforts were made to minimize the number of animals used and their suffering.

Histochemistry

The development of neuromuscular junctions was analysed in whole-mount diaphragm and intercostal muscles from E14, E15 and E18 embryos. Since no immuno-histochemical difference could be detected between embryos that were homozygous and heterozygous for munc18-1, both groups were pooled as controls for munc18-1 deficient embryos. After caesarean section, the embryos were immediately decapitated, and the rib cage, with spinal cord and diaphragm still attached, was dissected out and fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 90 minutes. All procedures were carried out at room temperature unless otherwise stated). After blocking of the PFA with 0.1 M PBS-glycine for 20 minutes, the diaphragm and intercostal muscles were isolated and further processed individually. The muscles were pre-incubated for 20 minutes in PBS containing 1% bovine serum albumin (BSA) and 0.5% Triton X-100. The muscles were now incubated overnight in a mixture of 1μg/ml α-bungarotoxin conjugated with Texas Red (Molecular Probes, Leiden, the Netherlands), 0.1% rabbit anti-synapsin (E028) and 0.05% rabbit anti-GAP-43 (9527) antisera in 1% BSA, at 4°C. After washing in PBS, 3 times 20 minutes, muscles were incubated in 1% FITC-conjugated donkey anti-rabbit antiserum (Jackson Immunoresearch) for 2 hours. After washing again in PBS, 3 times 20 minutes the muscles were mounted in Dabco-Mowiol and analysed with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). For AChE visualization the fixed muscles were incubated overnight at 37° in 0.5 mM 5-bromoindoxyl acetate (adapted from Holt and Withers, 1952). To analyze the spinal cord and dorsal root ganglions (DRGs), the embryos were frozen and transversal sections of 20 μm were cut with a cryotome, and mounted

directly on Superfrost plus slides (Menzel). The sections were postfixed in 4% PFA for 15 minutes, washed in PBS, preincubated in 4% fetal calf serum for 30 minutes and washed again in PBS. The sections were incubated overnight at 4° C with 0.1% rabbit anti-neurofilament-200 (Sigma) or 0.1% rabbit anti-syntaxin (I378) antisera in PBS with 0.1% Triton X-100. After washing in PBS the sections were incubated for 2 hours with 0.1% biotinylated goat anti-rabbit serum. The sections were washed again in PBS and incubated for 2 hours in 0.3% streptavidin-FITC conjugate. After washing in PBS the sections were covered with Dabco-Mowiol and coverslips and analyzed with a Zeiss microscope and MCID software (Imaging Research, Ontario, Canada).

Quantification

For the quantification of AChR cluster distribution a line was drawn through the region of highest cluster density. All clusters were counted over a region of approximately 2.5 mm (3 control vs 3 mutant diaphragms). Every cluster present within 125 μm of the central line was considered central. Every cluster between 125 and 250 μm was excentric and every cluster outside these lines was peripheral. The total amount of clusters was calculated per 1000 μm^2 of area averaged for the number of animals (+/- SEM) and compared with the other group through a bilateral t-test ($p<0.05$ was significant (*)).
For the quantification of cluster shape and size we measured 50 clusters per animal. From each diaphragm a 920 x 920 μm z-stack was made through the muscle with a Zeiss 510 Pascal confocal microscope. In each frame the central cluster band was horizontally centered and the first 50 clusters from left to right were measured during continuous animation through the z-stack. The largest span of each cluster was noted as its size. A cluster was considered long when it was three times as long as its width, round when it was less then twice as long as its width and round/long when either its shape did not fit the definition of long or round, or when the shape changed along the z-stack. The average size and shape (+/- SEM) was calculated for each animal and significant differences between the groups were tested with bilateral t-tests for each length or shape ($p<0.05$ was significant (*), $p<0.01$ was highly significant (**).

Electron microscopy

After caesarean section, 3 control and 3 mutant E 18 embryos were immediately decapitated, and diaphragms were dissected out and immediately fixed by in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB, pH 7.2; Merck, Darmstadt, Germany), for 2 h at 20 °C. After

rinsing in SCB, diaphragms were postfixed in 1% osmium tetroxide in SCB, for 2 h at 4 °C, rinsed in SCB, dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Semithin 0.5 μm sections were cut on a Reichert ultramicrotome and stained with toluidine blue to localize neuromuscular junctions with light microscopy. Ultrathin pale-gold sections of the junctions were contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope.

Electrophysiology

Electrophysiological measurements were performed at 26-28 °C at NMJs of diaphragm nerve-muscle preparations from E15 and E18 *munc18-1* null mutant and control mice using 30-40 MΩ micro-electrodes and standard recording equipment (Plomp et al., 1992). The analogue signal was high-pass filtered at 1 Hz. The phrenic nerve was stimulated electrically via a suction electrode and the resulting synaptic responses were recorded from muscle fibers near the endplate region. Thereafter, tetrodotoxin (1 μM, Sigma) was added to suppress the spontaneous contractions of fibers, which can occur in embryonic muscle. MEPPs, the postsynaptic responses resulting from spontaneous uniquantal ACh release, were recorded before and after application of 4 nM α-latrotoxin (Alomone Laboratories, Jerusalem, Israel). To test for the presence and function of postsynaptic AChRs in *munc18-1* null-mutant NMJs, a 1 mM carbachol-filled micro-pipette with a broken tip, allowing leakage of the acetylcholine analogue, was positioned at the muscle fiber near the place of impalement by the measuring electrode. The occurrence of depolarization and contraction of muscle fibers was monitored.

Results

Synaptic activity is absent in mutant neuromuscular junctions

Previously, we have shown that synaptic activity in the neuromuscular junction is absent at developmental stages close to birth (Verhage et al., 2000). In this study we first tested whether synaptic transmission was absent from the start. Therefore, synaptic events were analyzed at E15 and, as a control, at E18, using intracellular recording in diaphragm muscle (Fig. 1). Electrical stimulation of the phrenic nerve of control E15 diaphragm preparations resulted in muscle action potentials and subsequent muscle contraction, while *munc18-1* null-mutant preparations were unresponsive

(Fig. 1A). Intracellular recordings in muscle fibers from control embryos revealed the occurrence of MEPPs which varied considerably in amplitude, as observed by others in embryonic *Xenopus* NMJs (Poo et al., 1985). The MEPP frequency was 5.4 ± 0.6 min^{-1} (E15, 1 diaphragm, 9 NMJs) and 1.9 ± 0.4 min^{-1} (E18, 6 diaphragms, 6-10 NMJs per diaphragm). In contrast, in muscles from *munc18-1* null-mutant mice we did not detect a single MEPP

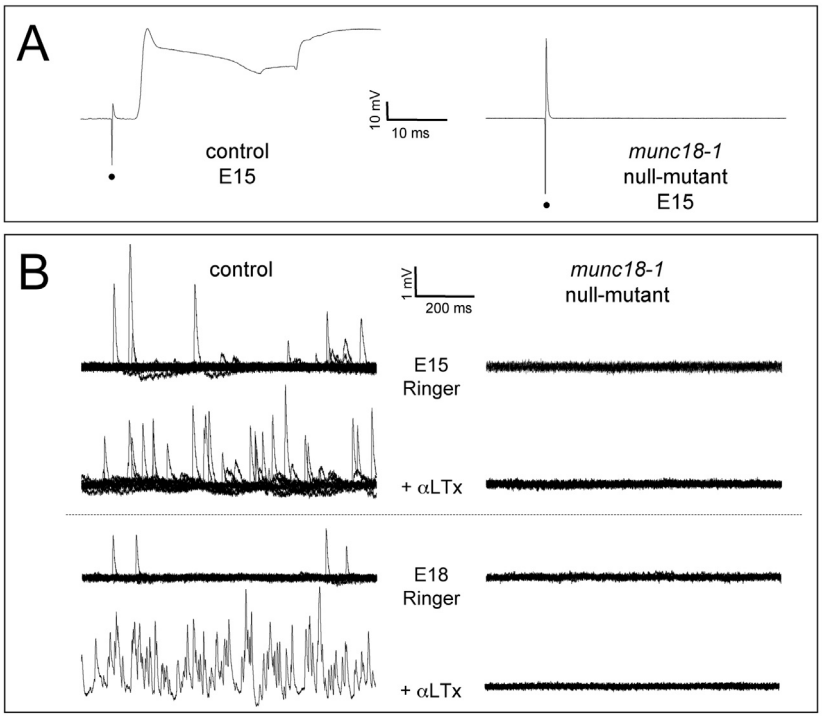

Figure 1. *Lack of neurotransmission in munc18-1 null-mutant diaphragm neuromuscular junctions of E15 and E18 embryos. (A) Electrical stimulation of the phrenic nerve of an E15 preparation via a suction electrode resulted in action potentials in control muscle fibres and was followed by muscle contraction, visible as an artifact on the recording signal, indicated with a black dot. Neither action potentials nor contractions were observed in munc18-1 null-mutant muscles. Identical observations were made in E18 preparations (not shown). (B) Spontaneous synaptic events were readily observed in control NMJs in Ringer medium but were absent in munc18-1 null-mutant neuromuscular junctions (NMJs). Shown is one min recording time, 60 superimposed 1 s traces. The neuro-excitatory toxin αLatrotoxin (4 nM) induced a dramatic increase in frequency of spontaneous events in control NMJs (10 and 1 s recording time displayed for E15 and E18 NMJs, respectively), but lacked effect on both E15 and E18 null-mutant synapses (60 superimposed 1 s recording traces displayed).*

during a total recording time of 19 min in 9 fibers of an E15 muscle and 35.5 min in 11 fibers from two E18 muscles (see also Verhage et al., 2000). In control E15 and E18 neuromuscular junctions, the MEPP frequency was strongly increased by application of 4 nM α-latrotoxin, a toxin known to stimulate neurotransmitter release (Fig. 1B). However, in *munc18-1* null-

mutant muscle fibers no MEPPs were observed after α-latrotoxin application. Functionality of postsynaptic AChRs was demonstrated by application of the cholinergic agonist carbachol. This elicited a strong postsynaptic potential and concomitant fiber contraction (data not shown, see Verhage et al., 2000). Thus, in the munc18-1 null mutant, neuromuscular transmission is absent throughout development, which is due to a lack of ACh release rather than postsynaptic defects.

Axonal outgrowth is normal but terminal branching is aberrant in the mutant

At E14, the diaphragm has developed from the pleuro-peritoneal fold and the characteristic motor neuronal innervation pattern is present. We investigated the innervation of the *munc18-1* null mutant diaphragm by staining for the growth cone marker GAP-43 and the synaptic vesicle marker synapsin. Primary branches (I in Fig. 2) of the phrenic nerve entered their respective hemi-diaphragms in both mutants and controls, and gave rise to several secondary branches (II in Fig. 2), which were perpendicularly oriented to the muscle fibers. Both primary and secondary branches were properly fasciculated and showed normal outgrowth in the mutant, but tertiary and terminal branching was aberrant. In control diaphragms, secondary branches gave rise to tertiary branches (III in Fig. 2) with regular intervals. From these tertiary branches, which lay parallel to the muscle fibers, several terminal branches arose (T in Fig. 2). In mutant diaphragms, tertiary branches were abnormally long and irregularly spaced. No terminal branches were observed at E14. Starting from E15, occasional terminal branching was observed (arrow in Fig. 4B). Hence, initial axon outgrowth and branching is normal in the *munc18-1* null mutant but the final stages of branching are aberrant.

Postsynaptic elements are present in the mutant

Recent findings suggest that postsynaptic elements like AChR and AChE clusters can be formed in future synaptic regions independent of neuronal signals (Lin et al., 2001; Yang et al., 2001). Concordantly, bromo-indoxyl acetate staining showed that AChE was present in *munc18-1* null mutant diaphragms (Fig. 3B) at E14, *i.e.* before terminal branching of the phrenic nerve was observed. AChE clusters were present both in controls (Fig. 3A) and mutants (Fig. 3B). In both groups, most AChE was present in a smear at the middle region of the muscle at this stage. The AChE that was clustered, occupied between 10 and 50% of the muscle span in both groups (data not

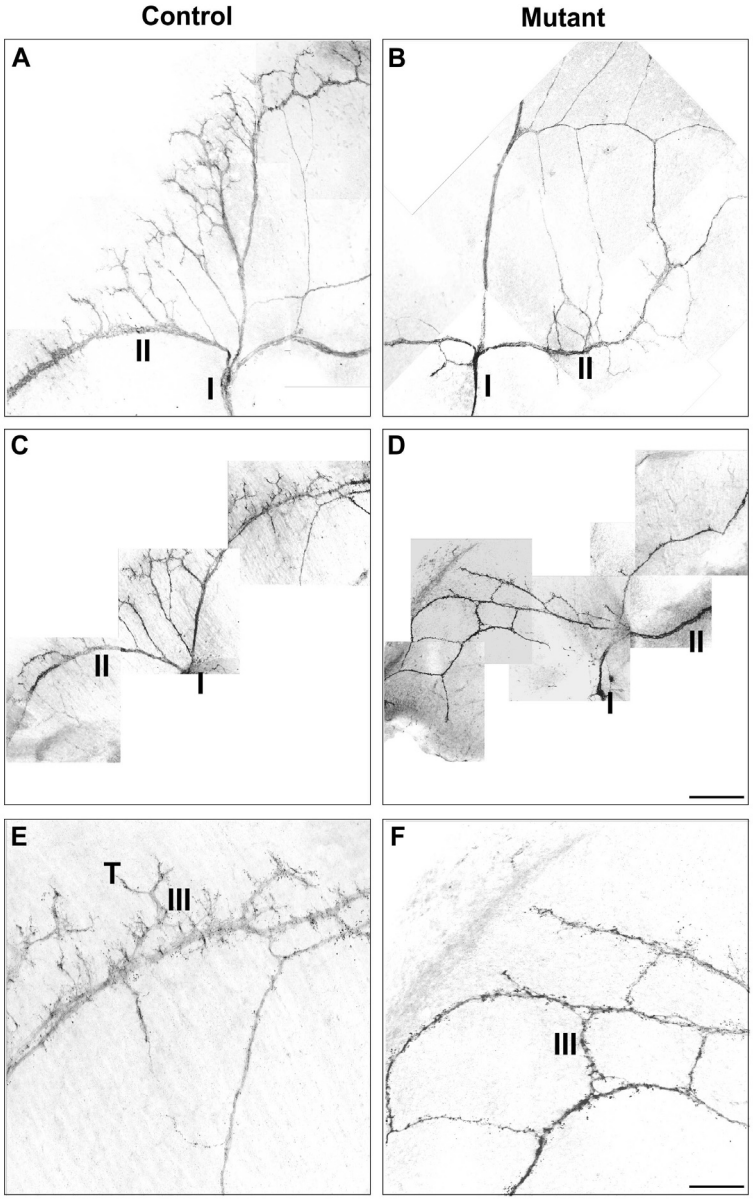

Figure 2. *Initial axonal pathfinding of the phrenic nerve is normal in Munc18-1 deficient diaphragms at E14. GAP-43/synapsin staining shows that both primary (I) and secondary (II) branches of the phrenic nerve are similar in control (A, C) and mutant (B, D) hemidiaphragms. Contrary to controls (E) tertiary branches (III) are different in the mutant (F) and terminal branches (T) are not observed. The scale bar is 300 µm (A, B, C, D) and 100 µm (E, F) respectively.*

shown). However, the amount of clusters and their relative intensity was always lower in mutant muscle (Fig. 3A-D).

Starting from E15, AChR clusters were readily observed in both groups,

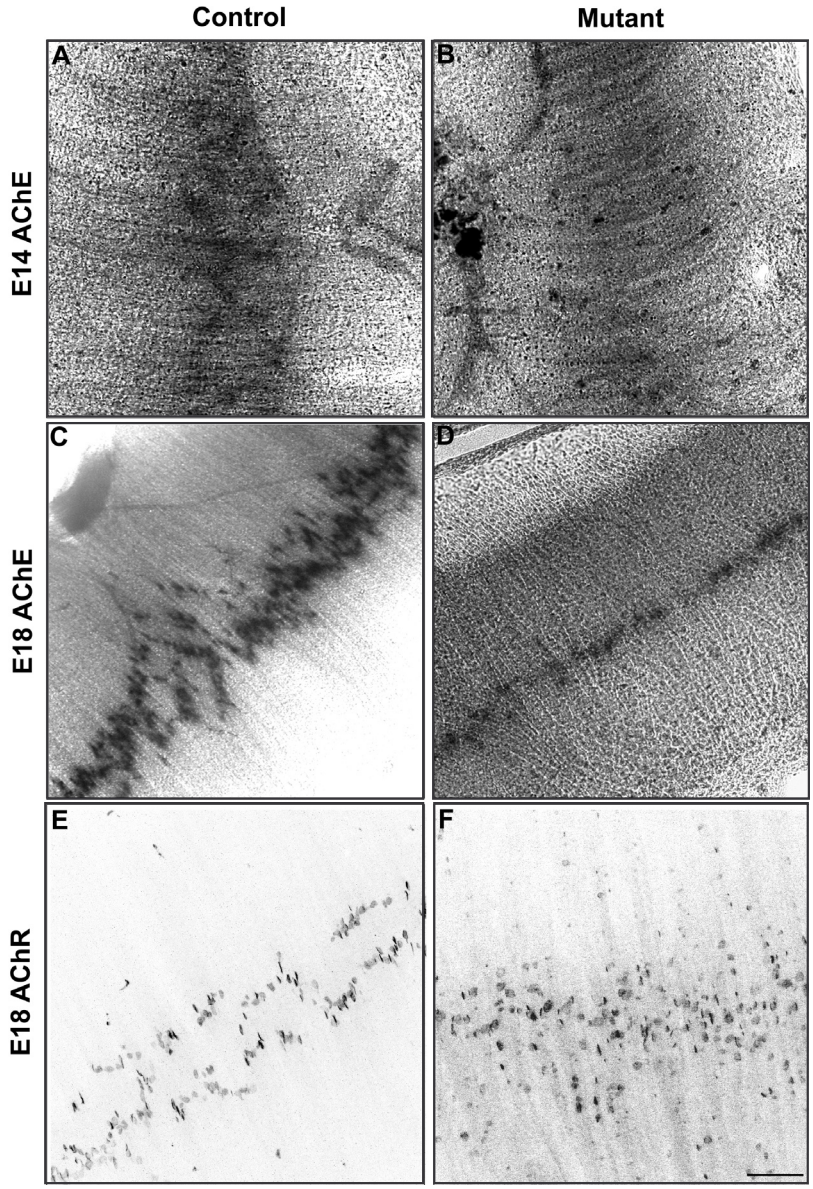

Figure 3. *AChR and AChE clusters are prepatterned. AChE staining in E14 diaphragms shows that AChE is present in a smear along the middle line of the muscle and that some clustering occurs both in controls (A) and in mutants (B). AChE staining in E18 diaphragms shows that AChE clusters are present both in controls (C) and in mutants (D), but that the amount of clusters is much lower in mutants. AChR staining in E18 diaphragms shows that, as in controls (E), the majority of AChR clusters are present in the middle region of the muscle (F). The scale bar is 100 µm.*

using rhodamine-conjugated α-bungarotoxin. Contrary to AChE, the amount of AChR clusters was not significantly different between mutant and control muscle (Table 1). However, the distribution of these clusters was different

Table 1

Avg. # of AChR clusters per 1000 μm² +/- SEM	Control	Mutant
Central	0.57 +/- 0.08	0.38 +/- 0.06
Excentric	0.1 +/- 0.03	0.30 +/- 0.05*
Peripheral	0.03 +/- 0.02	0.11 +/- 0.02*
Total	0.129 +/- 0.03	0.196 +/- 0.03

Table 1. *Quantification of AChR cluster distribution. A line was drawn through the region of highest cluster density. Every cluster present within 125 μm of this line was considered to be central, every cluster between 125 and 250 μm was excentric and every cluster outside these borders was peripheral. Bilateral t-tests show that although there is no significant difference between the amount of clusters in the central region there were significantly more clusters in the excentric and peripheral regions of mutant diaphragms (*p<0.05).*

in control and mutant diaphragms. In control diaphragms the majority of clusters was present in the central 250 μm of the muscle and relatively few clusters were present outside of this region (Fig. 3E, Table 1). In mutant diaphragms the AChR cluster distribution was more diffuse and more clusters were observed in the peripheral regions of the muscle (Fig. 3F, Table 1). Hence, both AChE- and AChR are clustered in the mutant, but the refinement/redistribution of these postsynaptic elements is abnormal.

Few synapses are observed in the mutant

To analyze whether neuromuscular synapses were formed in the absence neurotransmission, we used co-localization of synapsin or GAP-43 positive

Table 2

Size/shape average +/- SEM	Control	Mutant
0 - 5 μm	1 +/- 0.58	2.66 +/- 0.67
5 - 10 μm	11.67 +/- 1.67	9.17 +/- 4.20
10 - 15 μm	22 +/- 1.53	20.67 +/- 0.67
15 + μm	15.33 +/- 0.88	9.33 +/- 1.33
Round	11.67 +/- 1.33	31.67 +/- 2.19**
Round/long	10.33 +/- 0.67	11.67 +/- 0.88
Long	28 +/- 1.15	9.33 +/- 1.67*

Table 2. *Quantification of AChR cluster size and shape. For each diaphragm 50 clusters in the central region of the muscle were measured. Bilateral t-tests show that there is no significant difference in size distribution between controls and mutants. However, there is a significant increase in the amount of round clusters and decrease in the amount of long clusters in mutant diaphragms compared to controls (*p<0.05, **p<0.01).*

terminal branches with AChR clusters as a hallmark for synapse formation. In control muscle, this co-localization was readily observed. Figure 4A and D (see appendix) show that, in controls, the majority of AChR clusters were positioned directly underneath a terminal branch of the phrenic nerve at E15 and E18, respectively. In the mutant, co-localization of nerve terminal branches and AChR clusters was occasionally observed, suggesting that synapses are formed in the mutant. These synapses were present both at E15 and at E18 (Fig. 4B, C and E, F respectively (see appendix)). The number of terminal branches and the number of AChR clusters that co-localized with these branches, were much lower (on average 3 to 4 per mutant muscle),

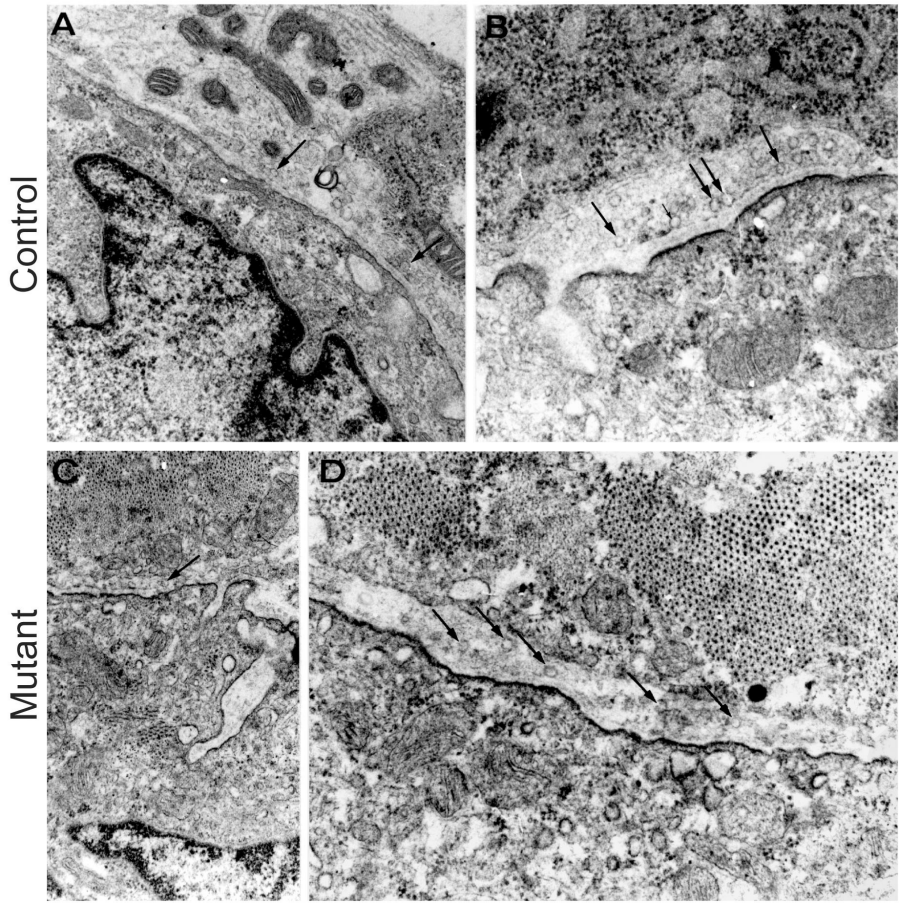

Figure 5. *Synapses are formed in the mutant. Electron micrographs show that both in control (A, B) and mutant (C, D) diaphragms, neuromuscular synapses are present at E18. The large arrows indicate synaptic vesicles in the vicinity of the electron-dense material of the postsynaptic density. The presynaptic active zone is demarcated by a row of docked vesicles in controls (B) and is less clearly visible in the mutant (D) because is has less (docked) vesicles. The small arrow in B indicates a hemi-fused vesicle in a control NMJ. Magnification is 28.000x (A, C) and 86.000x (B, D) respectively.*

leaving many AChR clusters a-neural in the mutant. Measurement of AChR clusters in the central region of the diaphragm revealed that the length of clusters was similar in controls and mutants. There was, however, a considerable difference in the shape of AChR clusters. The clusters were predominantly round in the mutant and elongated in controls (Table 2).
Because co-localization of pre- and postsynaptic staining could be coincidental, and therefore falsely suggest the presence of NMJs in the mutant, we confirmed the presence of NMJs in mutant diaphragms with electron microscopy. The arrows in figure 5 show that synaptic vesicles were present in close apposition to the postsynaptic density in both groups. However, the total amount of synaptic vesicles, and the number of morphologically docked vesicles appeared to be lower in mutant synapses. This is consistent with observations in the CNS (Maia et al., in preparation) and was not quantified further in the muscle. Together, these data suggest that synapses are formed in the mutant, but are much less abundant or less stable than in controls. In addition, mutant muscles show many a-neural AChR clusters.

Intercostal muscles show a similar phenotype as the diaphragm

Analysis of other mutant mice suggests that muscles derived from different precursor cells can respond differently to defects in synapse formation (Thaler et al., 1999; Lin et al., 2000; Wolpowitz et al., 2000; Yang et al., 2000). Synaptic development was therefore also analyzed in intercostal muscles.
At E15 both control and mutant intercostals had nerve bundles traversing the middle of the muscle suggesting a similar overall innervation pattern (data not shown). In control muscles these nerve bundles gave rise to synapsin and GAP-43 positive terminal branches with regular intervals (Fig. 6A ,see appendix), that all co-localized with AChR clusters (6B,C, see appendix). Intercostal nerves in the mutant at E15 occasionally showed nerve terminal branches (6D (see appendix)) that co-localized with AChR clusters (6E,F, see appendix), but, as in diaphragms, the amount of co-localization/synapses was much lower than in control muscles. At E18, synapsin and GAP-43 staining was still observed in the nerve bundle running through the middle of mutant intercostal muscles (Fig. 7B, see appendix), but this nerve showed abnormal branching and nerve terminal branches were no longer observed. In controls, the majority of AChR clusters was confined to the middle region of the muscle and in co-localization with nerve terminal branches (Fig. 7A, see appendix). As in the diaphragm, AChR clusters were more diffusely distributed over mutant intercostal muscles (Fig. 6B, see appendix) and some

clusters were present in the outer regions of the muscle (arrowheads in Fig. 7B, see appendix). However, we did not observe nerve terminal branches, or co-localization of pre- and postsynaptic staining at this stage.

Motor neurons defasciculate in the mutant

At E14, the general organization of the phrenic nerve was normal in the mutant but tertiary and terminal axonal branching were aberrant. Between E14 and E18 the branching pattern drastically changed in the mutant. Figure 8 (see appendix) shows wholemount control and mutant diaphragms. In control diaphragms, motor neurons ran exclusively through the middle of the muscle fibers (arrow in Fig. 8A). In the E18 mutant diaphragm the characteristic motor neuronal pattern had disappeared and the central branches of the phrenic nerve could no longer be detected. Many GAP-43- and synapsin-positive fibers were still observed in the mutant diaphragm but these were heavily de-fasciculated and formed a meshwork with no apparent organization (Fig. 8B). A number of these projections emanate from the normal point of entry of the phrenic nerve, and co-localize with AChR clusters, suggesting that at least some of these fibers are of motor neuronal origin. The neuronal processes at the margin of the diaphragm, presumably of sensory origin (Yang et al., 2000), appeared to be normal in the mutant (arrowheads in Fig. 8A, B). Due to a lack of selective staining for motor- or sensory neurons in embryonic muscle it was not possible to unequivocally determine the identity of the neurons in the mutant diaphragm. Still, these results suggest that massive axonal resprouting occurs in the mutant.

Motor neurons, but not sensory neurons, degenerate in the mutant

Synaptic transmission and synaptic competition also have marked effect on the survival of motor neurons. Therefore, we analyzed sections from cervical regions 3-5 of the spinal cord where the somata of these motor neurons lay at E14 and E18, using cryosections stained for neurofilament. At E14 no difference was detected between control and mutant spinal cords (data not shown). However, starting from E15, we observed considerable degeneration of the spinal cord (data not shown) and by E18 most of the (motor-) neurons of the spinal cord had degenerated (Fig. 9C). In contrast, up to birth, no obvious degeneration was observed in the sensory neurons that innervate the diaphragm muscle. The gross morphology of the mutant dorsal root ganglia (DRG) was normal (Fig. 9B, D). Hence, the mutant is characterized by a selective degeneration of spinal cord neurons, not the sensory neurons of the DRG.

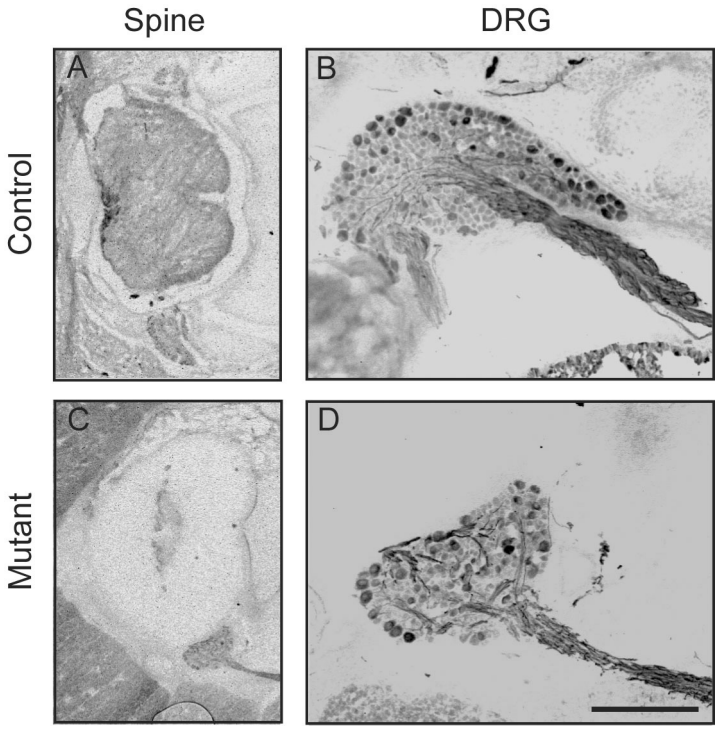

Figure 9. *Staining for neurofilament shows that at E18 the spinal cord (A) has almost completely degenerated (C) in the mutant. DRGs have similar morphology in control (B) and mutant (D) embryos. The scale bar is 1000 µm (A, C) and 250 µm (B, D) respectively.*

Discussion

In this study, we have characterized the development of neuromuscular synapses in the *munc18-1* null mutant mouse. We show that this mouse lacks synaptic secretion throughout prenatal development. We found that secretion is not essential for initial axon pathfinding, clustering of postsynaptic elements or the formation of basic NMJs. However, in the mutant, the terminal axonal branches were absent at E14, the organized pattern of branches was not maintained, postsynaptic elements were not properly refined/redistributed, synapses were rare and spinal cord neurons, but not DRG neurons degenerated.

It has been suggested that vesicular neurotransmitter secretion provides signals for pathfinding and membrane for outgrowth in the developing brain. This suggestion was largely based on observations that manipulation

of proteins involved in neurotransmitter secretion, such as SNAP-25 (synaptosomal associated protein of 25 kD), appeared to interfere with neurite outgrowth (Han et al., 1991; Osen-Sand et al., 1993; Osen-Sand et al., 1996; Kabayama et al., 1999). Our finding that axons grow out and find their way to the muscle in the *munc18-1* null mutant, contradicts this. The major branches of the phrenic nerve showed a similar pattern in mutant and control diaphragms until E15, while at this stage synaptic secretion was completely absent. This is in agreement with our observations in the CNS (Verhage et al., 2000), where axonal projections and segregation of brain areas were normal during early development. Furthermore, no evidence for impaired outgrowth was recently reported in the SNAP-25 null mutant mouse (Washbourne et al., 2002).
The absence of terminal nerve branches at E14 and their low incidence during further development suggests that the final stages of axonal pathfinding in the PNS are suppressed in the mutant. One possibility is that the later stages of axonal pathfinding are partially dependent on munc18-1 dependent processes, for instance neurotransmitter secretion. Alternatively, terminal branches may be less stable in the mutant.

We found less AChE clusters in mutant muscles, whereas AChR clusters were present at similar levels in controls and mutants. It was previously reported that denervation or action potential blockade results in a rapid decrease in AChE mRNA and protein, which can be reversed by direct electrical stimulation of the muscle (Cangiano et al., 1980; Lomo and Slater, 1980; Gaspersic et al., 1999; Boudreau-Lariviere et al., 2000). The opposite was observed for AChR mRNA, which was upregulated upon denervation (Boudreau-Lariviere et al., 2000). It is therefore likely that the observed difference in AChE and AChR levels in the *munc18-1* mutant is caused by differential, activity-dependent regulation of the expression of the two genes.

In mutant muscles, co-localization of pre- and postsynaptic elements was observed both in diaphragm and intercostal muscles, abeit with much lower incidence than in controls. This, together with the electron microscopical data showing synaptic structures in the mutant, indicates that synapses are formed in the mutant. Since the mutant displayed neither MEPPs nor evoked postsynaptic potentials throughout prenatal development, we conclude that neurotransmitter secretion is not essential to form basic synapses at the NMJ. This is in agreement with earlier observations in the CNS (Verhage et al., 2000). The total number of synapses, both in the CNS (A.S. Maia, Rudolf Magnus Institute, University of Utrecht Medical Center,

personal communication) and in the NMJ (this study) appears to be lower in the mutant. This suggests that either synapse formation is impaired or synapses are less stable. The observed reduction in the number of synaptic vesicles suggests that maturation of synapses is also impaired probably as a consequence of the lack of synaptic activity in the mutant.

Recently, it was proposed that AChE and AChR cluster in the future endplate region, independent of the nerve, and that these clusters are subsequently refined by positive and negative signals from the nerve (Lin et al., 2001; Yang et al., 2001). There is good evidence that neuronal agrin provides a positive/aggregating signal (Lin et al., 2001). Neurotransmitter secretion was proposed as a negative/dispersing signal. Our data are in full agreement with this concept. Firstly, because AChE clusters were present in the *munc18-1* mutant at E14, while no terminal branches of the phrenic nerve were present. Secondly, because in the mutant many a-neural AChE and AChR clusters do not disappear during further development, as in controls, where the phrenic nerve secretes neurotransmitter. Hence, these data are consistent with a negative/dispersing function of neurotransmission on clustering of postsynaptic elements at the NMJ. However, we cannot exclude that the persistence of a-neural AChE and AChR clusters in the *munc18-1* mutant is the consequence of the lack of another unknown clearance process that requires munc18-1.

In the *munc18-1* null mutant muscles most of the AChR clusters were present in the central half of the muscle, as in controls, but their distribution was more diffuse and some ectopic clusters were also observed. Potentially, this broad band of AChR clusters reflects the diffuse prepatterned endplate region as described by Yang *et al.* (2000), suggesting that in the *munc18-1* mutant no neuronal influence was exerted on the muscle. However, in the absence of neuronal input, the average size of AChR clusters was significantly smaller than normally innervated clusters (Lin *et al.*, 2001). In our study, we found no difference in average cluster size between mutant and control diaphragms. This indicates that in the *munc18-1* mutant, AChR clusters have grown in late embryonic development, suggesting some neuronal influence on AChR clusters in the mutant. Alternatively, these clusters may result from attempts of motor neurons to resprout and form new synapses after the initial synapses proved dysfunctional. This was also proposed to occur after neurotransmission is impaired by botulinum toxin treatment (de Paiva et al., 1999).

In normal NMJ development, muscle cells are often innervated by more than one motor neuron. During maturation, activity-based competition eliminates

weak, and stabilizes strong synapses (for a review see Lichtman and Colman, 2000), and approximately half of the motor neurons degenerate. In the *rapsyn*, *musk* and *choline acetyl transferase* (*chat*) null mutant mice, more motor neurons appear to survive than in wildtypes (Brandon et al., 2000, soc. neurosci., abstract; Banks et al., 2001; Terrado et al., 2001;. This abnormally high survival is likely to be due to the defect in neuromuscular transmission in these mutants and the resultant lack of activity-based competition. In the *munc18-1* mutant, activity and therefore activity-based competition are also absent. Still, most motor neurons die, as well as other spinal cord neurons. This is consistent with our findings in higher brain areas, where early-formed areas progressively degenerate approaching birth (Verhage et al., 2000). The difference in motor neuron survival between *munc18-1* null mutants and the *rapsyn*, *musk* and *chat* mutants is probably due to the fact that munc18-1 is expressed in all types of neurons and is required for synaptic secretion of various neurotransmitters (Verhage et al., 2000). Hence, in contrast to the motor neurons in the *rapsyn*, *musk* and *chat* mutants, the motor neurons in the *munc18-1* mutants probably lack synaptic input themselves. Interestingly, the DRG neurons in this mutant do not degenerate. DRG neurons probably are among the few neurons that receive normal input in this mutant because in primary sensory neurons this input is not dependent on neurotransmitter secretion, but on (mechano-) sensory stimulation. These considerations are consistent with the general concept that receiving synaptic input is important for neuronal survival and that activity-based competition at the target is secondary to this.

At birth, *munc18-1* mutant muscles contain an elaborate and disorganized meshwork of neuronal processes, probably resulting from axonal defasciculation and aberrant axonal sprouting. Interestingly, these abnormalities were different from the abnormal branching patterns observed in null mutants for three known synapse-organizing genes, *agrin*, *musk*, and *rapsyn*. Here, primary and secondary branching were relatively normal, tertiary branches grew excessively long, parallel to myotubes and only few (terminal) arbors were formed (Gautam et al., 1995; DeChiara et al., 1996; Gautam et al., 1996). The *munc18-1* null mutant differs from these mutants in that all branching hierarchy and organisation with respect to myotubes had disappeared. The innervation of the *munc18-1* mutant muscle shows more resemblance to the muscular dysgenesis (*mdg*) mutant and E14 diaphragms

in *nrg-1* and *erbb-2* mutants (Rieger and Pincon-Raymond, 1981; Powell et al., 1984; Rieger et al., 1984; Morris et al., 1999; Lin et al., 2000; Wolpowitz et al., 2000). It is not known what causes the difference in axonal branching patterns between the two groups. One consistent finding is that *mdg*, *nrg-1*, *erbb2* and *munc18-1* mutant muscles all contain uninnervated AChR clusters while *agrin*, *musk* and *rapsyn* mutants do not. These AChR clusters may be involved in retrograde signaling that induces aberrant/excessive sprouting.

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Abbreviations

AChE	acetylcholinesterase
AChR	acetylcholine receptor
BSA	bovine serum albumin
CNS	central nervous system
DRG	dorsal root ganglion
E	embryonic day
GAP-43	growth cone associated protein-43
Mdg	muscular dysgenesis
MEPP	miniature endplate potential
Munc18-1	mammalian uncoordinated 18-1
MuSK	muscle specific kinase
NMJ	neuromuscular junction
PBS	phosphate buffered saline
PFA	paraformaldehyde
PNS	peripheral nervous system
SNAP-25	synaptosomal associated protein of 25 kD
Unc18	uncoordinated 18

References

Banks, G. B., Chau, T. N., Bartlett, S. E. and Noakes, P. G., 2001. Promotion of motoneuron survival and branching in rapsyn-deficient mice. JComp Neurol. 429, 156-165.

Bezakova, G., Rabben, I., Sefland, I., Fumagalli, G. and Lomo, T., 2001. Neural agrin controls acetylcholine receptor stability in skeletal muscle fibers. ProcNatlAcadSciUSA. 98, 9924-

9929.
Boudreau-Lariviere, C., Chan, R. Y., Wu, J. and Jasmin, B. J., 2000. Molecular mechanisms underlying the activity-linked alterations in acetylcholinesterase mRNAs in developing versus adult rat skeletal muscles. JNeurochem. 74, 2250-2258.
Brandon, E. P., Lin, W., D'Amour, K. A., Pizzo, D. P., Thode, S., Dominguez, B., Thal, L. J., Lee, K. F. and Gage, F. H., 2000. Developmental defects in choline acetyltransferase knockout mice. soc neurosci abstract.
Cangiano, A., Lomo, T., Lutzemberger, L. and Sveen, O., 1980. Effects of chronic nerve conduction block on formation of neuromuscular junctions and junctional AChE in the rat. Acta Physiol Scand. 109, 283-296.
de Paiva, A., Meunier, F. A., Molgo, J., Aoki, K. R. and Dolly, J. O., 1999. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. ProcNatlAcadSciUSA. 96, 3200-3205.
DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., Kinetz, E., Compton, D. L., Rojas, E., Park, J. S., Smith, C., DiStefano, P. S., Glass, D. J., Burden, S. J. and Yancopoulos, G. D., 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell. 85, 501-512.
Featherstone, D. E., Rushton, E. and Broadie, K., 2002. Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. NatNeurosci. 5, 141-146.
Gaspersic, R., Koritnik, B., Crne-Finderle, N. and Sketelj, J., 1999. Acetylcholinesterase in the neuromuscular junction. ChemBiolInteract. 119-120, 301-308.
Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P. and Sanes, J. R., 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell. 85, 525-535.
Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P., 1995. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature. 377, 232-236.
Han, H. Q., Nichols, R. A., Rubin, M. R., Bahler, M. and Greengard, P., 1991. Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. Nature. 349, 697-700.
Holt, S. J. and Withers, R. F. J., 1952. Cytochemical localization of esterases using indoxyl derivatives. Nature. 170, 1012-1014.
Houenou, L. J., Pincon-Raymond, M., Garcia, L., Harris, A. J. and Rieger, F., 1990. Neuromuscular development following tetrodotoxin-induced inactivity in mouse embryos. JNeurobiol. 21, 1249-1261.
Kabayama, H., Takei, K., Fukuda, M., Ibata, K. and Mikoshiba, K., 1999. Functional involvement of synaptotagmin I/II C2A domain in neurite outgrowth of chick dorsal root ganglion neuron. Neuroscience. 88, 999-1003.
Lichtman, J. W. and Colman, H., 2000. Synapse elimination and indelible memory. Neuron. 25, 269-278.
Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R. and Lee, K. F., 2001. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature. 410, 1057-1064.
Lin, W., Sanchez, H. B., Deerinck, T., Morris, J. K., Ellisman, M. and Lee, K. F., 2000. Aberrant development of motor axons and neuromuscular synapses in erbB2- deficient mice. ProcNatlAcadSciUSA. 97, 1299-1304.
Lomo, T. and Slater, C. R., 1980. Control of junctional acetylcholinesterase by neural and muscular influences in the rat. JPhysiol. 303, 191-202.
Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F., 1999. Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron. 23, 273-283.
Osen-Sand, A., Catsicas, M., Staple, J. K., Jones, K. A., Ayala, G., Knowles, J., Grenningloh, G. and Catsicas, S., 1993. Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature. 364, 445-448.
Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A., Montecucco, C. and Catsicas, S., 1996. Common and distinct fusion proteins in axonal growth and transmitter release. JComp Neurol. 367, 222-234.

Plomp, J. J., van Kempen, G. T. and Molenaar, P. C., 1992. Adaptation of quantal content to decreased postsynaptic sensitivity at single endplates in alpha-bungarotoxin-treated rats. JPhysiol. 458, 487-499.
Poo, M. M., Sun, Y. A. and Young, S. H., 1985. Three types of transmitter release from embryonic neurons. J Physiol. 80, 283-289.
Powell, J. A., Rieger, F., Blondet, B., Dreyfus, P. and Pincon-Raymond, M., 1984. Distribution and quantification of ACh receptors and innervation in diaphragm muscle of normal and mdg mouse embryos. DevBiol. 101, 168-180.
Rieger, F. and Pincon-Raymond, M., 1981. Muscle and nerve in muscular dysgenesis in the mouse at birth: sprouting and multiple innervation. DevBiol. 87, 85-101.
Rieger, F., Powell, J. A. and Pincon-Raymond, M., 1984. Extensive nerve overgrowth and paucity of the tailed asymmetric form (16 S) of acetylcholinesterase in the developing skeletal neuromuscular system of the dysgenic (mdg/mdg) mouse. DevBiol. 101, 181-191.
Saitoe, M., Schwarz, T. L., Umbach, J. A., Gundersen, C. B. and Kidokoro, Y., 2001. Absence of junctional glutamate receptor clusters in Drosophila mutants lacking spontaneous transmitter release. Science. 293, 514-517.
Terrado, J., Burgess, R. W., DeChiara, T., Yancopoulos, G., Sanes, J. R. and Kato, A. C., 2001. Motoneuron Survival Is Enhanced in the Absence of Neuromuscular Junction Formation in Embryos. JNeurosci. 21, 3144-3150.
Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J. and Pfaff, S. L., 1999. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9 [see comments]. Neuron. 23, 675-687.
Verhage, M., Maia, A. S., Plomp, J. J., Brussaard, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., van den Berg, T. K., Missler, M., Geuze, H. J. and Sudhof, T. C., 2000. Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science. 287, 864-869.
Washbourne, P., Thompson, P. M., Carta, M., Costa, E. T., Mathews, J. R., Lopez-Bendito, G., Molnar, Z., Becher, M. W., Valenzuela, C. F., Partridge, L. D. and Wilson, M. C., 2002. Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci. 5, 19-26.
Westerfield, M., Liu, D. W., Kimmel, C. B. and Walker, C., 1990. Pathfinding and synapse formation in a zebrafish mutant lacking functional acetylcholine receptors. Neuron. 4, 867-874.
Wolpowitz, D., Mason, T. B., Dietrich, P., Mendelsohn, M., Talmage, D. A. and Role, L. W., 2000. Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron. 25, 79-91.
Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C. and Burden, S. J., 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron. 30, 399-410.
Yang, X., Li, W., Prescott, E. D., Burden, S. J. and Wang, J. C., 2000. DNA topoisomerase IIbeta and neural development. Science. 287, 131-134.
Zhao, H. and Nonet, M. L., 2000. A retrograde signal is involved in activity-dependent remodeling at a C. elegans neuromuscular junction. Development. 127, 1253-1266.

Chapter 3

Trophic support delays, but does not prevent cell-intrinsic degeneration of neurons deficient for munc18-1

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Abstract

The stability of neuronal networks is thought to depend on synaptic transmission which provides activity-dependent maintenance signals for both synapses and neurons. Here, we tested the relation between presynaptic secretion and neuronal maintenance using *munc18-1* null mutant mice as a model. These mutants have a specific defect in secretion from synaptic and large dense core vesicles (Verhage *et al*., 2000; Voets *et al*., 2001). Neuronal networks in these mutants develop normally up to synapse formation, but eventually degenerate. The proposed relation between secretion and neuronal maintenance was tested in low density- and organotypic cultures and *in vivo*, by conditional, cell-specific inactivation of the munc18-1 gene. Dissociated *munc18-1* deficient neurons died within 4 days in vitro (DIV). Application of trophic factors, insulin or BDNF, delayed degeneration up to 7DIV. In organotypic cultures, *munc18-1* deficient neurons survived until

9 DIV. On glial feeders, these neurons survived up to 10DIV and 14DIV when insulin was applied. Co-culturing dissociated mutant neurons with wild-type neurons did not prolong survival beyond 4 DIV, but co-culturing mutant slices with wild-type slices prolonged survival up to 19DIV. Cell-specific deletion of *munc18-1* expression in cerebellar Purkinje cells *in vivo* resulted in the specific loss of these neurons, without affecting connected or surrounding neurons. Together, these data allow three conclusions. First, the lack of synaptic activity cannot explain the degeneration in *munc18-1* mutants. Second, trophic support delays but cannot prevent degeneration and, third, a cell-intrinsic, yet unknown function of munc18-1 is essential for prolonged survival.

Introduction

During brain development, a surplus of neurons and synapses is formed after which many neurons and synapses are eliminated. In various model systems, synaptic activity is a major determinant in synapse elimination (for review see: Lichtman & Colman, 2000; Katz & Crowley, 2002). For instance blocking neuromuscular transmission during the critical period, prevents neuromuscular synapse elimination (Duxson, 1982). Blocking synaptic transmission in a subset of motor neurons also selectively eliminates the inactive synapses (Buffelli *et al*., 2003). Similarly, inhibiting synaptic activity in the visual cortex, prevents synapse elimination, while selectively suppressing activity in a subset of synapses (for instance by closing one eye) results in the elimination of the less active synapses (for review see: Katz & Crowley, 2002). For neurons, similar rules apply, since blocking neuromuscular transmission, either genetically or pharmacologically, also prevents naturally occurring motor neuronal elimination (Houenou *et al*., 1990; Banks *et al*., 2001). Activity-based competition for target-derived trophic substances has been proposed to underlie these elimination processes (Katz & Shatz, 1996; Sanes & Lichtman, 1999), however, the exact mechanism underlying synaptic and neuronal elimination still remains unknown.

We have previously generated *munc18-1* null mutant mice that completely lack spontaneous and evoked neurotransmitter secretion and die at birth (Verhage *et al*., 2000). Brain development in *munc18-1* null mutant mice is normal up to the point of synapse formation, after which widespread neurodegeneration occurs in the same order as initial development; i.e. early-

maturing brain regions degenerate first. Degenerating cells in the mutant brain have electron dense nuclei and cytoplasm, condensed chromatin and are terminal dUTP nick end labelling positive, suggesting that *munc18-1* deficient neurons die through apoptosis (Verhage *et al*., 2000). We proposed that this degeneration is due to the absence of synaptic activity, and in line with this, we found that sensory dorsal root ganglia, which are unique in not depending on synapses for their activity, are spared from degeneration (Heeroma *et al*., 2003). Secretion from large dense core vesicles (LDCV) is also severely compromised in the *munc18-1* null mutant (Voets *et al*., 2001, Korteweg et al, in preparation). As LDCV secretion is probably the source of many neurotrophic substances (Berg *et al*., 2000; Wang *et al*., 2003), reduced LDCV secretion may also contribute to the degeneration of neuronal networks.

We exploited the *munc18-1* null mutant as a model to characterize the role of synaptic input and trophic support for the survival of neurons and neuronal networks *in vitro* and *in vivo*. We used neocortical and hippocampal neurons, of *munc18-1* null mutant and control embryos, in low-density, micro island and organotypic cultures, subjected these neurons to different conditions of activation and trophic support and analysed the effects on survival. Moreover, we also generated a conditional null allelle of the *munc18-1* gene in order to perform cell-specific deletion of *munc18-1* expression *in vivo* and analyse the fate of *munc18-1* deficient neurons in an otherwise normal brain. We used L7-Cre mice (Barski *et al*., 2000) to specifically delete expression in the Purkinje cells of the cerebellum. We found that co-culturing with control neurons delayed degeneration of *munc18-1* mutant neurons in organotypic, but not low density or micro island cultures. Providing trophic support with insulin, BDNF, glial feeder cells or organotypic cultures, delayed but could not prevent degeneration of mutant neurons. Finally, cell-specific deletion of *munc18-1*, *in vivo*, resulted in specific degeneration of *munc18-1* deficient neurons, suggesting that *munc18-1* deficient neurons do not degenerate due to a lack of synaptic input or trophic support, but because munc18-1 is essential for long-term neuronal survival, regardless of its role in regulated secretion.

Materials and methods

Laboratory animals

Munc18-1 deficient mice were described previously (Verhage *et al*., 2000). For various experiments, *munc18-1* deficient mice were crossed with either mice expressing *soluble green fluorescent protein* (*gfp*) (a kind gift of Dr. A. Nagy) or mice expressing a GFP-based neuronal surface marker (*gfptkras*, Roelandse *et al*., 2003). Mouse embryos were obtained by caesarian section of pregnant females from timed heterozygous matings. Purkinje cell specific knockout mice were created using Cre/loxP-mediated recombination. *L7-cre* transgenic mice (Barski *et al*., 2000) were crossed with *loxp-munc18-1* mutants. *Loxp-munc18-1* mutants were created by insertion of loxP sites flanking exon 2 of the *munc18-1* gene by homologous recombination in ES-cells. The mouse lines and crosses that were used in each experiment are listed in table 1. Animals were housed and bred in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All animal experiments were approved of by the Dutch Committee for Experiments on Animals and effort was made to minimize animal discomfort.

Crosses	Experimental group	Control group	Experiment
Munc18-1$^{+/-}$ x *munc18-1*$^{+/-}$	*Munc18-1*$^{-/-}$	*Munc18-1*$^{+/-}$, *munc18-1*$^{+/+}$	Micro island culture, low density (co-culture)
Munc18-1$^{+/-}$; *gfp* x *munc18-1*$^{+/-}$; *gfp*	*Munc18-1*$^{-/-}$; *gfp*	*Munc18-1*$^{+/-}$; *gfp*, *munc18-1*$^{+/+}$; *gfp*	Micro island culture, low density (co-culture)
Munc18-1$^{+/-}$; *gfptkras* x *munc18-1*$^{+/-}$; *gfptkras*	*Munc18-1*$^{-/-}$; *gfptkras*	*Munc18-1*$^{+/-}$; *gfptkras*, *munc18-1*$^{+/+}$; *gfptkras*	Organotypic culture)
Loxp-munc18-1$^{+/+}$ x *loxp-munc18-1*$^{+/-}$; *l7cre*$^{+/+}$	*Loxp-munc18-1*$^{+/+}$; *l7cre*$^{+/-}$	*Loxp-munc18-1*$^{+/-}$; *l7cre*$^{+/-}$	Conditional cerebellar Purkinje cell specific *munc18-1* knockout

Table 1: *Mouse lines and crosses that were used in this study.*

Cortical cultures

Cortices were dissected from embryonic day 18 (E18) mice and collected in Hanks Buffered Salts Solution (HBSS)(Sigma), buffered with 7 mM Hepes.

After removal of the meninges, the cortices were minced and incubated for 20 minutes in trypsinated HBSS at 37 °C. After washing, neurons were triturated with fire polished Pasteur pipettes, counted with a hemacytometer and plated in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% Hepes, 1% glutamax (Invitrogen), 1% Pen/Strep (Invitrogen) and 0.2% 14.3 mM β-mercapto-ethanol. Low density cultures were plated on poly-L-lysin coated glass coverslips at 25,000/cm^2. For island cultures, neurons were plated at 6,000/cm^2 on islands of rat glia. Glial islands were obtained by spraying a 0.25 mg/ml rat tail collagen solution (BD Biosciences, Bedford, USA) on glass coverslips. After drying and UV sterilization glial cells were plated at 600/cm^2. In some experiments, 100 nM insulin (Sigma) or 50 nM BDNF (Sigma) was added to prolong the lifespan of munc18-1 deficient neurons. 50% of the medium was refreshed every week.

Organotypic cultures

Organotypic slice cultures from E18 hippocampi were prepared as follows. Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous mating. GFP-expressing animals were identified by direct inspection using a Leica MZ12 dissection microscope fitted with fluorescence optics. Brains were dissected in ice-cold dissection Gey's balanced salt solution (dGBSS, consisting of GBSS (Invitrogen) with 0.65 g glucose and 200μM kynurenate, pH 7.4) and cut into 400 μm thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK). After separating individual slices, hippocampal region was dissected out and separated. These hippocampal slices were kept at 4˚C in dGBSS for 45 min to recuperate. All subsequent procedures were identical to those described for organotypic slice cultures from P8 mice (Gahwiler, 1998).For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous perfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) saturated with 95% O2 /5% CO2 using a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany) and analyzed with MetaMorph software (Universal Imaging Corp., West Chester PA).

Electrophysiological Recordings

Whole cell current-clamp recordings were performed on cultured neurons at

DIV 8 and 9. The patch pipette contained the following solution (in mM): 125 K^+-gluconic acid, 10 NaCl, 4.6 $MgCl_2$, 4 K_2-ATP, 15 Creatine Phosphate, 1 EGTA and 20 U/ml Phospocreatine Kinase (pH 7.30). External medium contained (in mM): 140 NaCl, 2.4 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.30). Glutamate (1 mM) was added using an application pipette for the duration of 4 seconds (with picospritzer). Axopatch 200A was used for whole-cell recordings. Signal was acquired using Digidata 1322A and Clampex 8.1. Clampfit 8.0 was used for offline analysis.

Immunocytochemical procedures

Dissociated cultures were fixed by adding 4% paraformaldehyde (PFA) to the medium in a 1/1 ratio. After 15 minutes the PFA/medium mixture was exchanged for 4% PFA, for 15 minutes. After washing with PBS the cells were permeated with 0.1% Triton X-100 for 5 minutes. After washing with PBS the cells were ready for processing or storage. Before staining the cells were incubated in 4% fetal calf serum for 20 minutes to block a-specific reactions. After washing with PBS the cells were incubated in a mixture of antibodies and 0.1% Triton X-100 for 1 hour at room temperature or overnight at 4 °C. The antibodies used were: mouse anti-MAP2 (1:200, Boehringer Mannheim), mouse anti-HPC1 (syntaxin) (1:1000, Sigma), rabbit anti S100β (1:400, Dako, Glostrup, Denmark). After washing 3 times 5 minutes with PBS, the cells were incubated for 1 hour at room temperature in goat anti-mouse Alexa546 (1: 1000, Molecular Probes, Oregon, USA). After washing in 3 times 5 minutes in PBS the coverslips were mounted on microscopic slides with Dabco-Mowiol and analysed with a Zeiss 510 Meta Confocal microscope.

Cultures slices were prepared by microwave-assisted fixation (Jensen & Harris, 1989). Briefly, slices were transferred into pre-warmed fixative (4% PFA, 0.5% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer (CCB), pH 7.3) and irradiated at maximum power (1000 W) for 9 sec in a Bio-Rad H2500 microwave processor. Post-irradiation temperature, measured by the built-in temperature probe, was 35 - 50˚ C. Slices were washed 5 x 10 min in 0.1M CCB and incubated overnight at room temperature in 0.3% Triton X-100 5% normal goat serum (NGS; Invitrogen) and 1% bovine serum albumin (BSA; Sigma) in 0.1M CCB. Slices were then incubated overnight at room temperature in 0.1M CCB with 0.3% Triton X-100, 5% NGS, 1% BSA, mouse anti-MAP2C (1:5, Weisshaar *et al.*, 1992) and rabbit anti-GFP (1:800, Novartis, Basel, Switzerland). After washing 5 times for 10 min each in 0.1M CCB slices were incubated overnight at room temperature in 0.1M CCB with 0.3% Triton X-100, 5% NGS, 1% BSA, goat anti-mouse Alexa546 (1:200, Molecular Probes)

and goat anti-rabbit Alexa488 (1:200, Molecular Probes). After washing, slices were stored at 4 °C in 0.1M CCB and mounted for imaging in purpose-built chambers, as described above.
L7-cre x *loxp-munc18-1* mutant and wild-type mice were anesthetized by Nembutal injection and transcardially perfused with 4% PFA. Histochemistry was performed by incubating cryosections (25 µm thickness) for 3 min in 0.05% Thionine. Sections were analyzed with a light microscope (DM-RB, Leica) equipped with a digital camera. Immunocytochemistry was performed on cryosections (25 µm thickness), rinsed in 0.05 M Tris (Ph 7.6), and preincubated in blocking buffer (5% normal horse serum and 0.25% Triton X-100 in 0.5 M Tris) for 60 min. GAD antibody (sh-a-GAD 1:2000, kind gift of Dr Oertel, Department of Neurology, Philipps University Marburg, Germany) and calbindin antibody (r-a-CaBp 1:10000, Swant, Bellinzona, Switserland) were diluted in blocking buffer, and sections were incubated for 24hrs at room temperature. After incubation, samples were rinsed in Tris, followed by 15 min incubation in 0.125% Glutaraldehyde in 0.5M Tris. Incubation with the secondary antibodies Cy3 (d-a-sh, 1:200) and Fitc (d-a-r, 1:200, both from Jackson Immuno Res. Lab. Inc, West grove, USA) was performed in blocking buffer for 2 hr at room temperature. Fluorescence was documented using confocal imaging microscopy (LSM 510 inverted confocal microscope, argon/krypton laser; Zeiss).

Results

Munc18-1 deficient neurons in culture lack neurotransmitter secretion but are otherwise biophysically normal.

Munc18-1 deficient neurons in the neocortex and spinal cord lack neurotransmitter secretion at E15, 16 and 18 (Verhage *et al.*, 2000). Here, we investigated whether this holds true for neurons *in vitro*, cultured beyond E18 and whether the absence of *munc18-1* affects other biophysical properties of cultured neurons. The resting membrane potential (V_{rest}) is generally accepted as an informative parameter for the viability/health of (cultured) neurons (Pancrazio *et al.*, 2001). The V_{rest} of *munc18-1* deficient neurons in autaptic island cultures of 8 days *in vitro* (8DIV) was similar to control neurons ($V_{rest, control}$= -52.0 +/- 2.0 mV, n = 5; $V_{rest, mutant}$= -51.1 +/- 5.4 mV, n = 5, insets fig. 1A). Current injection into neurons (DIV 8) induced action potentials in both control and mutant neurons (n = 5, Fig. 1A). Current injection varied between recordings, because injection was manual

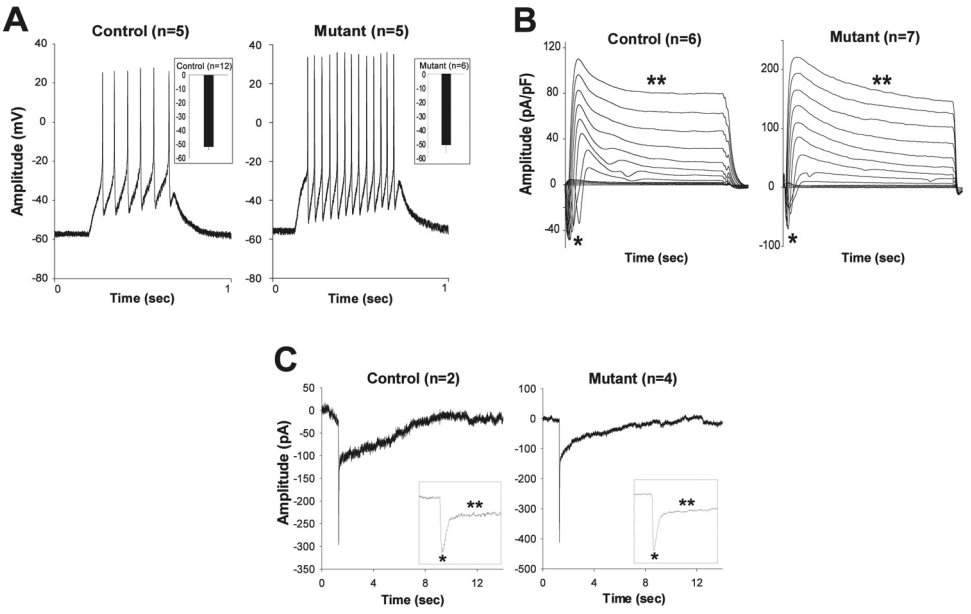

Figure 1: *Electrophysiological characterization of munc18-1 deficient neurons in culture (A) Current injection into cultured neurons induced action potentials in both control and munc18-1 mutant neurons (n = 5). The amount of current injection was not monitored during the recordings. The difference in action potential frequency can thus be explained by the variable amount current injection between recordings. The V_{rest} of autaptic islands of munc18-1 deficient neurons is very similar to control autaptic neurons (p=0.86; see inset in panel A). (B) The Na^+ and K^+ channel expression was studied using repetitive block-pulse stimulation (200 ms block pulse; -70 to 40 mV; 10 mV increments). Both control (n=6) and mutant (n=7) neurons show Na^+-current (single asterisk, tetrodotoxin-sensitive) and K^+-currents (double asterisk, compound current). (C) Example traces of glutamate application inducing inward current in both wild type (n=9) and mutant (n=11) neurons (V_m=-70 mV). Inset: First 6 seconds of amplification. A fast AMPA component (single asterisk) and a slow NMDA component (double asterisk) were observed in recordings from both control and mutant neurons.*

and not standardized between recordings. This explains the different action potential frequency between the recordings of *munc18-1* deficient neurons and control neurons. However, in whole cell voltage clamp recordings both spontaneous and evoked release were absent in mutant neurons (data not shown, n=10), whereas control neurons exhibited spontaneous events at 0.5-2 Hz, with 10-200 pA amplitudes, and evoked responses of 0.1-2 nA amplitudes. Na^+ and K^+-currents were comparable between control and mutant neurons (Fig. 1B).

We have previously shown that *munc18-1* deficient neurons express functional GABA and acetylcholine receptors (Verhage, *et al.*, 2000). As the majority of central synapses are driven by glutamate, we tested whether *munc18-1* deficient neurons express functional glutamate receptors. Glutamate application (1 mM, 4 sec) elicited both a fast (AMPA-R mediated) and a slow (NMDA-R mediated) response in control and mutant neurons

(DIV 7/8) indicating that both types of glutamate receptors are present and functional in mutant neurons (n = 9 ; n = 11, respectively; Fig. 1C). Thus, apart from the lack of synaptic vesicle secretion in *munc18-1* deficient neurons, we did not detect additional biophysical differences between mutant and control neurons.

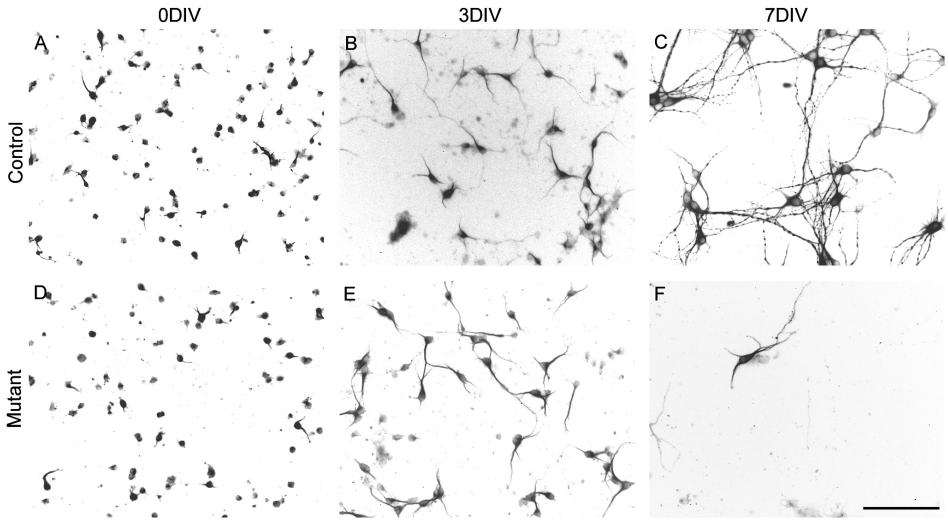

Figure 2: Survival in vitro of munc18-1 null mutant neurons and their controls. Control (A - C) and mutant (D - F) neurons, identified by MAP2 staining, were plated at equal densities. At 3DIV, control (B) and mutant (E) neurons were still in culture at equal densities. At 7DIV, only a fraction of mutant neurons (F) was left in culture compared to the control situation (C). Scale bar is 50 μm.

Munc18-1 is essential for neuronal viability

We analyzed survival of *munc18-1* null mutant neurons from neocortex and hippocampus in culture. Cortical neurons were plated at a density of 20k/ cm^2 (Fig. 2A, D). Trypan blue staining indicated that the viability of mutant and control neurons after dissociation was similar (data not shown). As another measure for viability after dissociation, neuronal attachment to the substrate was analysed at 2, 6 and 24 hours after plating. At all three time-points, more than 95% of both mutant and control neurons were attached (data not shown), suggesting equal initial viability in both groups.

After 3DIV, the amount of control and mutant neurons, identified by microtubule associated protein 2 (MAP2) staining, was counted in 24 randomly chosen fields of 0.137 mm^2 (Fig. 2B, E). For control neurons the average count (+/- standard deviation) was 21.5 +/- 6.7, compared to 22.2 +/- 5.4 per field for mutant neurons. This corresponds to a density of

approximately 16k/cm^2, suggesting a 20% loss between 0 and 3DIV for both groups.
After 7DIV, neurons were counted in 48 randomly chosen fields of 0.137 mm^2 (Fig. 2C, F). Control culture average density was 15.4 +/- 4.9 neurons whereas in mutant cultures neurons were rarely encountered (average 0.7 +/- 0.7 neurons per field). Thus, while 7DIV control neurons were still present at a density of 11.2k/cm^2, the majority of mutant neurons had died by 7DIV. This indicates that *munc18-1* deficient neurons, obtained from E18 neocortex, are equally viable as control neurons during the first 3DIV. However the massive loss of *munc18-1* deficient neurons between 3 and 7DIV suggests a critical role for munc18-1 dependent processes in neuronal viability in later stages.

Co-culturing munc18-1 deficient neurons with wild type neurons does not improve neuronal viability

The degeneration of *munc18-1* deficient neurons may be due to a lack of synaptic input and may thus be prevented by culturing mutant and wild type neurons together in a low density culture so that mutant neurons receive synaptic input from wild type neurons. To identify the different neuronal populations, the *munc18-1* null mutation was crossed into transgenic mice expressing *green fluorescent protein* (*gfp*) under control of the cytomegalovirus (CMV) promoter (see table 1). Co-cultures were prepared using *munc18-1* deficient, *gfp* expressing, neurons with non-fluorescent control neurons and, vice versa, of *gfp* expressing control neurons together with non-fluorescent, *munc18-1* deficient, neurons.
At 3DIV, the development of munc18-1 deficient neurons was indistinguishable from gfp expressing wild-type neurons. Neurons of both genotype had formed mixed neuronal networks with dense, reciprocal innervation (Fig. 3A-I, see appendix). At 4DIV, in co-cultures of munc18-1 deficient, gfp expressing neurons with non-fluorescent control neurons, few mutant neurons, identified by MAP2 staining, remained in co-culture (Fig. 3J-L, see appendix). At 5DIV, approximately 0.001 % of mutant neurons remained in co-culture, similar to mutant neurons in mono-culture. At 14DIV, some GFP-positive cells were still in culture, but these cells lacked MAP2 staining (Fig. 3M-O), and were probably glial cells (discussed below). Therefore our data suggests that culturing *munc18-1* deficient neurons with wild type neurons has no noticeable effect on neuronal viability.

Trophic factors delay the degeneration of munc18-1 deficient neurons

Munc18-1 deficient neurons may degenerate due to a lack of trophic support. Addition of trophic and/or neuromodulatory substances to the culturing medium might therefore prevent this degeneration. Brain derived neurotrophic factor (BDNF) and insulin are known to have a trophic and survival promoting effect on neurons (Lindholm *et al.*, 1996; Yamada *et al.*, 2001). Addition of 100 nM of insulin to the culturing medium prevented the massive loss of *munc18-1* deficient neurons at 4DIV and allowed their survival up to 7DIV (data not shown). However, the number of *munc18-1* deficient neurons that reached 7DIV, was lower (approximately 1%) than in control cultures (approximately 50%) and between 7 and 11DIV, the remaining neurons degenerated. Addition of BDNF to the culturing medium had a similar effect on mutant neuron viability (data not shown). Thus, application of insulin or BDNF delays, but does not prevent degeneration of *munc18-1* deficient neurons.

Culturing on glial cells delays the degeneration of munc18-1 deficient neurons

Most neuronal support in the brain is provided by glial cells (Barres, 1991). Therefore, glial support might compensate for the loss of *munc18-1*. To test this hypothesis, control and *munc18-1* deficient neurons were cultured on a glial feeder layer. At 7DIV, the number of *munc18-1* deficient neurons in culture (Fig. 4B) was comparable with control cultures (Fig. 4A) indicating that glial support indeed had a positive effect on the viability of *munc18-1* deficient neurons. After 10DIV on a glial feeder layer, approximately 1% of *munc18-1* deficient neurons were still in culture (Fig. 4C). Similar to the situation at 7DIV, these neurons appeared morphologically underdeveloped and none survived past 10DIV. Interestingly, when insulin was added to *munc18-1* mutant neurons cultured on glia, neurite branching and outgrowth and neuronal survival rate were still normal at 10DIV (Fig. 4D). However, beyond 10DIV, degeneration still occurred and no mutant neuron reached 14DIV. Together, these data suggest that glial cells and insulin have a synergistic effect in promoting the viability of *munc18-1* deficient neurons. In addition, insulin aids *munc18-1* deficient neurons to obtain/maintain normal neuronal morphology. Nevertheless, glial cells and insulin, neither separate nor in combination, prevent eventual degeneration of mutant neurons.

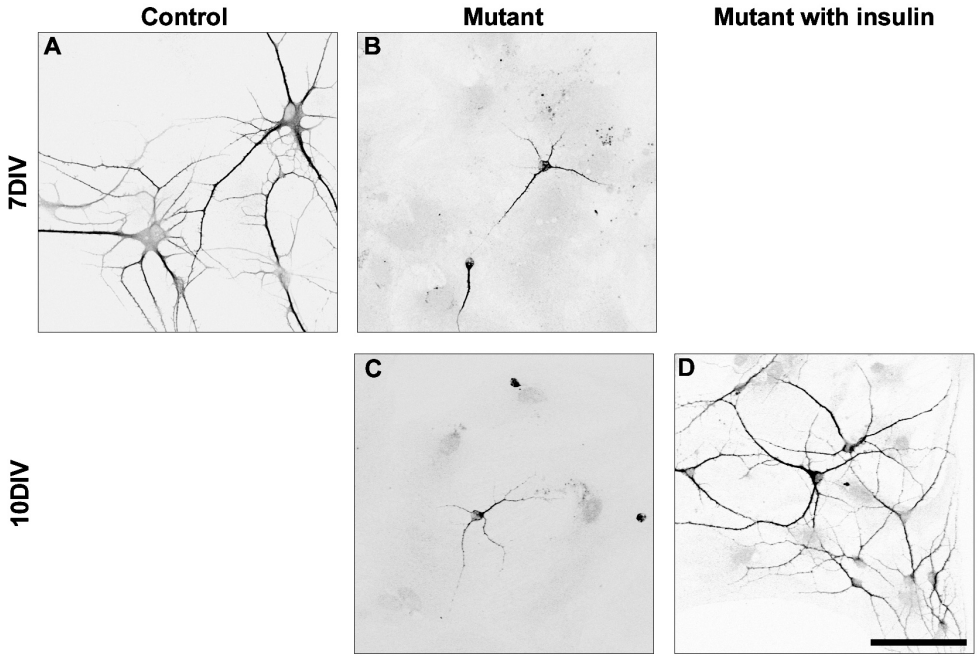

Figure 4: *The effect of glial support and insulin application on the degeneration of munc18-1 deficient neurons. Neurons were plated on glial feeder layers and stained for MAP2 for identification. At 7DIV, munc18-1 deficient neurons (B) were still present in numbers comparable to control neurons (A). Mutant neurons had shorter neurites and less branching points. At 10DIV, some mutant neurons (C) were still present, but most had degenerated. When insulin was added to these cultures (D), normal morphology was preserved and mutant neurons were viable for approximately 13DIV. The scale bar is 50 μm.*

Organotypic culture with wild type slices delays the degeneration of munc18-1 deficient slices

As trophic substances and glial cells worked synergistically in delaying degeneration of *munc18-1* deficient neurons, embedding of these neurons in a more natural trophic environment might delay degeneration further. To test this, we used organotypic slice cultures of E18 hippocampus. The *munc18-1* null allele was crossed into mice transgenically expressing *gfp*-linked to a plasma membrane targeting sequence (*gfptkras*, see table 1), and therefore enables detailed morphological analysis in living neurons (Roelandse *et al*., 2003). Previous publications have shown that postnatal brain slices can be kept *in vitro* for months and that neuronal development in these slices closely resembles *in vivo* maturation (Gahwiler, 1984). Since *munc18-1* deficient mice are postnatally lethal, E18 mice were used for generating the slice cultures. Co-cultures of fluorescent and non-fluorescent wild type slices are known to cross-innervate and to develop mature neuronal characteristics

as dendritic spines and presynaptic varicosities (Roelandse and Matus, in preparation). Such a culture at 32 DIV is shown in figure 5A (mid panel, see appendix); both hippocampi were equal in size and retained morphological features characteristic for the mature hippocampus.
Cultures of single *munc18-1* deficient slices expressing *gfptkras* rapidly degraded during the first week (Fig. 5B, mid panel, see appendix). At 9DIV, some MAP2 positive neurons were still present (Fig. 5B, right panel, see appendix). However, at later stages, all neurons had degenerated, suggesting that organotypic embedding, in itself, is not more successful in preventing degeneration of *munc18-1* deficient neurons than combined glial and trophic support in island mono-cultures.
Low-density co-cultures of *munc18-1* deficient and control neurons did not delay the degeneration of mutant neurons. Interestingly, co-culturing *munc18-1* deficient slices, expressing *gfptkras*, with non-fluorescent control slices did delay the degradation of the fluorescently labelled *munc18-1* deficient slice (compare Figs. 5B and C, mid panels, see appendix). At 9DIV in co-culture, there was no sign of neuronal degeneration in the *munc18-1* deficient slice (Fig. 5C, right panel, see appendix) whereas in the single mutant slice, neurons were virtually absent at the same stage (Fig. 5B, right panel, see appendix). However, at 19DIV, the *gfp* expressing *munc18-1* deficient slice had degenerated, despite the presence of a control slice (Fig. 5D, mid panel, see appendix). Still, some MAP2 positive neurons were present in the vicinity of the non-fluorescent wild type culture (Fig. 5D, right panel, see appendix). No mutant neurons were observed beyond 25 DIV. Quadra-cultures of 3 gfp expressing wild-type slices and 1 munc18-1 deficient slice (Fig. 5E, mid panel, see appendix) showed a dense invasion of gfp expressing wild-type axons surrounding a munc18-1 deficient neuron (identified by the red MAP2C counterstaining; fig. 5E, right panel, see appendix) suggesting that synaptic connections had been established. Together, this suggests that not organotypic slice culturing per se, but co-culturing with control slices prolongs the lifespan of the *munc18-1* deficient neurons.

Surviving munc18-1 deficient cells in culture are glial cells

Eventually every neuron that lacked *munc18-1*, died prematurely. Some *munc18-1* deficient, GFP positive and MAP2 negative cells survived to 14DIV in mixed cultures (Fig. 3D-F, see appendix). Figure 6B (see appendix) shows munc18-1 deficient cells of 21DIV that were mitotic (data not shown) and syntaxin-1 negative (Fig. 6A, C, see appendix). By visual inspection, two cell types could be distinguished among the remaining cells (Fig. 6D,

se appendix). One cell type had the morphology of type 2 astrocytes and indeed was S100β positive (Fig. 6E, see appendix). The other cell type morphologically resembled oligodendrocytes. Hence, in contrast to neurons, glial cell viability seems not to be affected by the absence of *munc18-1*.

Cell-specific deletion of munc18-1 in vivo causes specific loss of munc18-1 deficient neurons

To analyse degeneration of *munc18-1* deficient neurons beyond birth and without the complication of widespread apoptosis, we generated a mutant mouse in which *munc18-1* expression could be conditionally deleted using the Cre-lox system. This munc18-1-lox mouse was crossed with a mouse, transgenically expressing *cre* under control of the L7 promoter (see table 1). *Cre* expression is specific for cerebellar Purkinje cells, starts at postnatal day 6 and is present in all Purkinje cells at postnatal week 3 (Barski *et al*., 2000). To allow depletion of remnant munc18-1 protein, the effect of *munc18-1* deletion was analysed after 8 postnatal weeks. Animals homozygous for the *munc18-1-lox* allele and crossed to L7-Cre, had developed severe ataxia by this time, suggesting a cerebellar defect. Nissl staining showed that overall cerebellar morphology of mutant mice (Fig. 7E, see appendix) was indistinguishable from control mice (Fig. 7A, see appendix), but closer examination showed that Purkinje cell bodies were absent in the mutant cerebellum (Fig. 7B, F, see apendix). Staining for the Purkinje cell marker calbindin showed immunoreactivity in layer 4 of the cerebellum of control mice (Fig. 7C, see appendix) but not in the cerebellum of mutant mice (Fig. 7G, see appendix). Finally, staining for glutamic acid decarboxylase (GAD), a general marker for inhibitory neurons, showed increased immunoreactivity in the Purkinje cell layer of control cerebelli (Fig. 7D), but not of mutant cerebelli (Fig. 7H, see appendix). Apart from Purkinje cells, no obvious signs of degeneration were observed in the cerebellum. Together, these results suggest that cell-specific deletion of *munc18-1* results in the specific loss of those neurons that lack *munc18-1* without affecting connected or surrounding cells.

Discussion

In the present study, we tested the relation between presynaptic secretion and neuronal maintenance *in vitro* and *in vivo* using neurons with *munc18-1* null alleles as a model. We found that *munc18-1* deficient neurons have no

synaptic secretion but have otherwise normal biophysical properties. These neurons degenerated in micro island, low density and organotypical cultures and *in vivo*. We found no evidence that synaptic activity delays or prevents degeneration. Trophic support either by exogenous application of trophic substances, glial support, or co-culturing with wild-type organotypic slices, delayed, but did not prevent degeneration. The effects of various treatments on survival of the mutant neurons are summarized in Fig. 8. Cell-specific deletion of *munc18-1* expression in cerebellar Purkinje cells *in vivo* led to the specific loss of these neurons without affecting connected or surrounding cells.

Munc18-1 deficient neurons are healthy at early developmental stages

Degeneration of *munc18-1* deficient neurons starts in the embryonic brain. Those brain areas that form synapses first, also show the first signs of degeneration (Verhage *et al.*, 2000). However, the viability of mutant neocortical neurons was unimpaired during plating and the first 3DIV. Mutant neurons displayed normal resting membrane potentials, K^+ and Na^+ currents at 8DIV and expressed functional neurotransmitter receptors. Furthermore, mutant hippocampal neurons remained viable for up to 19DIV when co-cultured with control hippocampal slices. This suggests that mutant neurons were healthy at the time of plating and shortly thereafter and thus that *munc18-1* expression is dispensable during early development and differentiation phases.

The lack of synaptic activity is an unlikely explanation for the degeneration of munc18-1 deficient neurons

We previously suggested that the degeneration in *munc18-1* null mutant brain could be the consequence of the lack of synaptic activity in these mutants. This suggestion was based on a large body of (indirect) evidence that synaptic activity regulates synapse stability (see Verhage *et al.*, 2000 and references herein) and, more specifically, the fact that *munc18-1* deficient primary sensory neurons were selectively spared from degeneration (Heeroma *et al.*, 2003). These neurons are different from the degenerating central neurons in the sense that they do not rely on synaptic transmission for their input, and are therefore expected to be the only neurons in the mutant that receive normal input. However, we show here that co-culturing *munc18-1* deficient neurons with control neurons did not prevent or even delay degeneration, although we cannot rule out that the synapses onto the *munc18-1* deficient

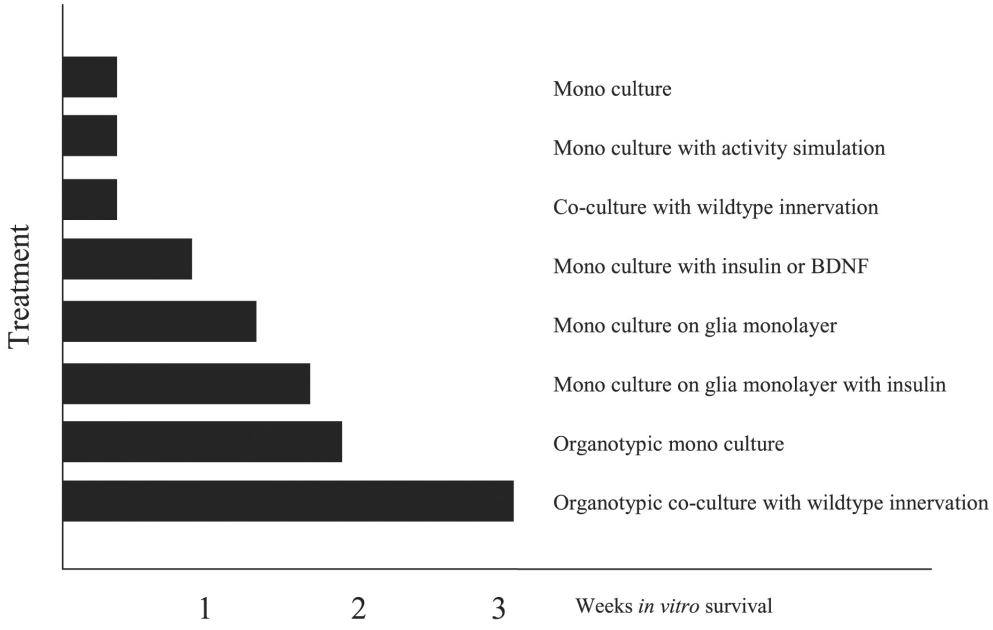

Figure 8: *Summary of the effects of various treatments on the survival of munc18-1 null mutant neurons.*

neurons may not have been functional. Still, specific deletion of *munc18-1* expression *in vivo* in cerebellar Purkinje cells, i.e., without affecting the synaptic input onto these cells, led to a specific degeneration of the Purkinje cells. Hence, providing synaptic input is not sufficient to prevent degeneration of *munc18-1* deficient neurons. Recently, a second genetic deletion was described, which also renders neurons completely silent: null mutation of *munc13-1* and *munc13-2* expression. In contrast to the *munc18-1* null mutant neurons, these *munc13* double null mutant neurons survived *in vivo* and *in vitro* without signs of degeneration (Varoqueaux *et al*., 2002). Therefore, we conclude that receiving synaptic input *per se* is not important for neuronal survival and the lack of synaptic activity is an unlikely explanation for the degeneration of *munc18-1* deficient neurons.
For motor neurons, there is clear evidence that synaptic activity at the terminals influences survival of the neurons (which have their soma in the spinal cord). Genetic (Banks *et al*., 2001) and pharmacological (Houenou *et al*., 1990) inhibition of transmission at the neuromuscular junction led to increased survival of motor neurons in the spinal cord. This suggests that neuronal output is an important factor in neuronal survival in this system, probably via a retrograde signal from the neuromuscular junction. In principle such a mechanism could explain the observed degeneration

in *munc18-1* null mutant neurons. However, it is unknown if such output dependency also applies to other systems than the neuromuscular junction and the lack of degeneration in *munc13* double null mutant neurons argues against this.

Trophic support delays, but does not prevent degeneration of munc18-1 deficient neurons

In addition to the lack of synaptic vesicle secretion, *munc18-1* null mutants also have severe impairments in the secretion from other secretory vesicles, for instance in chromaffin cells and the pituitary (Voets *et al.*, 2001; Korteweg *et al.*, in preparation). As such vesicles are known to contain neurotrophic substances (Berg *et al.*, 2000; Wang *et al.*, 2003), degeneration of *munc18-1* deficient neurons might be due to a lack of trophic support. The ability to properly secrete the content of such secretory vesicles could also explain the difference between the degenerating *munc18-1* deficient neurons and the viable *munc13-1/2* deficient neurons. It is unknown if *munc13-1/2* deficient neurons are also impaired in other secretory routes than synaptic transmission. Indeed, treatment of *munc18-1* deficient neurons with insulin or BDNF delayed degeneration. Culturing on glial cells allowed *munc18-1* deficient neurons to survive up to 10DIV or even 14DIV when insulin was added exogenously. In organotypic slices, *munc18-1* deficient neurons were viable up to 19DIV, provided they were co-cultured with control slices. Still, eventually all mutant neurons degenerated. Also *in vivo*, cell-specific deletion of *munc18-1*, resulted in specific degeneration of cerebellar Purkinje cells. As the rest of the brain is unaffected, also connecting and surrounding neurons, it is unlikely that the trophic environment for these Purkinje cells was fundamentally different from control cells. Together, this indicates that neither trophic support nor the combination of trophic support and synaptic activity is sufficient to explain the degeneration of *munc18-1* deficient neurons.

The observation that trophic support delayed degeneration of *munc18-1* deficient neurons may be explained in several ways. The trophic actions of substances like insulin and BDNF are multiple. Both substances reportedly stimulate neurite outgrowth, synapse formation, synapse activation and synaptic strength (Schulingkamp *et al.*, 2000; Vicario-Abejon *et al.*, 2002). Both substances are also known to exert survival-promoting effects, promoting survival under a-physiological conditions such as serum deprivation in culture (Yamada *et al.*, 2001; Hamabe *et al.*, 2003). Therefore, the observed delay in *munc18-1* deficient neuronal degeneration might be

explained by suppression of apoptosis as well as promoting connectivity or both.

An additional cellular function of munc18-1 is essential for prolonged neuronal survival

Taken together, the currently available data suggest that absence of release from synaptic vesicles by itself has little effect on neuronal maintenance. The loss of secretion from other secretory vesicles is probably a negative factor, but still an insufficient explanation for the observed degeneration after deletion of *munc18-1* expression. In this respect the *munc18-1* gene is different from most other genes involved in secretion. Deletion of their expression dramatically reduces secretion and synaptic transmission in a number of cases (e.g. Schoch *et al.*, 2001; Varoqueaux *et al.*, 2002 ; Washbourne *et al.*, 2002; Harrison *et al.*, 1994; Deitcher *et al.*, 1998; Zhao & Nonet, 2000; Misgeld *et al.*, 2002; Brandon *et al.*, 2003), but among these, very few show an equally strong phenotype as the *munc18-1* null mutation, or have completely silent synapses: only the *munc13-1/2* double null mutation in mice and probably *syntaxin* mutations in Drosophila (Verhage *et al.*, 2000; Saitoe *et al.*, 2001; Featherstone *et al.*, 2002; Varoqueaux *et al.*, 2002). Massive neuronal degeneration is only reported in *munc18-1* null mutant mice and *syntaxin* null mutant flies (Schulze & Bellen, 1996; Verhage *et al.*, 2000). The most likely explanation for this aspect of the phenotype is that munc18-1 has another, cell-intrinsic and essential function, distinct from its established role in secretion. One possibility is that munc18-1 is not only required to keep syntaxin-1 available for synaptic vesicle fusion, but also to prevent (other) neuronal syntaxins from promiscuous interactions in other cellular processes involving vesicular trafficking (Toonen & Verhage, 2003). Second, munc18-1 might be involved in the recognition of potential synaptic partners through MINT, CASK and neurexin (Biederer & Sudhof, 2000). Third, munc18-1 might be necessary for the proper targeting of ion channels and transporter proteins (Naren *et al.*, 1997; Khan *et al.*, 2001). Fourth, munc18-1 has recently been shown to bind to cytoskeletal proteins, suggesting a role for munc18-1 in cytoskeletal rearrangement (Bhaskar *et al.*, 2004). Finally, munc18-1 might exert another, yet unknown, function that is indispensable for long-term neuronal survival. Irrespective of what the additional role of munc18 proves to be, the results reported here demonstrate that the molecular mechanisms underlying the survival and maintenance of central nervous system neurons is less activity-dependent than those that have been inferred from studies of the neuromuscular junction or neurotrophin-

dependent than has previously been thought and instead involve novel cell intrinsic mechanisms.

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Abbreviations

BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CMV	Cytomegalovirus
dGBSS	dissection Gey's balanced salts solution
DIV	days in vitro
E18	embryonic day 18
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
HBSS	hanks balanced salts solution
LDCV	large dense core vesicle
MAP2	microtubule associated protein 2
PFA	Paraformaldehyde
TUNEL	Terminal dUTP nick end labeling

References

Banks, G.B., Chau, T.N., Bartlett, S.E. & Noakes, P.G. (2001) Promotion of motoneuron survival and branching in rapsyn-deficient mice. *J.Comp Neurol*., 429, 156-165.

Barres, B.A. (1991) New roles for glia. *J Neurosci*, 11, 3685-3694.

Barski, J.J., Dethleffsen, K. & Meyer, M. (2000) Cre recombinase expression in cerebellar Purkinje cells. *Genesis*, 28, 93-98.

Berg, E.A., Johnson, R.J., Leeman, S.E., Boyd, N., Kimerer, L. & Fine, R.E. (2000) Isolation and characterization of substance P-containing dense core vesicles from rabbit optic nerve and termini. *J Neurosci Res*, 62, 830-839.

Bhaskar, K., Shareef, M.M., Sharma, V.M., Shetty, A.P., Ramamohan, Y., Pant, H.C., Raju, T.R. & Shetty, K.T. (2004) Co-purification and localization of Munc18-1 (p67) and Cdk5 with neuronal cytoskeletal proteins. *Neurochem Int*, 44, 35-44.

Biederer, T. & Sudhof, T.C. (2000) Mints as adaptors. Direct binding to neurexins and recruitment of munc18. *J Biol Chem*, 275, 39803-39806.

Brandon, E.P., Lin, W., D'Amour, K.A., Pizzo, D.P., Dominguez, B., Sugiura, Y., Thode, S., Ko, C.P., Thal, L.J., Gage, F.H. & Lee, K.F. (2003) Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. *J Neurosci*, 23, 539-549.

Buffelli, M., Burgess, R.W., Feng, G., Lobe, C.G., Lichtman, J.W. & Sanes, J.R. (2003) Genetic

evidence that relative synaptic efficacy biases the outcome of synaptic competition. *Nature*, 424, 430-434.
Deitcher, D.L., Ueda, A., Stewart, B.A., Burgess, R.W., Kidokoro, Y. & Schwarz, T.L. (1998) Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin. *J Neurosci*, 18, 2028-2039.
Duxson, M.J. (1982) The effect of postsynaptic block on development of the neuromuscular junction in postnatal rats. *J Neurocytol*, 11, 395-408.
Featherstone, D.E., Rushton, E. & Broadie, K. (2002) Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. *Nat Neurosci*, 5, 141-146.
Gahwiler, B.H. (1984) Slice cultures of cerebellar, hippocampal and hypothalamic tissue. *Experientia*, 40, 235-243.
Gahwiler, B.H., Thompson, S. M., McKinney, R. A., Debanne, D. and Robertson, R. T. (1998) Organotypic slice cultures of neural tissue. In Goslin, K.a.B., G. (ed.) *Culturing nerve cells*. MIT press, Cambridge, pp. 461-498.
Hamabe, W., Fujita, R. & Ueda, H. (2003) Neuronal necrosis inhibition by insulin through protein kinase C-activation. *J Pharmacol Exp Ther*.
Harrison, S.D., Broadie, K., van de Goor, J. & Rubin, G.M. (1994) Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission. *Neuron*, 13, 555-566.
Heeroma, J.H., Plomp, J.J., Roubos, E.W. & Verhage, M. (2003) Development of the mouse neuromuscular junction in the absence of regulated secretion. *Neuroscience*, 120, 733-744.
Houenou, L.J., Pincon-Raymond, M., Garcia, L., Harris, A.J. & Rieger, F. (1990) Neuromuscular development following tetrodotoxin-induced inactivity in mouse embryos. *J.Neurobiol.*, 21, 1249-1261.
Jensen, F.E. & Harris, K.M. (1989) Preservation of neuronal ultrastructure in hippocampal slices using rapid microwave-enhanced fixation. *J Neurosci Methods*, 29, 217-230.
Katz, L.C. & Crowley, J.C. (2002) Development of cortical circuits: lessons from ocular dominance columns. *Nat Rev Neurosci*, 3, 34-42.
Katz, L.C. & Shatz, C.J. (1996) Synaptic activity and the construction of cortical circuits. *Science*, 274, 1133-1138.
Khan, A.H., Thurmond, D.C., Yang, C., Ceresa, B.P., Sigmund, C.D. & Pessin, J.E. (2001) Munc18c regulates insulin-stimulated glut4 translocation to the transverse tubules in skeletal muscle. *J Biol Chem*, 276, 4063-4069.
Lichtman, J.W. & Colman, H. (2000) Synapse elimination and indelible memory. *Neuron*, 25, 269-278.
Lindholm, D., Carroll, P., Tzimagiogis, G. & Thoenen, H. (1996) Autocrine-paracrine regulation of hippocampal neuron survival by IGF-1 and the neurotrophins BDNF, NT-3 and NT-4. *Eur J Neurosci*, 8, 1452-1460.
Misgeld, T., Burgess, R.W., Lewis, R.M., Cunningham, J.M., Lichtman, J.W. & Sanes, J.R. (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron*, 36, 635-648.
Naren, A.P., Nelson, D.J., Xie, W., Jovov, B., Pevsner, J., Bennett, M.K., Benos, D.J., Quick, M.W. & Kirk, K.L. (1997) Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature*, 390, 302-305.
Pancrazio, J.J., Keefer, E.W., Ma, W., Stenger, D.A. & Gross, G.W. (2001) Neurophysiologic effects of chemical agent hydrolysis products on cortical neurons in vitro. *Neurotoxicology*, 22, 393-400.
Roelandse, M., Welman, A., Wagner, U., Hagmann, J. & Matus, A. (2003) Focal motility determines the geometry of dendritic spines. *Neuroscience*, 121, 39-49.
Saitoe, M., Schwarz, T.L., Umbach, J.A., Gundersen, C.B. & Kidokoro, Y. (2001) Absence of junctional glutamate receptor clusters in Drosophila mutants lacking spontaneous transmitter release. *Science*, 293, 514-517.
Sanes, J.R. & Lichtman, J.W. (1999) Development of the vertebrate neuromuscular junction. *Annu.Rev.Neurosci.*, 22, 389-442.
Schulingkamp, R.J., Pagano, T.C., Hung, D. & Raffa, R.B. (2000) Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci Biobehav Rev*, 24, 855-872.

Schulze, K.L. & Bellen, H.J. (1996) Drosophila syntaxin is required for cell viability and may function in membrane formation and stabilization. *Genetics*, 144, 1713-1724.
Toonen, R.F. & Verhage, M. (2003) Vesicle trafficking: pleasure and pain from SM genes. *Trends Cell Biol*, 13, 177-186.
Usiak, M.F. & Landmesser, L.T. (1999) Neuromuscular activity blockade induced by muscimol and d-tubocurarine differentially affects the survival of embryonic chick motoneurons. *J Neurosci*, 19, 7925-7939.
Varoqueaux, F., Sigler, A., Rhee, J.S., Brose, N., Enk, C., Reim, K. & Rosenmund, C. (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc Natl Acad Sci U S A*, 99, 9037-9042.
Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J. & Sudhof, T.C. (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science*, 287, 864-869.
Vicario-Abejon, C., Owens, D., McKay, R. & Segal, M. (2002) Role of neurotrophins in central synapse formation and stabilization. *Nat Rev Neurosci*, 3, 965-974.
Voets, T., Toonen, R.F., Brian, E.C., de Wit, H., Moser, T., Rettig, J., Sudhof, T.C., Neher, E. & Verhage, M. (2001b) Munc18-1 promotes large dense-core vesicle docking. *Neuron*, 31, 581-591.
Wang, X., Butowt, R. & von Bartheld, C.S. (2003) Presynaptic neurotrophin-3 increases the number of tectal synapses, vesicle density, and number of docked vesicles in chick embryos. *J Comp Neurol*, 458, 62-77.
Weisshaar, B., Doll, T. & Matus, A. (1992) Reorganisation of the microtubular cytoskeleton by embryonic microtubule-associated-protein 2 (MAP2c). *Development*, 116, 1151-1161.
Yamada, M., Tanabe, K., Wada, K., Shimoke, K., Ishikawa, Y., Ikeuchi, T., Koizumi, S. & Hatanaka, H. (2001) Differences in survival-promoting effects and intracellular signaling properties of BDNF and IGF-1 in cultured cerebral cortical neurons. *J Neurochem*, 78, 940-951.
Zhao, H. & Nonet, M.L. (2000) A retrograde signal is involved in activity-dependent remodeling at a C. elegans neuromuscular junction. *Development*, 127, 1253-1266.

Chapter 4

Altered neurite outgrowth and branching in munc18-1 deficient neurons with inhibited regulated secretion

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Abstract

Genetic and pharmacological studies of neuronal SNARE proteins have raised controversy on whether synaptic vesicle fusion contributes membrane to outgrowing neurites. Here we aimed to clarify this issue by analysing neurite outgrowth and branching of *munc18-1* deficient neurons which are incapable of neurotransmitter secretion. We found that neurite outgrowth is normal during the first 3 days *in vitro* (DIV) and *munc18-1* deficient neurons form a normal number of contacts. However, dynamic growth cone analysis revealed that after 3DIV, net neurite outgrowth is slower, while more and longer growth cone filopodia are formed. After 7DIV, *munc18-1* deficient dendrites are shorter and have fewer branches and contact points. These results suggest that while initial neurite outgrowth and network formation is independent of *munc18-1*/synaptic activity, later stages of dendritic tree formation are regulated by *munc18-1*, possibly through its role in synaptic activity.

Introduction

SNAREs are important catalysts of regulated secretion. For instance, genetic deletion of *snap-25* or *synaptobrevin-2*, dramatically reduces evoked neurotransmitter release (Schoch et al., 2001; Washbourne et al., 2002). During the process of regulated secretion, not only a cargo is delivered to the extracellular environment, but also the vesicular and plasmamembrane of the cell are fused. Therefore, regulated secretion could play a role in processes requiring membrane expansion, such as neurite outgrowth. In line with this, expanding growth cones were shown to secrete neurotransmitter (Poo et al., 1985; Young and Poo, 1983). However, the role of, SNARE-mediated, regulated secretion in neurite outgrowth has been under debate; gross brain morphology of mouse embryos deficient for *snap-25* or *synaptobrevin-2*, is normal, suggesting these SNAREs are dispensable for neurite outgrowth (Schoch et al., 2001; Washbourne et al., 2002).

Still, exposing neurons to antisense oligonucleotides against SNAP-25 or cleaving SNAP-25 with botulinum neurotoxin A, greatly reduces axonal outgrowth, suggesting that indeed SNARE-mediated vesicular fusion is required for neurite extension (Osen-Sand et al., 1993; Osen-Sand et al., 1996). This discrepancy might be explained by the presence of several functional homologues of SNAP-25 that can compensate for the genetic loss of *snap-25* but are sensitive to *snap-25* antisense and cleavage with BoNT-A. Indeed, for synaptobrevin, a redundant protein, TI-VAMP, has been identified that is involved in neurite outgrowth (Martinez-Arca et al., 2001).

When SNARE proteins are involved in neurite outgrowth, the proteins that regulate vesicular secretion may be involved as well. One of the most potent regulators of neuronal secretion is *munc18-1*. Genetic deletion of *munc18-1* in mice completely abolishes spontaneous and evoked neurotransmitter release in neurons, and greatly reduces large dense core vesicle secretion in chromaffin cells (Verhage et al., 2000; Voets et al., 2001). Vice versa, overexpression of *munc18-1* in micro island cultures of cortical neurons increases the frequency of spontaneous fusion events and the recovery rate of the readily releasable pool after high frequency stimulation and sucrose shock (Toonen et al., in preparation). Together, this suggests that *munc18-1* is positively correlated to the number of synaptic vesicles that can fuse to the presynaptic membrane, and thus theoretically, to the amount of membrane that can be added to a growing neurite/the neuritogenic potential of a neuron.

Here we tested the correlation between *munc18-1* and neurite outgrowth by comparing control and *munc18-1* null mutant neurons in culture. We found that neurite outgrowth in mutant neurons was normal during the first 3 days *in vitro* (DIV). Dynamic growth cone analysis after 3DIV, however, indicated that net neurite outgrowth was reduced in mutant neurons while filopodial outgrowth was increased. After 7DIV, mutant neurons had significantly shorter neurites with less branching points, suggesting that *munc18-1* indeed is positively correlated with neurite outgrowth.

Materials and Methods

Laboratory animals

Munc18-1 deficient mice were generated as described before (Verhage et al., 2000). Mouse embryos were obtained by caesarian section of pregnant females from timed heterozygous matings. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

Cortical cultures

Cortices were dissected from embryonic day 18 mice and collected in Hanks Buffered Salts Solution (HBSS)(Sigma, St. Louis, USA), buffered with 7 mM Hepes. After removal of the meninges, the cortices were minced and incubated for 20 minutes in trypsinated HBSS at 37 °C. After washing the neurons were triturated with fire polished Pasteur pipettes, counted with a hemacytometer and plated in Neurobasal medium (Invitrogen, Carlsbad, USA) supplemented with 2% B-27 (Invitrogen), 1.8% Hepes, 1% glutamax (Invitrogen), 1% Pen/Strep (Invitrogen) and 0.2% β-mercaptoethanol. Low density cultures were plated on poly-L-lysin coated glass coverslips at 25,000/cm^2. For island cultures, neurons were plated at 6,000/cm^2 on islands of rat glia. Glial islands were obtained by spraying a 0.25 mg/ml rat tail collagen solution (BD Biosciences, Bedford, USA) on glass coverslips. After drying and UV sterilization glial cells were plated at 600/cm^2. For electrophysiological experiments, cultures were treated with 100 nM insulin to promote mutant neuron viability up to 9DIV. 50% of the medium was refreshed every week.

Organotypic cultures

Organotypic slice cultures from E18 hippocampi were prepared as follows. Mouse embryos were obtained by caesarean section of pregnant females

from timed heterozygous mating. GFP-expressing animals were identified by direct inspection using a Leica MZ12 dissection microscope fitted with fluorescence optics. Brains were dissected in ice-cold dissection Gey's balanced salt solution (dGBSS, consisting of GBSS (Invitrogen) with 0.65 g glucose and 200µM kynurenate, pH 7.4) and cut into 400 µm thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK). After separating individual slices, hippocampal region was dissected out and separated. These hippocampal slices were kept at 4˚C in dGBSS for 45 min to recuperate. All subsequent procedures were identical to those described for organotypic slice cultures from P8 mice (Gahwiler et al., 1998).For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous superfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) saturated with 95% O2 /5% CO2 using a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany)

Electrophysiological Recordings

Whole cell recordings were performed on cultured neurons on DIV 8 and 9. The patch pipette solution contained the following (in mM): 125 K^+-gluconic acid, 10 NaCl, 4.6 $MgCl_2$, 4 K_2-ATP, 15 Creatine Phosphate, 1 EGTA and 20 U/ml Phospocreatine Kinase (pH 7.30). The external medium used contained the following components (in mM): 140 NaCl, 2.4 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 10 Glucose (pH 7.30). For sucrose application 500 mOsm sucrose was added to normal external medium. Application of 500 mOsm sucrose was performed with an application pipette for a duration of 4 seconds (with picospritzer). α-latrotoxin was bath-applied at a final concentration of 1 nM. Axopatch 200A was used for whole-cell recordings. Signal was acquired using Digidata 1322A and Clampex 8.1. Clampfit 8.0 was used for offline analysis.

Immunocytochemical procedures

Cultures were fixed by adding 4% paraformaldehyde (PFA) to the medium in a 1/1 ratio. After 15 minutes the PFA/medium mixture was exchanged for 4% PFA, for 15 minutes. After washing with PBS the cells were permeated with 0.1% Triton X-100 for 5 minutes. After washing with PBS the cells were ready for processing or storage. Before staining the cells were incubated in 4% fetal

calf serum for 20 minutes to block a-specific reactions. After washing with PBS the cells were incubated in a mixture of antibodies and 0.1% Triton X-100 for 1 hour at room temperature of overnight at 4 °C. The antibodies used were: mouse anti MAP2, 1/200 (Boehringer Mannheim, Germany) and rabbit anti synapsin1, 1/1000 (E028). After washing 3 times 5 minutes with PBS, the cells were incubated for 1 hour at room temperature in goat anti rabbit Alexa488 and/or goat anti mouse Alexa546 (Molecular Probes, Oregon, USA). After washing in 3 times 5 minutes in PBS the coverslips were mounted on microscopic slides with Dabco-Mowiol and analysed with a Zeiss 510 Meta Confocal microscope (Heidelberg, Germany).

Data analysis

For the analysis of neurite outgrowth and network formation in 7DIV neurons a number of parameters were analysed; the number of primary dendrites and the total number of dendritic branching points were counted and the length of the longest primary dendrite was measured. The average and standard deviation of these parameters were compared (for control and *munc18-1* deficient neurons) with 2-tailed paired t-tests.

Results

Electrophysiological properties of munc18-1 deficient neurons

A previous study pointed out that acute cortical slices of munc18-1 deficient mice are unable to secrete GABA in response to direct electrical stimulation (Verhage et al., 2000). To further study the secretion phenotype of *munc18-1* null neurons we used whole-cell patch clamp recordings of control and *munc18-1* mutant neurons in low-density cultures of 9 days *in vitro* (DIV). One method to induce massive release of synaptic vesicles is the application of a high concentration of sucrose (500 mOsm) to the external medium, which is sufficient to trigger fusion of the entire readily releasable pool (Rosenmund and Stevens, 1996). Application of high sucrose to control neurons induced a large inward current (Fig. 1A). This inward current could be blocked by GABA-A receptor blocker bicucculine (data not shown), suggesting the current was evoked by GABA release. Sucrose application did not induce any current in *munc18-1* deficient neurons, suggesting that sucrose does not elicit transmitter release in mutant neurons (Fig. 1B). The most potent induction of synaptic vesicle release that is currently known is by application of α-latrotoxin, a toxin derived from the black widow spider

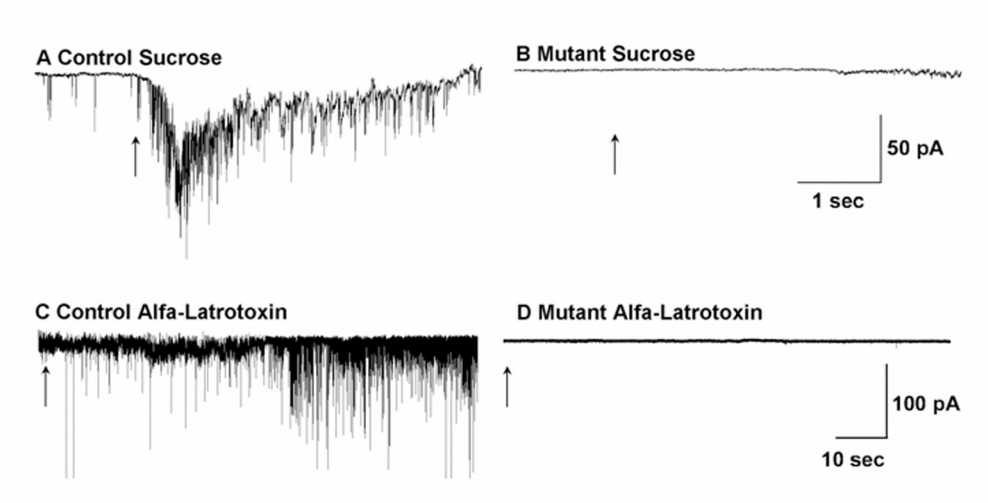

Figure 1: *Munc18-1 deficient neurons are unable to release synaptic vesicles. Control and mutant neurons were recorded in low-density cultures (DIV 8). Application of high sucrose solution releases the ready releasable pool (Rosenmund and Stevens, 1996). Application of sucrose (indicated by black arrow in A and B) induced release in control neurons (n=3). However, no release is induced by sucrose in mutant neurons (n=2). Application of α-latrotoxin to cultured control neurons (DIV 8) induced massive release (C, n=4)). However, mutant neurons do not show any release after α-latrotoxin application (D, n=3).*

(Schiavo et al., 2000). Recordings from control neurons show a massive increase in synaptic input following α-latrotoxin application (Fig. 1C). *Munc18-1* deficient neurons, however, show no postsynaptic response to α-latrotoxin application (Fig. 1D). Since *munc18-1* mutant neurons do have functional receptors (GABA, Acetylcholine, (Verhage et al., 2000); Glutamate, Heeroma *et al*., 2004), this suggests that the lack of postsynaptic responses in mutant neurons is due to the disability of synaptic vesicles to fuse with the presynaptic membrane.

Neurite outgrowth, network formation and dendritic branching

The data above support our previous findings that regulated secretion, and in particular, neurotransmitter secretion, is severely disturbed in *munc18-1* deficient neurons (Verhage et al., 2000). We have previously shown that basic neurite outgrowth and synapse formation occurs in the absence of *munc18-1*/regulated secretion but also found evidence that the later stages of neurite outgrowth and synapse formation are hampered (Heeroma et al., 2003; Verhage et al., 2000). Here, the process of neurite outgrowth and network/synapse formation in the absence of *munc18-1* was studied in more detail.

Control and *munc18-1* deficient neurons were plated on poly-L-lysine coated coverslips and dendrite length was monitored at 0, 3 and 7DIV. At these time-points every dendrite, identified as any detectable protrusion positive for microtubule associated protein-2 (MAP2) staining, was measured relative to the diameter (D) of its soma and categorised into bins (0-1 D, 1-2 D or >2 D), enabling quick and robust analysis of many dendrites.

After 7 hours *in vitro* (0DIV), dendrite length was measured as a calibration/ starting point of later counts. For both control and mutant cultures, 96 randomly chosen fields of 0.137 mm^2 were analysed. As expected, the vast majority of control and mutant neurons had not yet grown dendrites (longer than 1 D, fig. 2). Relatively few neurons already had dendrites longer than 1 D (control: 5.6% +/- 0.06, mutant: 7.1% +/-0.08). A chi-square test revealed that the average dendrite on mutant neurons was slightly, but significantly longer ($p<0.001$).

After 3DIV, 24 randomly chosen fields of control and mutant cultures were

Neurite outgrowth	0DIV Control	0DIV Mutant	3DIV Control	3DIV Mutant	7DIV Control	7DIV Mutant
0-1D	1378	968	131	221	70	17
1D-2D	81	75	271	245	146	11
>2D	0	0	189	207	432	16

Figure 2: *Mutant neurons have normal neurite length at 3DIV but shorter neurites at 7DIV. Neurite length of control and mutant neurons was measured relative to cell body diameter (D). At 0DIV, 3DIV and 7DIV, 96, 24 and 48 randomly chosen field of 0.137 mm^2 were analysed. To aid comparison, average neurite numbers per 24 fields are depicted. A chi square test revealed that control neurites were significantly longer at 7DIV ($p<0.001$).*

analysed. In general, neurons in both groups had grown more primary dendrites (not shown) and those dendrites were longer; 22% of dendrite of control neurons were still shorter than 1 D, 46% were between 1 and 2 D and 32% was longer than 2 D (absolute values are listed under fig. 2). In mutant neurons, the length distribution was comparable with 33, 36 and 31% in the three categories respectively.

After 7DIV, 48 randomly chosen fields of control and mutant cultures were analysed. In control neurons, dendrite length had increased again, with 11% of dendrite still shorter than 1D, 22% between 1 and 2 D and 67% longer than 2 D (absolute values are listed under fig. 2). The average length of dendrites from *munc18-1* deficient neurons, however, had not dramatically changed with 40% still shorter than 1 D, 24% between 1 and 2 D and 36% longer than 2 D. Comparison with a chi-square test revealed that mutant

dendrites were highly significantly ($p<0.001$) shorter than control neurons. It must be noted, however, that by 7DIV most *munc18-1* deficient neurons had already degenerated (discussed in Heeroma *et al.,* 2004). These data suggest that dendrite length of control and *munc18-1* deficient neurons were not different during the first 3DIV. Between 3 and 7DIV, however, most mutant neuron were lost. The neurons that remained in culture until 7DIV had significantly shorter dendrites.

We also analysed the capacity of control and *munc18-1* deficient neurons to form networks. Since only few synapses are formed before 7DIV, the number of contacts with other neurons was counted (defined as physical overlap of dendrites). At 0DIV, the number of contact points of each neuron was noted (as 0, 1, 2 or >2). As both control and mutant neurons had only extended a few short dendrites by this time (see above), the number of contact points was concomitantly low (Fig. 3). At 3DIV, the number of contact points between neurons was nearly identical for both groups; 38% of control neurons still had no contact with other neurons, 32% had 1 contact, 22% had 2 contacts and 8% had more contacts. Of *munc18-1* deficient neurons, 38% had no contacts, 32% had 1 contact, 23% had 2 contacts and 7% had more than 2 contacts (the absolute values are listed under fig. 3). At 7DIV,

Contacts	0DIV		3DIV		7DIV	
	Control	Mutant	Control	Mutant	Control	Mutant
0	1049	783	193	199	17	13
1	139	72	165	171	71	3
2	9	5	115	124	112	0
>2	0	0	42	0	170	0

Figure 3: *Mutant neurons show normal network formation at 3DIV but have fewer contacts with other neurons at 7DIV. Network formation, defined as physical overlap of neurites was analysed for control and mutant neurons. At 0DIV, 3DIV and 7DIV, 96, 24 and 48 randomly chosen field of 0.137 mm^2 were analysed. To aid comparison, average neurite numbers per 24 fields are depicted.*

the begin of the synaptogenic period of cultured neurons, the number of contact points of control neurons had sharply increased; 5% still had no contact with other neurons, 19% had 1 contact, 30% had 2 contacts and 46% had more than 2 contacts. At this time point most *munc18-1* deficient neurons had degenerated (Heeroma *et al*., 2004). Of the remaining neurons the majority of approximately 80% had no contacts with other neurons and approximately 20% had one contact. These data suggest that the network forming capacity of *munc18-1* deficient neurons is normal up to 3DIV, but

is sharply declined at the onset of the synaptogenic period, which coincides with massive degeneration of *munc18-1* deficient neurons.

When *munc18-1* deficient neurons are grown on a glial feeder layer, these neurons can be maintained in culture up to 10DIV (Heeroma *et al.*, 2004). After 7DIV, the amount of primary dendrites, the length of the longest primary dendrite and the total number of branching points were measured for control (n=6) and munc18-1 deficient neurons (n=10). The number of primary dendrites in *munc18-1* deficient neurons (4,2 +/- 1,1) was not different from control neurons (4,5 +/- 0,5). The length of the longest dendrite, however, was significantly shorter (p=0.01) for *munc18-1* deficient neurons (93,6 +/- 44,4 μm) than for control neurons (153,0 +/- 18,9 μm). The number of branching points was highly significantly less ($p=10^{-7}$) for *munc18-1* deficient neurons (3,2 +/- 2,4) than for control neurons (14,2 +/- 2,0). Representative examples of control and mutant neurons are depicted in figure 4. Together, these results suggest that the presence of *munc18-1* is not of influence on the number of primary dendrites that are formed, their initial outgrowth or the formation of initial contacts between neurons.

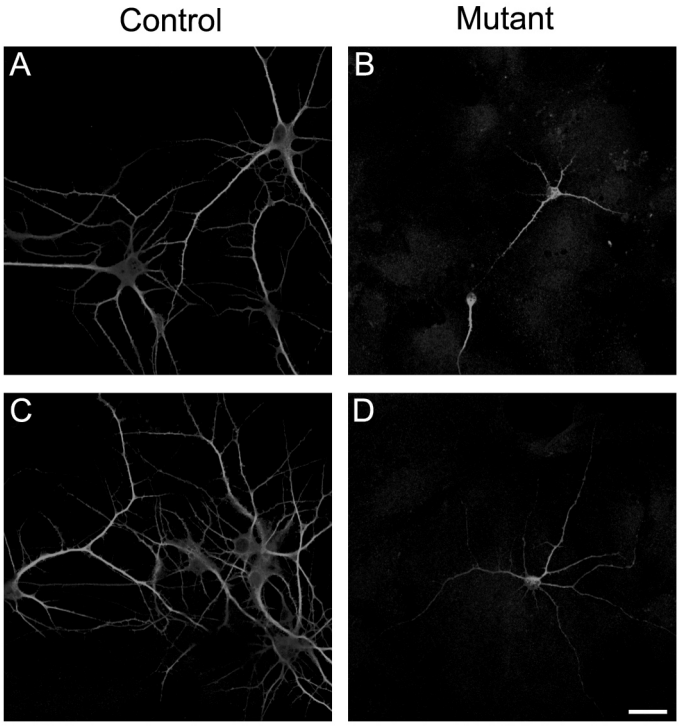

Figure 4: *Neurite outgrowth of control and munc18-1 deficient neurons after 7DIV. Control (A, C) and mutant (B, D) neurons were cultured on microglial islands and stained for dendrite marker MAP-2. Mutant neurons have shorter dendrites with fewer branching points. Scale bar is 25 μm.*

However, after 7DIV, the lack of *munc18-1* results in shorter dendrites and fewer branching points.

Growth cone dynamics

The data above suggest that *munc18-1* deficient neurons have normal neurite outgrowth and network forming characteristics during the first 3DIV, but later on have reduced neurite outgrowth and branching. However, these assumptions are based on static data and thus do not reveal the actual effect of *munc18-1* on neurite outgrowth. Therefore, we analysed the dynamic behaviour of control and mutant growth cones in 3DIV organotypic hippocampal cultures.

Contrary to the experiments above where the entire neuronal morphology was analyzed, here only growth cones were monitored enabling analysis in the more natural environment of organotypic slice cultures. To optimize growth cone visualization *munc18-1* deficient and control mice were crossed with mice transgenically expressing plasmamembrane marker *gfptkras* (Roelandse et al., 2003). Hippocampal slices were cultured in a plasma clot (Gahwiler et al., 1991) to promote adhesion and support outgrowth of neurites from the slice (Fig. 5A). After 3DIV several neurites had protruded into the surrounding plasma clot enabling analysis of their morphology. Using a spinning disk confocal microscope growth cones could be tracked through time with minimal risk of phototoxicity. First the average outgrowth rate from the hippocampal slice into the surrounding plasma clot was calculated. The position of 16 control and 18 mutant growth cones was determined each minute for one hour and compared with compared with the initial X and Y coordinates (Fig. 5B). The average control growth cone grew 5.8 +/- 0.7 (SEM) µm/min in X direction and 6.3 +/- 0.6 µm/min in Y direction. The average outgrowth of mutant growth cones was highly significantly less ($p(X)=10^{-5}$, $P(Y)=10^{-4}$) with 0.9 +/- 0.9 (X) and 2.4 +/- 0.8 (Y) µm/min outgrowth respectively (Fig. 5C). Second the number of newly appearing filopodia was determined on 21 control and mutant growth cones. Control growth cones on average formed 23.3 +/- 1 new filopodia per hour. Mutant growth cones formed significantly more ($p=10^{-11}$) filopodia with 48.8 +/- 2.2 per hour (Fig. 6). Third, the average length of filopodia was measured. In controls, filopodia were on average 2.9 +/- 0.1 µm, which was significantly shorter ($p=10^{-6}$) than mutant filopodia that on average were 3.4 +/- 1.0 µm (Fig. 6). These data suggest that mutant neurites of 3DIV show less net elongation while forming more and longer filopodia.

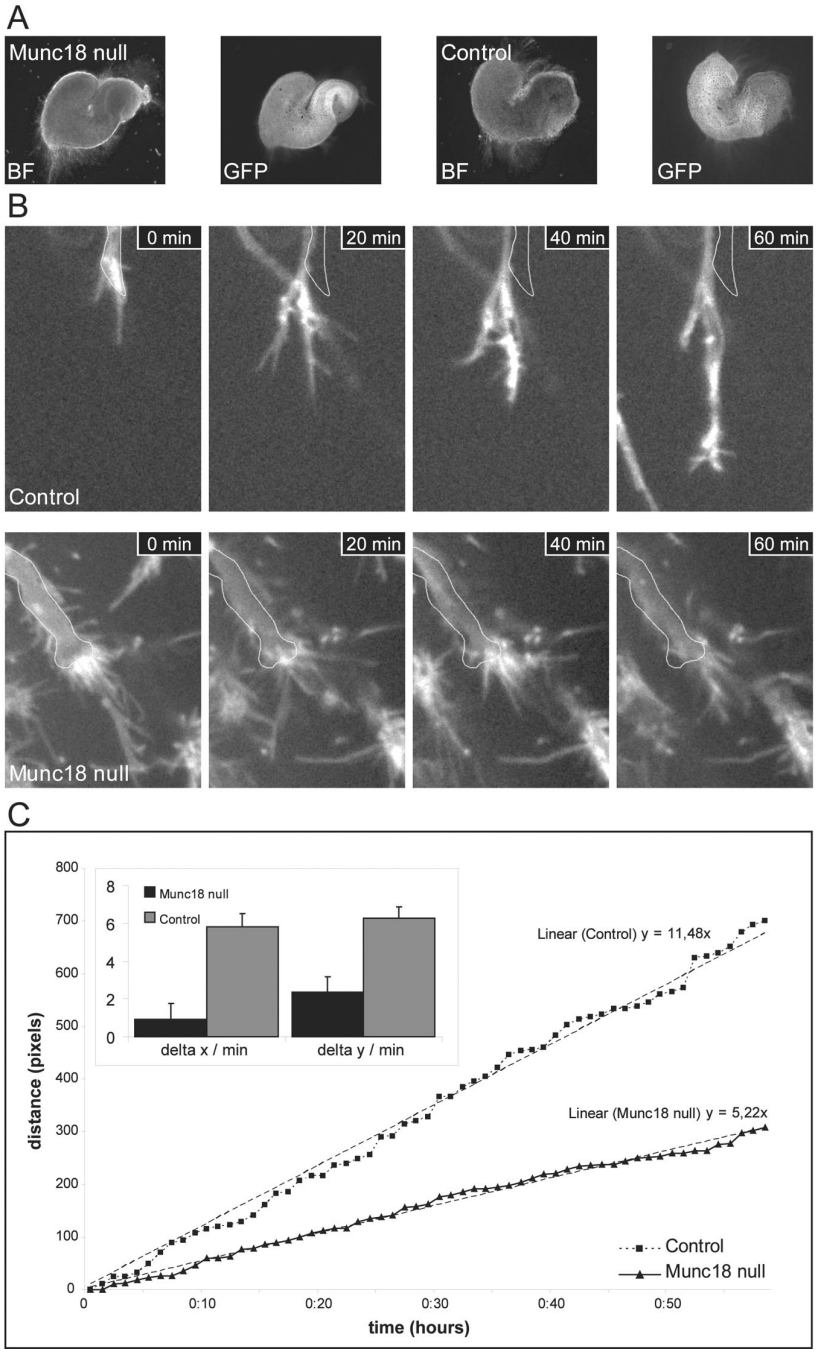

Figure 5: *Growth cone dynamics in mutant and control organotypic cultures. A: Brightfield (BF) and fluorescent (GFP) pictures show mutant and control hippocampal organotypic cultures of 3DIV. B: Tracking growthcones for 60 minutes shows extension of control neurites and virtually no net movement of mutant growthcones. C: statistical analysis shows that net neurite extension in mutant neurons is only 15-30% of control values.*

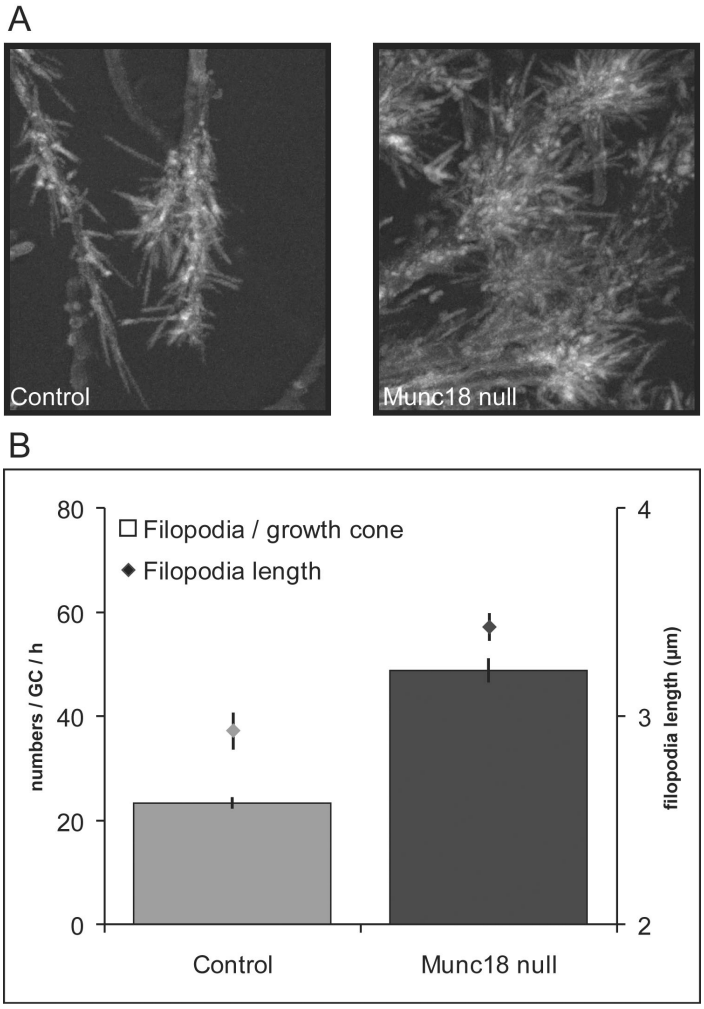

Figure 6: *Filopodial dynamics of control and mutant hippocampal organotypic cultures. A: Munc18-1 deficient growthcones are much more studded with filopodia than controls. B: The number of newly appearing filopodia on mutant growthcones is doubled compared to controls. The average length of filopodia is significantly greater in mutant cultures.*

Discussion

In the present study, we analysed neurite outgrowth, branching and network forming properties of *munc18-1* deficient neurons that lack neurotransmitter secretion. Up to 3DIV, neurite outgrowth and network formation of mutant neurons was identical to control neurons. Dynamic analysis of mutant neurons indicated that at 3DIV, net growth cone protrusion was reduced, while the formation and length of filopodia was increased. After 7DIV, mutant neurons had shorter dendrites, less branching points and fewer contacts than control neurons.

We have previously shown that *munc18-1* deficient neurons in an acute slice preparation do not release GABA after direct electrical stimulation (Verhage et al., 2000). Here, we applied sucrose and α-latrotoxin, the most potent stimulators of neurotransmitter secretion known at this point, to cultured cortical neurons. While control neurons showed a vigorous postsynaptic response after application of both substances, no postsynaptic response was measured in mutant neurons. Hence, we conclude that *munc18-1* deficient neurons are absolutely incapable of neurotransmitter secretion. This is, to date, the most dramatic secretion phenotype that has been described in mammalian neurons, rendering *munc18-1* as a key regulator of neurotransmitter secretion.

Our finding that dendrite outgrowth and network formation are normal in *munc18-1* deficient neurons during the first 3DIV suggests that *munc18-1*, and thus neurotransmitter secretion, is dispensable for membrane addition to outgrowing neurites in this period. This contradicts earlier suggestions, based on experiments with botulinum toxin which cleaves SNARE proteins (Williamson and Neale, 1998; Osen-Sand et al., 1993). A possible explanation for this discrepancy is that the SNARE proteins responsible for the regulated secretion of neurotransmitter are also involved in other membrane fusion processes such as the addition of membrane to growing neurites. *Munc18-1*, on the other hand appears to be a specific regulator of neurotransmitter secretion and is does not influence dendrite outgrowth during the first 3DIV.

In later developmental stages, however, *munc18-1* does appear to be involved in neurite elongation; net elongation of 3DIV mutant neuronal growth cones was slower and 7DIV *munc18-1* deficient neurons had shorter

dendrites with fewer branches and formed less contacts with other neurons. This is in agreement with a study in cultured hippocampal neurons where overexpression of *munc18-1* led to more branching and a larger neuritic tree (Steiner et al., 2002). It has previously been proposed that neurotransmitter mediated activity is a positive regulator of neurite outgrowth and branching (Lipton and Kater, 1989; Brewer and Cotman, 1989). Therefore, the effect of *munc18-1* on neurite branching and outgrowth is possibly more related to its role in neurotransmitter secretion/neuronal activity than to its role in membrane fusion. In the initial phases of neuronal differentiation and neurite outgrowth, synapses and thus synaptic activity are scarce and presumably of little influence. For this reason, early neurite outgrowth is more likely to be directed by innate genetic programs rather than synaptic activity.This might therefore explain the discrepancy between the negative effect of *munc18-1* deficiency on dendrite outgrowth and branching in 7DIV neurons and the lack of this effect in 3DIV neurons and in the developing brain (Verhage et al., 2000).
Apart from our experimental data there is also a theoretical constraint against synaptic vesicles subserving growth cone expansion. Given that an axon of 1μm diameter can grow up to 40 μm per hour this would take 100.000 synaptic vesicles of 20 nm diameter (per hour) when these vesicles were the sole source of membrane at the growth cone. Fusion of 100.000 vesicles per hour, or ~25 per second would be pushing the limits of the fastest mature synapses and are therefore extremely unlikely to occur at a growth cone that lacks fully differentiated synaptic vesicle fusion machinery. We therefore propose that if *munc18-1* contributes to neurite outgrowth by regulating the addition of vesicles to the plasma membrane, this contribution is only marginal and only applies to the terminal stages of outgrowth. The mechanism by which *munc18-1* mediated neuronal activity accounts for the remainder of neurite outgrowth remains elusive however.

We found that *munc18-1* deficient neurons formed more new filopodia on their growth cones than control neurons, and that these filopodia were longer. Possibly, filopodia are formed by mechanical stretching of the present plasmamembrane. Alternatively, additional membrane for these protrusions might be delivered in a munc18-1 independent fashion. The higher incidence and larger size of growth cone filopodia might even be a combined consequence of constitutive, munc18-1 independent, plasmamembrane delivery to neurites and reduced neurite outgrowth which is correlated with the absence of munc18-1. In addition, the longer net length of filopodia suggests that mutant neurons have more active filopodia

than control neurons and thus that the lack of neurotransmitter secretion has a positive effect on filopodial movement. This agrees with a study of presynaptic filopodia of hippocampal mossy fiber terminals where high concentrations of kainic acid slowed filopodial movement down (Tashiro et al., 2003). Postsynaptically, similar principles apply since glutamate receptor activation was found to inhibit dendritic spine movement (Fischer et al., 2000).

Data from a recent study by Niell *et al.* suggest that dendritic filopodia probe their environment for suitable synaptic partners and upon synapse stabilization serve as starting points for further dendrite outgrowth (Niell et al., 2004). Synaptic activity/neurotransmitter secretion is a likely candidate to be involved in synapse stabilization (Ackermann and Matus, 2003; Wong and Wong, 2000). Following this line of thought, the complete lack of neurotransmitter secretion in *munc18-1* mutant neurons would impair synapse stabilization and thus subsequent extension of dendrites. The rudimental neurite outgrowth and synapse formation that is observed in *munc18-1* deficient neurons is probably part of an innate genetic program that serves as a starting point for further, activity based, *munc18-1*-dependent, formation of dendrites and synapses.

In this study we used *munc18-1* deficient neurons to investigate the role of synaptic vesicle secretion in neurite outgrowth. We concluded that if synaptic vesicle secretion contributes to neurite expansion, this effect is marginal, restricted to late stages of outgrowth and probably mediated by neurotransmitters/synaptic activity rather than synaptic vesicles *per se*. In chapter 3, however, we speculated that *munc18-1* might have additional cellular functions that are distinct from its role in regulated secretion. Indeed, recent studies have shown interactions between munc18-1 and cytoskeletal elements and proteins involved in synapse stabilization (Bhaskar et al., 2004; Biederer and Sudhof, 2000). Perhaps, (mutant forms of) munc18-1 can be used to resolve the respective roles of synaptic activity, cytoskeletal proteins etc. in neuronal network formation.

Reference List

Ackermann,M. and Matus,A. (2003). Activity-induced targeting of profilin and stabilization of dendritic spine morphology. Nat. Neurosci. 6, 1194-1200.

Brewer,G.J. and Cotman,C.W. (1989). NMDA receptor regulation of neuronal morphology in cultured hippocampal neurons. Neurosci Lett 99, 268-73.

Fischer,M., Kaech,S., Wagner,U., Brinkhaus,H., and Matus,A. (2000). Glutamate receptors

regulate actin-based plasticity in dendritic spines. Nat Neurosci 3, 887-94.
Gahwiler,B.H., Rietschin,L., and Gerber,U. (1991). Cholinergic fiber growth in co-cultures of CNS tissue. J Physiol (Paris) 85, 154-157.
Heeroma,J.H., Plomp,J.J., Roubos,E.W., and Verhage,M. (2003). Development of the mouse neuromuscular junction in the absence of regulated secretion. Neuroscience 120, 733-44.
Lipton,S.A. and Kater,S.B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. Trends Neurosci 12, 265-70.
Martinez-Arca,S., Coco,S., Mainguy,G., Schenk,U., Alberts,P., Bouille,P., Mezzina,M., Prochiantz,A., Matteoli,M., Louvard,D., and Galli,T. (2001). A common exocytotic mechanism mediates axonal and dendritic outgrowth. J. Neurosci. 21, 3830-3838.
Niell,C.M., Meyer,M.P., and Smith,S.J. (2004). In vivo imaging of synapse formation on a growing dendritic arbor. Nat. Neurosci. 7, 254-260.
Osen-Sand,A., Catsicas,M., Staple,J.K., Jones,K.A., Ayala,G., Knowles,J., Grenningloh,G., and Catsicas,S. (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature 364, 445-448.
Osen-Sand,A., Staple,J.K., Naldi,E., Schiavo,G., Rossetto,O., Petitpierre,S., Malgaroli,A., Montecucco,C., and Catsicas,S. (1996). Common and distinct fusion proteins in axonal growth and transmitter release. J. Comp Neurol. 367, 222-234.
Poo,M.M., Sun,Y.A., and Young,S.H. (1985). Three types of transmitter release from embryonic neurons. J Physiol 80, 283-9.
Roelandse,M., Welman,A., Wagner,U., Hagmann,J., and Matus,A. (2003). Focal motility determines the geometry of dendritic spines. Neuroscience 121, 39-49.
Rosenmund,C. and Stevens,C.F. (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16, 1197-207.
Schiavo,G., Matteoli,M., and Montecucco,C. (2000). Neurotoxins affecting neuroexocytosis. Physiol Rev 80, 717-66.
Schoch,S., Deak,F., Konigstorfer,A., Mozhayeva,M., Sara,Y., Sudhof,T.C., and Kavalali,E.T. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294, 1117-22.
Steiner,P., Sarria,J.C., Huni,B., Marsault,R., Catsicas,S., and Hirling,H. (2002). Overexpression of neuronal Sec1 enhances axonal branching in hippocampal neurons. Neuroscience 113, 893-905.
Tashiro,A., Dunaevsky,A., Blazeski,R., Mason,C.A., and Yuste,R. (2003). Bidirectional regulation of hippocampal mossy fiber filopodial motility by kainate receptors: a two-step model of synaptogenesis. Neuron 38, 773-84.
Verhage,M., Maia,A.S., Plomp,J.J., Brussaard,A.B., Heeroma,J.H., Vermeer,H., Toonen,R.F., Hammer,R.E., van den Berg,T.K., Missler,M., Geuze,H.J., and Sudhof,T.C. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287, 864-869.
Voets,T., Toonen,R.F., Brian,E.C., de Wit,H., Moser,T., Rettig,J., Sudhof,T.C., Neher,E., and Verhage,M. (2001). Munc18-1 promotes large dense-core vesicle docking. Neuron 31, 581-91.
Washbourne,P., Thompson,P.M., Carta,M., Costa,E.T., Mathews,J.R., Lopez-Bendito,G., Molnar,Z., Becher,M.W., Valenzuela,C.F., Partridge,L.D., and Wilson,M.C. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci 5, 19-26.
Williamson,L.C. and Neale,E.A. (1998). Syntaxin and 25-kDa synaptosomal-associated protein: differential effects of botulinum neurotoxins C1 and A on neuronal survival. J. Neurosci. Res. 52, 569-583.
Wong,W.T. and Wong,R.O. (2000). Rapid dendritic movements during synapse formation and rearrangement. Curr Opin Neurobiol 10, 118-24.
Young,S.H. and Poo,M.M. (1983). Spontaneous release of transmitter from growth cones of embryonic neurones. Nature 305, 634-7.

Chapter 5

Introduction of munc18-1 in cultured late-embryonic null mutant neurons prevents degeneration and restores neurite outgrowth, branching and neurotransmitter secretion

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Abstract

Apart from a lack of neurotransmitter secretion, *munc18-1* deficient neurons are characterized by improper neurite outgrowth and premature degeneration (Heeroma et al., 2003; Heeroma et al., 2004 and chapter 4). In this study, we aimed to test whether these characteristics are due to a latent developmental defect and to generate a test system to analyze munc18-1 mutant variants. Hereto, neocortical neurons from embryonic day 18 (E18), *munc18-1* deficient mice were cultured and subsequently infected with lenti viruses that were mutated to drive the expression of *munc18-1-egfp*. We monitored these neurons up to 3 weeks *in vitro* and found that neuronal viability, dendrite outgrowth and neurotransmitter secretion were completely restored. Thus, *munc18-1* deficient neurons do not degenerate due to latent developmental defects and lenti viruses could successfully transfer *munc18-1* and rescue the mutant phenotype. Therefore, lenti viruses can also be used to transfer mutant forms of *munc18-1* on a null mutant background which should enable a proper analysis of the mutant phenotype. Here, we present the first preliminary data from mutants with suspected altered binding to syntaxin-1 and mint-1. We found that mutations to munc18-1 severely reduced affinity for syntaxin-1 and mint-1 (D34N and D34N;M38V) or syntaxin alone (S313D). Pilot data suggest that these mutants are all capable

of basic neurotransmitter secretion and are not compromised in neurite outgrowth or viability. In conclusion, cultured *munc18-1* deficient neurons derived from E18 embryos are not prone to degenerate and, in conjunction with lenti viruses, form a promising tool to dissect the molecular function of munc18-1.

Introduction

Munc18-1 is essential for neurotransmitter secretion (Heeroma et al., 2003; Verhage et al., 2000) and a strong positive regulator of large dense core vesicle secretion (Voets et al., 2001). After normal initial development *munc18-1* deficient neurons degenerate both *in vivo* and in culture (Heeroma et al., 2003; Heeroma et al., 2004). This degeneration might be related to the lack of *munc18-1* mediated synaptic secretion or neurotrophic support. The lack of neurotransmitter secretion alone is unlikely to cause neuronal degeneration as neurons deficient for both *munc13-1* and *2*, which have highly impaired synaptic vesicle fusion, can survive in culture for weeks (Varoqueaux et al., 2002). In culture, providing trophic support, either direct, or by co-culturing with wildtype hippocampal slices or glial cells, delayed but could not prevent degeneration of *munc18-1* deficient neurons (Heeroma et al., 2004). Possibly *munc18-1* has a function in long term neuronal survival that is distinct from its established function in synaptic secretion. Alternatively, the lack of *munc18-1 in utero*, might have rendered *munc18-1* mutant neurons prone to degenerate.

Apart from its role in synaptic secretion, *munc18-1* also has a marked effect on neurite outgrowth and synapse formation. In developing neocortex, the amount of synapses is less in mutants than in control littermates (Bouwman et al., 2004). In the peripheral nervous system, mutant mice have dramatically less neuromuscular junctions and show severely aberrant neurite branching and elongation (Heeroma et al., 2003). In cultured hippocampal neurons, overexpression of *munc18-1* increases total neurite length by increasing the number of neurite branches (Steiner et al., 2002). Conversely, the absence of *munc18-1* significantly decreases dendrite length and branching (chapter 4). Thus, apart from synaptic secretion *munc18-1* appears to regulate neurite outgrowth and synapse formation. One possibility is that *munc18-1* regulates membrane addition to growing neurites via synaptic vesicle fusion or a similar mechanism.

Munc18-1 interacts with many partners ranging from synaptic proteins such

as DOC2 (Verhage et al., 1997), to structural proteins such as neurofilaments and microtubules (Bhaskar et al., 2004). Mint-1 and syntaxin-1 are probably the best known binding partners of munc18-1 and are primarily known for their involvement in regulated secretion (Biederer and Sudhof, 2000; Garcia et al., 1994; Hata et al., 1993; Wu et al., 1998). Possibly the various interactions of munc18-1 are involved in different aspects of the null mutant phenotype. Specific mutations of *munc18-1* might therefore differentially affect regulated secretion, neurite outgrowth and neuronal viability.
In this study we tested if the impaired neurite outgrowth and degeneration of *munc18-1* deficient neurons are the result of an acute lack of *munc18-1* function or latent defects from earlier development. In addition, we aimed to create a system in which we could test various mutant forms of *munc18-1*. Hereto, we cultured late-embryonic neocortical neurons from *munc18-1* deficient mice and used mutated lenti viruses to induce expression of *munc18-1-egfp*. We found that *munc18-1* restored neurite outgrowth, neurotransmitter secretion and neuronal survival. Thus, *munc18-1* deficient neurons do not degenerate due to latent developmental defects and can be used together with lenti viruses to analyze the phenotype of *munc18-1* mutants on a null background. Preliminary data suggest that mutations to munc18-1 changed the affinity for syntaxin-1 and mint-1 (D34N and D34N;M38V) or syntaxin alone (S313D) but that this has no obvious effect on either neuronal survival, neurite outgrowth or basic neurotransmitter secretion.

Materials and Methods

Laboratory animals

Munc18-1 deficient mice were generated as described before (Verhage et al., 2000). Mouse embryos were obtained by caesarian section of pregnant females from timed heterozygous matings. Embryos, heterozygous for *munc18-1*, were indistinguishable from, and therefore pooled with, wildtype embryos to serve as control animals. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

Generation and biochemical analysis of constructs

Munc18-1 mutants were generated using Quickchange (Stratagene) and subcloned in pcDNA3 (Invitrogen) expression plasmids. HEK293 cells were transfected with Munc18-1 wild-type or mutant cDNAs, metabolically

labeled with [^{35}S]methionine/cysteine (Dupont New England Nuclear) for 24 hours and lysed according to standard procedures. The amount of Munc18-1 expression in each lysate was analysed on SDS-PAGE and lysate volume was adjusted to correct for any expression differences. Hundred µg of cell lysate was incubated overnight at 4°C with syntaxin-1 coupled to glutathione-S-transferase (GST). One hour after addition of glutathione-agarose beads, lysates were washed thoroughly with buffered saline. Beads were resuspended in SDS loading buffer, boiled and run on 12% SDS-PAGE. Gels were blotted onto PVDF membranes and radioactive proteins were quantified on a phosphoimager (Fuji, BAS1000).

Cortical cultures and lenti viral transduction

Cortices were dissected from embryonic day 18 (E18) mice and collected in Hanks Buffered Salts Solution (HBSS; Sigma), buffered with 7 mM Hepes. After removal of the meninges, cortices were minced and incubated for 20 minutes in 0.25%? trypsine in HBSS at 37 °C. After washing the neurons were triturated with fire polished Pasteur pipettes, counted and plated in Neurobasal medium (Invitrogen, Carlsbad, USA) supplemented with 2% B-27 (Invitrogen), 1.8% Hepes, 1% glutamax (Invitrogen), 1% Pen/Strep (Invitrogen) and 0.2% β-mercaptoethanol. Neurons were plated at 6,000/cm^2 on micro islands of rat glia. Glial islands were obtained by spraying a 0.25 mg/ml rat tail collagen solution (BD Biosciences, Bedford, USA) on glass coverslips. After drying and UV sterilization glial cells were plated at 600/cm^2. 50% of the medium was refreshed every week. Munc18-1 wild-type and mutant cDNAs linked to enhanced green fluorescent protein (EGFP) via an internal ribosomal entry site (IRES) were subcloned into pLenti vectors with pCMV promotors and viral particles were produced as described (Naldini et al., 1996a; Naldini et al., 1996b). Neurons were transduceed at 1DIV and EGFP fluorescence was detectable after 2 to 3DIV. Transduction efficiencies between 40 and 100% were routinely obtained.

Immunocytochemical procedures

Cultures were fixed by adding 4% paraformaldehyde (PFA) to the medium in a 1/1 ratio. After 15 minutes the PFA/medium mixture was exchanged for 4% PFA, for 15 minutes. After washing with PBS the cells were permeated with 0.1% Triton X-100 for 5 minutes. After washing with PBS the cells were ready for processing or storage. Before staining the cells were incubated in 4% fetal calf serum for 20 minutes to block a-specific reactions. After washing with PBS the cells were incubated in a mixture of mouse monoclonal

anti-MAP2, 1/200 (Boehringer) and rabbit polyclonal anti-synapsin1, 1/1000 (E028) antibodies and 0.1% Triton X-100 for 1 hour at room temperature or overnight at 4 °C. After washing 3 times with PBS, the cells were incubated for 1 hour at room temperature in secondary antibodies conjugated to Cy5 or Alexa546 (Molecular Probes, Oregon, USA). After washing in PBS the coverslips were mounted with Dabco-Mowiol and analysed with a Zeiss 510 Meta Confocal microscope (Heidelberg, Germany).

Electrophysiological Recordings

Whole cell voltage-clamp (V_m=-70 mV) recordings were performed on cultured neurons on DIV 10-15. The patch pipette solution contained the following (in mM): 125 K^+-gluconic acid, 10 NaCl, 4.6 $MgCl_2$, 4 K_2-ATP, 15 Creatine Phosphate, 1 EGTA and 20 U/ml Phospocreatine Kinase (pH 7.30). The external medium used contained the following components (in mM): 140 NaCl, 2.4 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 10 Glucose (pH 7.30). Axopatch 200A was used for whole-cell recordings. Signal was acquired using Digidata 1322A and Clampex 8.1. Clampfit 8.0 was used for offline analysis.

Results

Introduction of munc18-1 on a null background restores neuronal viability and neurite outgrowth

Munc18-1 null mutant neurons can survive up to 10 days *in vitro* (DIV) when cultured on a glial feeder layer, but eventually degenerate (Heeroma et al., 2004). Here we tested whether *munc18-1* mutant neurons are prone to degenerate due to a temporary lack of *munc18-1* during embryonic development. In addition, we aimed to generate a test system to analyze mutant forms of *munc18-1*. Directly after plating we introduced a fusion protein of enhanced green fluorescent protein and munc18-1 (munc18-1-EGFP) in *munc18-1* null mutant neurons using lenti virus as a vector. After 7DIV, *munc18-1* deficient, neurons (Fig. 1A-C, see appendix) and *munc18-1-egfp* expressing neurons (Fig. 1D-F, see appendix) were present in culture. At this point there is no obvious difference in survival rate of mutant and control neurons (Heeroma et al., 2004). After 10DIV, few *munc18-1* deficient neurons (Fig. 1G-I, see appendix) were left in culture while *munc18-1-EGFP* expressing neurons (Fig. 1J-L, see appendix) were still abundant. After 18DIV, only *munc18-1-EGFP* expressing neurons were left in culture (Fig. 1M-O, see appendix).

Apart from neuronal survival, introduction of *munc18-1* had a marked effect on neuronal morphology. Both after 7 and 10DIV, MAP2-positive dendrites (red) of *munc18-1-egfp* transduced neurons (Fig. 1D-F and J-L, see appendix) are dramatically longer than *munc18-1* deficient neurons (Fig. 1A-C and G-I, see appendix). Also the number of dendrite branches appears to be higher in *munc18-1* expressing neurons. No difference was observed in the number of primary dendrites in both groups of neurons. These data suggest that introduction of *munc18-1* in cultured E18 null mutant neurons successfully rescued these neurons from degeneration and restored dendrite outgrowth. As introduction of wildtype *munc18-1*(egfp) rescued the null mutant phenotype, this system may also be suitable to test the phenotype of mutant forms of *munc18-1*.

Munc18-1 (mutants) restore neurotransmitter secretion in cultured cortical neurons

Munc18-1 deficient neurons show neither evoked nor spontaneous

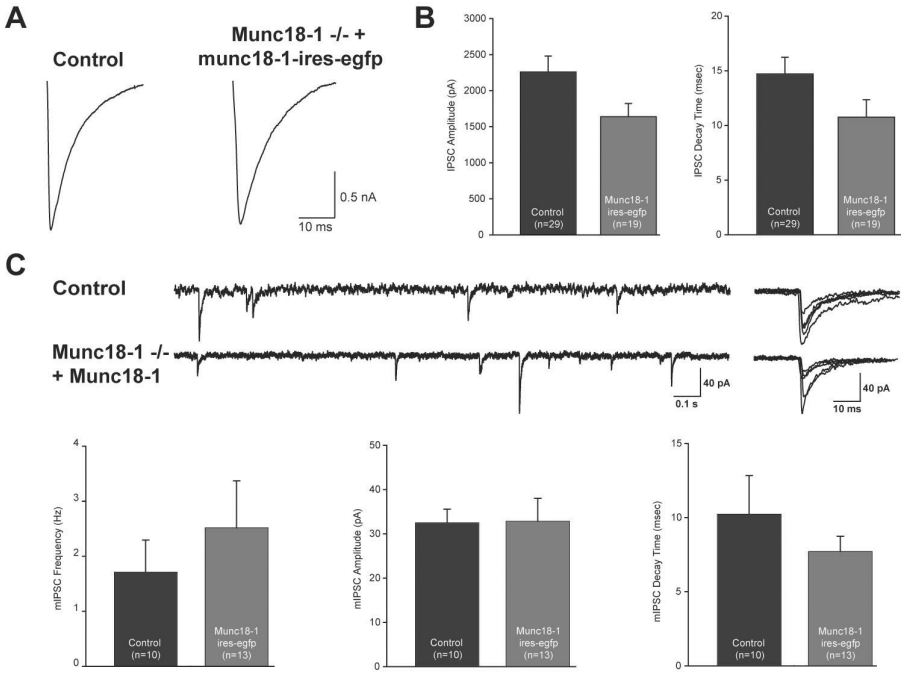

Figure 2: *Basic electrophysiological characterization of munc18-1 deficient neurons rescued with munc18-1-ires-egfp. Transduction of munc18-1 deficient neurons with ≠munc18-1-ires-egfp restored both miniature and single evoked release to control levels. A: Example traces of single evoked IPSCs in both control and rescued neurons. B: Both amplitude and decay time are restored, although the amplitude seems to be smaller in rescued munc18-1 deficient neurons as compared to control (p=0.08). C: Miniature event frequency, amplitude and decay time are all fully restored in rescued neurons.*

neurotransmitter secretion (Heeroma et al., 2004). To test if delayed expression of *munc18-1* could restore synaptic function we expressed *munc18-1-ires-egfp* in autaptic island cultures of E18 *munc18-1* deficient neurons and analyzed the electrophysiological properties in parallel with wild-type neurons.

Whole cell patch clamp analysis of *munc18-1* deficient neurons, rescued with *munc18-1-ires-egfp*, indicated that basic neurotransmitter secretion is restored. Single electrical stimulation showed complete recovery of the autaptic response as compared to control recordings (Fig. 2A and B). Comparison of miniature events revealed that properties like amplitude, decay time and frequency were completely restored to control levels (Fig. 2C). To test whether delayed expression of *munc18-1* could also restore continuous neurotransmitter secretion, synaptic rundown was analyzed during a stimulation train of 10 Hz. Figure 3 shows that rescued *munc18-1* deficient neurons had a similar rundown pattern as control neurons, indicating that also the maintenance of the readily releasable pool of synaptic vesicles was fully restored.

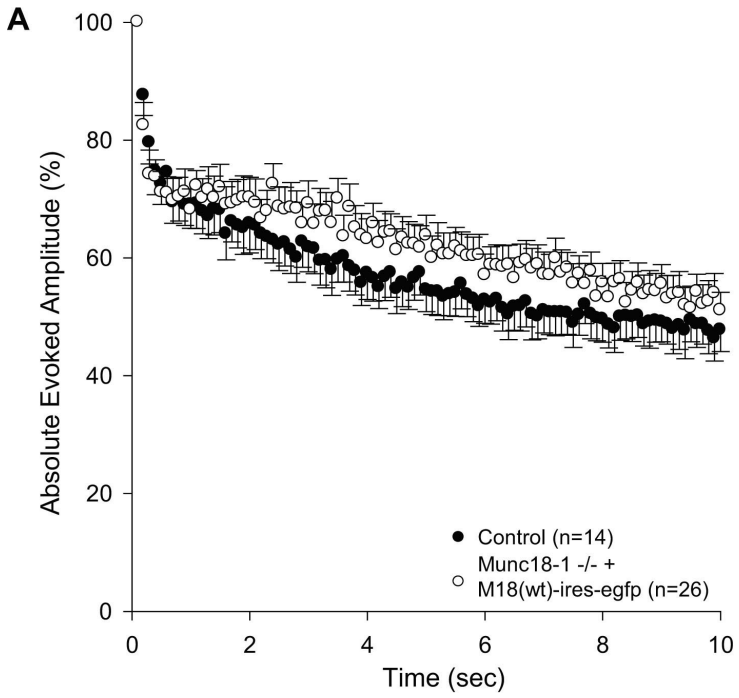

Figure 3: *Characterization of synaptic vesicle recycling in munc18-1 deficient neurons rescued with munc18-1-ires-egfp. Synaptic rundown was investigated using a 10 Hz stimulation train. Munc18-1 deficient neurons rescued with munc18-1-ires-egfp showed complete recovery of synaptic rundown as compared to control neurons.*

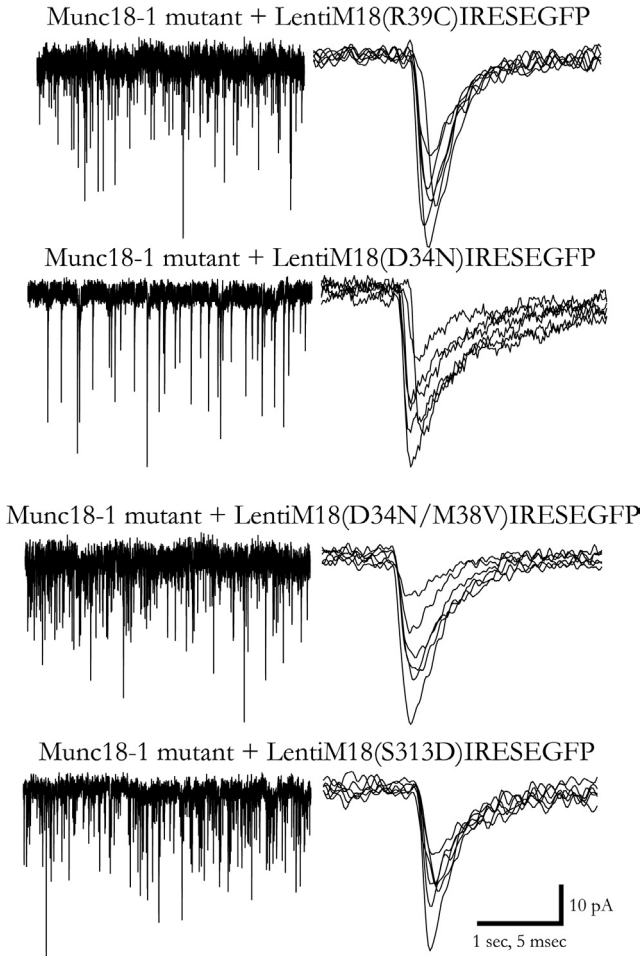

Figure 4: Mutant forms of *munc18-1* restore basic neurotransmitter secretion in null mutant neurons. Null mutant neurons were transduced with *r39c-ires-egfp, d34n-ires-egfp, d34n;m38v-ires-egfp* or *s313d-ires-egfp* directly after plating. Preliminary analysis of spontaneous events at 10DIV revealed that every mutant, in principle, was capable of neurotransmitter secretion.

To test whether we could successfully introduce and analyze mutant forms of *munc18-1* on a null mutant background we also expressed the mutants r39c, d34n, d34n;m38v and s313d which are reported to have altered affinity for syntaxin-1 and mint-1 (Harrison et al., 1994; Naren et al., 1997). Preliminary analysis of neurons expressing *r39c-ires-egfp*, *d34n-ires-egfp, d34n;m38v-ires-egfp* and *s313d-ires-egfp* indicated that all these variants restored basic (spontaneous) synaptic transmission (Fig. 4). More detailed analysis will reveal whether more demanding stimulation protocols do affect neurotransmitter secretion in these mutants. Nonetheless, these

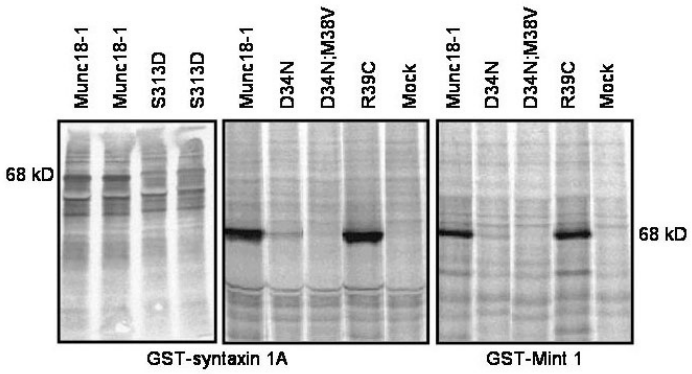

Figure 5: *A) Effect of different point mutations in munc18-1 on affinity for syntaxin-1 and mint 1. GST pull-down assay with GST-syntaxin-1 and GST-mint 1 from cell lysates of metabolically labeled HEK293 cells expressing either wild-type munc18-1 or different mutant forms of munc18-1. Captured [^{35}S] labeled munc18-1 protein (68 kD) was visualized by phopsho-imaging. Syntaxin-1 and Mint-1 show reduced affinity for D34N and no affinity for D34N;M38V, while affinity for R39C appears normal). Syntaxin binding to S313D was severely reduced in vitro.*

data suggest that delayed expression of *munc18-1* completely restores neurotransmitter secretion and that all tested mutants forms of *munc18-1* are capable of mediating synaptic vesicle fusion.

Mutations alter munc18-1 affinity for syntaxin-1 and mint-1

Munc18-1 interacts with syntaxin-1 and mint-1, proteins that are implicated in synaptic secretion and plasticity. Previously published data have described a number of mutant forms of munc18-1 that affect neurotransmitter secretion (Harrison et al., 1994; Naren et al., 1997). Here we tested the binding affinity of these mutant forms of munc18-1 for syntaxin-1 and mint-1. GST pull-down experiments suggested that the D34N;M38V mutation abolished the interactions between munc18-1 and syntaxin-1 and mint-1 (Fig. 5). However, a more sensitive yeast two hybrid analysis revealed that there was a weak interaction between D34N;M38V and syntaxin-1 and mint-1 (Toonen *et al*., in preparation). The D34N mutation reduced the affinity for both syntaxin-1 and mint-1. The interaction of syntaxin-1 and mint-1 with R39C was normal. The *s313d* mutation strongly reduced, but did not abolish, the interaction with syntaxin-1 (Fig. 5). Phosphorylation of munc18-1, which is simulated by the *s313d* mutation, does not affect mint-1 binding (E.C. Brian, personal communication). This suggests that every mutation of munc18-1 tested here is capable, in principle, of interactions with syntaxin-1 and mint-1, but the strength of these interactions varies.

Preliminary analysis: the affinity of munc18-1 for syntaxin-1 and mint-1 is not crucial for neuronal viability or outgrowth

To test whether the strength of the interaction between munc18-1 and syntaxin-1 and mint-1 influences neuronal viability, we analyzed *munc18-1* null mutant neurons expressing *egfp, munc18-1-egfp, munc18-1-ires-egfp, r39c-ires-egfp, d34n-ires-egfp, d34n;m38v-ires-egfp* or *s313d-ires-egfp*. Null mutant neurons were transduced with lenti virus after plating and fixed after 8 and 21DIV respectively. For morphological analysis, all cultures were stained with dendrite marker MAP2 (red in figures 6 and 7, see appendix) and synaptic vesicle marker synapsin (blue in figures 5 and 6, see appendix).

Conform the data above *munc18-1* deficient, *egfp* expressing neurons were present in culture after 8DIV (Fig. 6A-D, see appendix) but no *munc18-1* deficient neuron survived up to 21DIV. Neurons expressing any variant of *munc18-1* were present in culture both after 8DIV (Fig. 6, see appendix) and after 21DIV (Fig. 7, see appendix). MAP2 staining in 8DIV neurons (Fig. 6A, E, I, M, Q, U, Y, see appendix) shows that all neurons, either expressing no *munc18-1* or a mutant form of *munc18-1* had a similar amount of primary dendrites. *Munc18-1* null neurons (Figs. 1A-C, 1G-I and 6A, see appendix) have shorter dendrites and fewer branches that neurons expressing *munc18-1* (Figs. 1D-F, 1J-L and 6E, see appendix). No obvious difference was observed in general dendrite tree morphology of neurons expressing the various mutant forms of *munc18-1* suggesting that every *munc18-1* mutant was able to restore dendrite length and branching (Fig. 6I, M, Q, U, Y, see appendix).

We used the presence of synapsin-positive clusters (1 to 2 μm) in the direct vicinity of MAP2 staining as an indication of synapses (Fig. 6D, H, L, P, T, X, AB, see appendix). After 8DIV, synapses were found on every neuron, either *munc18-1* deficient or expressing (a mutant form of) *munc18-1*. Apart from the apparent difference in the size of the dendrite tree, no evident difference was observed in the synaptic density of neurons expressing only *egfp* or (a variant of) *munc18-1*.

The binding affinity of munc18-1 for syntaxin-1 and mint-1 had no obvious effect on neuronal morphology after 8DIV. Possibly munc18-1 binding to syntaxin-1 and mint-1 only has a mild or long-term effect on neurite outgrowth and synapse formation. We therefore screened for differences in dendrite number, length, branching and synaptic density between neurons expressing *munc18-1-egfp, munc18-1-ires-egfp, r39c-ires-egfp, d34n-ires-egfp, d34n;m38v-ires-egfp* or *s313d-ires-egfp* after 21DIV (Fig. 7, see appendix). However, no clear differences were observed for any of these parameters. Although preliminary, these data suggest that in neocortical

neurons the presence of *munc18-1* is required for long term neuronal survival, neurite elongation and branching, but not synaptogenesis *per se*. The affinity of munc18-1 for two of its main binding partners, syntaxin-1 and mint-1, however, does not seem to be critically important for neuronal survival or morphology.

Discussion

The aim of this study was to test whether reduced neurite outgrowth and degeneration of *munc18-1* deficient neurons are due to latent developmental or acute defects and to create a system to test various mutant forms of *munc18-1* on a null mutant background. We modified lenti viruses to express *munc18-1-egfp, r39c-ires-egfp, d34n-ires-egfp, d34n;m38v-ires-egfp* and *s313d-ires-egfp* in late-embryonic *munc18-1* null mutant neurons and analyzed neuronal survival, dendrite outgrowth and neurotransmitter secretion. We found that *munc18-1* could restore neuronal viability, dendrite outgrowth and neurotransmitter secretion and thus that *munc18-1* deficient neurons can be used in combination with lenti viruses to analyze mutant forms of *munc18-1*. Preliminary data suggest that synaptic density is not severely influenced by the presence of *munc18-1*. In addition, mutations to munc18-1 could reduce the interaction with syntaxin-1 (S313D) alone or both syntaxin-1 and mint-1 (D34N and D34N;M38V), but the strength of these interactions did not appear to affect neuronal viability, morphology or basic neurotransmitter secretion.

We found that degeneration of *munc18-1* deficient neurons in culture could be prevented by introduction of *munc18-1* in cultured late-embryonic neurons. Therefore, neurons obtained from *munc18-1* deficient embryos are not prone to degenerate and thus presumably healthy at the time of plating. The finding that delayed expression of *munc18-1* on a null background also restored dendrite outgrowth and neurotransmitter secretion supports this. This suggests that the degeneration of *munc18-1* deficient neurons that occurs both *in vivo* and in culture results from an acute *munc18-1* related dysfunction, not from a latent defect.

As neither the presence of *munc18-1* nor its particular mutant form is of influence on the number of primary dendrites *munc18-1*-mediated processes such as synaptic secretion and neuronal activity do not influence this parameter. Therefore, basic neurite outgrowth is probably innate and regulated by other proteins, such as TI-VAMP and the exocyst complex (Martinez-Arca et al., 2001; Murthy et al., 2003).

The presence of *munc18-1* is positively correlated with dendrite length and branching. This suggests that, contrary to basic neurite outgrowth, the growth of secondary or tertiary dendrites is regulated by *munc18-1* (mediated processes). Whether *munc18-1* regulates neurite growth directly via fusion of vesicular and plasma membranes or indirectly, for instance through neuronal activity remains unknown. However, since *munc18-1* deficient motor neurons actually extended longer tertiary neurite branches (Heeroma et al., 2003) it is more likely that *munc18-1* indirectly influences dendrite outgrowth.

Unlike the size of the dendrite tree, our first indications are that the presence of *munc18-1* does not dramatically influence synaptic density. Therefore, the observed reduction in synapse number in developing mutant neocortex (Bouwman et al., 2004) might reflect reduced neurite outgrowth rather than impaired synaptogenesis *per se*. In a recent real-time imaging study of dendrite outgrowth in zebrafish it was shown that dendrites continuously extend filopodia in search of synaptic partners. A fraction of these filopodia, presumably opposing presynaptic terminals, stabilizes and form a branching/starting point for further dendrite outgrowth (Niell et al., 2004). One of the proposed criteria for synapse stabilization is the occurrence of synaptic transmission (Ackermann and Matus, 2003). This view is very compatible with our data; we observed a normal density of presynaptic synapsin clusters in the vicinity of dendritic MAP2 staining, indicating (the initiation of) synapse formation in *munc18-1* deficient neurons. The lack of neurotransmitter secretion would prevent stabilization of these synapses and as a consequence impair further dendrite outgrowth. In line with this, we found that organotypic cultures of *munc18-1* deficient neurons have reduced net neurite outgrowth, but increased filopodial activity, suggesting a lack of synaptic stabilization (chapter 4).

Munc18-1 is predominantly known as an essential protein for neurotransmitter secretion and the observed degeneration of *munc18-1* deficient neurons was therefore intuitively attributed to a lack of synaptic secretion (Verhage et al., 2000). As munc18-1 is proposed to mediate synaptic secretion via interaction with syntaxin-1 and mint-1 (Biederer and Sudhof, 2000; Garcia et al., 1994; Hata et al., 1993; Wu et al., 1998), manipulation of these interactions could also be expected to interfere with neurotransmitter secretion and neuronal survival. Although preliminary, the results of the present study suggest that the affinity of munc18-1 for syntaxin-1 and mint-1 does not affect long-term neuronal survival. Residual binding of munc18-1 with syntaxin-1 and mint-1 cannot be excluded however, leaving open the possibility that this is sufficient to promote neuronal survival and drive at least basic synaptic

secretion. Alternatively, the binding affinity of munc18-1 for syntaxin-1 or mint-1 might not be a critical determinant for synaptic vesicle fusion. In *C. elegans* replacement of wildtype syntaxin with a constitutively open form of syntaxin (LE) suppresses the phenotype of the *unc18* mutant, increasing neurotransmitter secretion at the neuromuscular junction (Weimer et al., 2003). On the one hand this illustrates the significance of the unc18-syntaxin interaction in neurotransmitter secretion, but on the other hand this shows that, at least in worms, this interaction is not essential for secretion. However, in *C. elegans unc18* itself is not essential for neurotransmitter secretion, while its orthologues *rop* in *Drosophila* and *munc18-1* in mice are. Future experiments should elucidate whether the affinity of munc18-1 for mint-1 and syntaxin-1 is of influence on neuronal survival and more demanding synaptic events such as sustained evoked secretion.

Similar to neurotransmitter secretion, our first indications are that dendrite outgrowth and branching are not strictly dependent on the interaction of munc18-1 with syntaxin-1 or mint-1. Possibly, *munc18-1* regulates neurite outgrowth in a way distinct from synaptic secretion. Munc18-1 was recently found to bind microtubules (Bhaskar et al., 2004) offering the possibility that *munc18-1* is directly involved in structural reorganisation of the cytoskeleton. As cytoskeletal dynamics are involved in many basic cellular processes, this could not only explain defective neurite outgrowth but also sheds new light on the inevitable degeneration neurons that lack *munc18-1*.

We used mutated lenti viruses to rescue *munc18-1* deficient neurons and express a range of mutant forms of *munc18-1* on a null background. This provides an excellent tool to dissect the different functions of *munc18-1* and explain the various aspects of the null mutant phenotype. Eventually, this approach might help to resolve whether the loss of regulated secretion, decreased dendrite outgrowth and neuronal degeneration, are unrelated phenomena caused by the lack of *munc18-1*, or are interconnected processes that actively regulate each other.

The main goal of these experiments was to validate the model system used in this thesis and test whether cultured *munc18-1* deficient neurons are healthy at the time of plating. The qualitative description of neuronal viability, neurite outgrowth and synaptic function used here was sufficient to serve this purpose. In addition, these experiments yielded interesting data about the role of munc18-1, and the potential lack of relevance of mint-1 and syntaxin-1 affinity in these processes. Therefore, a quantitative study of the mutants used here could provide insight in the mechanism by which *munc18-1* regulates neuronal viability, neurite outgrowth and synapse formation, as was described in this thesis.

Reference List

Ackermann,M. and Matus,A. (2003). Activity-induced targeting of profilin and stabilization of dendritic spine morphology. Nat. Neurosci. 6, 1194-1200.

Bhaskar,K., Shareef,M.M., Sharma,V.M., Shetty,A.P., Ramamohan,Y., Pant,H.C., Raju,T.R., and Shetty,K.T. (2004). Co-purification and localization of Munc18-1 (p67) and Cdk5 with neuronal cytoskeletal proteins. Neurochem Int 44, 35-44.

Biederer,T. and Sudhof,T.C. (2000). Mints as adaptors. Direct binding to neurexins and recruitment of munc18. J Biol Chem 275, 39803-6.

Bouwman,J., Maia,A.S., Camoletto,P.G., Posthuma,G., Roubos,E.W., Oorschot,V.M.J., Klumperman,J., and Verhage,M. (2004). Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. Neuroscience 126, 115-126.

Garcia,E.P., Gatti,E., Butler,M., Burton,J., and De Camilli,P. (1994). A rat brain Sec1 homologue related to Rop and UNC18 interacts with syntaxin. Proc Natl Acad Sci U S A 91, 2003-7.

Harrison,S.D., Broadie,K., van de Goor,J., and Rubin,G.M. (1994). Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission. Neuron 13, 555-66.

Hata,Y., Slaughter,C.A., and Sudhof,T.C. (1993). Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature 366, 347-51.

Heeroma,J.H., Plomp,J.J., Roubos,E.W., and Verhage,M. (2003). Development of the mouse neuromuscular junction in the absence of regulated secretion. Neuroscience 120, 733-44.

Heeroma,J.H., Roelandse,M., Wierda,K., Van Aerde,K.I., Toonen,R.F., Hensbroek,R.A., Brussaard,A., Matus,A., and Verhage,M. (2004). Trophic support delays but does not prevent cell-intrinsic degeneration of neurons deficient for munc18-1. Eur. J Neurosci 20, 623-634.

Martinez-Arca,S., Coco,S., Mainguy,G., Schenk,U., Alberts,P., Bouille,P., Mezzina,M., Prochiantz,A., Matteoli,M., Louvard,D., and Galli,T. (2001). A common exocytotic mechanism mediates axonal and dendritic outgrowth. J. Neurosci. 21, 3830-3838.

Murthy,M., Garza,D., Scheller,R.H., and Schwarz,T.L. (2003). Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. Neuron 37, 433-47.

Naldini,L., Blomer,U., Gage,F.H., Trono,D., and Verma,I.M. (1996a). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A 93, 11382-8.

Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M., and Trono,D. (1996b). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263-7.

Naren,A.P., Nelson,D.J., Xie,W., Jovov,B., Pevsner,J., Bennett,M.K., Benos,D.J., Quick,M.W., and Kirk,K.L. (1997). Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. Nature 390, 302-5.

Niell,C.M., Meyer,M.P., and Smith,S.J. (2004). In vivo imaging of synapse formation on a growing dendritic arbor. Nat. Neurosci. 7, 254-260.

Steiner,P., Sarria,J.C., Huni,B., Marsault,R., Catsicas,S., and Hirling,H. (2002). Overexpression of neuronal Sec1 enhances axonal branching in hippocampal neurons. Neuroscience 113, 893-905.

Varoqueaux,F., Sigler,A., Rhee,J.S., Brose,N., Enk,C., Reim,K., and Rosenmund,C. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A 99, 9037-42.

Verhage,M., de Vries,K.J., Roshol,H., Burbach,J.P., Gispen,W.H., and Sudhof,T.C. (1997). DOC2 proteins in rat brain: complementary distribution and proposed function as vesicular adapter proteins in early stages of secretion. Neuron 18, 453-61.

Verhage,M., Maia,A.S., Plomp,J.J., Brussaard,A.B., Heeroma,J.H., Vermeer,H., Toonen,R.F., Hammer,R.E., van den Berg,T.K., Missler,M., Geuze,H.J., and Sudhof,T.C. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287, 864-869.

Voets,T., Toonen,R.F., Brian,E.C., de Wit,H., Moser,T., Rettig,J., Sudhof,T.C., Neher,E., and

Verhage,M. (2001). Munc18-1 promotes large dense-core vesicle docking. Neuron 31, 581-91.
Weimer,R.M., Richmond,J.E., Davis,W.S., Hadwiger,G., Nonet,M.L., and Jorgensen,E.M. (2003). Defects in synaptic vesicle docking in unc-18 mutants. Nat. Neurosci. 6, 1023-1030.
Wu,M.N., Littleton,J.T., Bhat,M.A., Prokop,A., and Bellen,H.J. (1998). ROP, the Drosophila Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. Embo J 17, 127-39.

Chapter 6

General discussion

The aim of this thesis was to elucidate the role of regulated secretion in neurite outgrowth, synapse formation and neuronal viability. As genetic deletion of *munc18-1* results in the most severe neurosecretory phenotype described to date (Verhage *et al.*, 2000;Voets *et al.*, 2001), the *munc18-1* null mutant mouse served as a model system for our experiments.

In chapter 2 we aimed to describe motor neuronal survival, outgrowth, branching and synaptic development in diaphragm and intercostal muscles of *munc18-1* deficient mouse embryos. We found that motor neurons projected to and branched at the muscle where synapses were formed. However, the final stages of branching were severely aberrant and synapses were scarce. In late embryonic development, motor neurons, but not sensory DRGs degenerated. Postsynaptically, AChRs were present, but receptor clusters were less uniformly shaped and distributed. Despite the presence of synapses, neither α-latrotoxin nor direct phrenic nerve stimulation elicited any postsynaptic response.

The aim of chapter 3 was to focus on the relationship between presynaptic regulated secretion and neuronal survival. We found that also in culture *munc18-1* deficient neurons did not secrete neurotransmitter but displayed otherwise normal biophysical properties. As was observed *in utero*, mutant neurons degenerated in culture and *in vivo* when *munc18-1* was deleted in a cell specific manner. In dissociated cultures this degeneration was not prevented or delayed by co-culturing with wildtype neurons. Providing neurotrophic substances, glial support or co-culturing with wildtype organotypic slices delayed, but did not prevent mutant neuron degeneration.

In chapter 4 the aim was to analyse dendrite outgrowth and branching and growth cone dynamics of *munc18-1* deficient neurons in culture. After plating, mutant neurons initially displayed normal dendrite outgrowth and network formation. After 3DIV, mutant growth cones showed reduced net outgrowth, while filopodial length and formation were increased. In later stages, mutant neurons had shorter dendrites with fewer branching points.

In chapter 5 we aimed to rescue cultured *munc18-1* deficient neurons from degeneration and to create a system to analyze *munc18-1* mutants on a null

background. Expression of *munc18-1* in cultured late-embryonic neurons restored neuronal survival, dendrite outgrowth and neurotransmitter secretion, indicating that indeed, cultured *munc18-1* deficient neurons are healthy at the moment of plating and degenerate due to acute *munc18-1* related defects. Preliminary data of *munc18-1* (mutants) on a null background suggested that *munc18-1* is not essential for synapse formation and reduced binding to syntaxin-1 and mint-1 does not affect dendrite outgrowth or basic neurotransmitter secretion.

Neurotransmitter secretion defect in munc18-1 deficient neurons

In chapter 2 we found that NMJs were present in *munc18-1* deficient muscles at the time of electrophysiological analysis (E15 and E18). Direct stimulation of the phrenic nerve did not elicit any responses in the mutant muscle. Application of α-latrotoxin, the most potent stimulator of synaptic vesicle secretion known thus far, did not result in a postsynaptic response either. However, direct stimulation of AChRs elicited a muscle response in the mutant, suggesting normal development of the postsynaptic apparatus (Verhage et al., 2000).
Apart from functional GABA and ACh receptors (Verhage et al., 2000), *munc18-1* deficient neurons in culture also express functional AMPA and NMDA receptors (chapter 3). In chapter 4 we found that mutant neocortical neurons in culture did not release neurotransmitter upon α-latrotoxin stimulation or sucrose application, which normally induces release of the entire readily releasable pool (Rosenmund & Stevens, 1996). Together these results indicate that the lack of synaptic transmission in *munc18-1* deficient neurons is due to a lack of vesicular release and not a defect in postsynaptic signal reception.
Munc18-1 was the first gene to be identified as essential for "spontaneous" neurotransmitter vesicle fusion. In mice, a whole range of mutations of synaptic proteins have been described that depress neurotransmitter secretion to some extent. However, none of these mutants completely abolished both evoked and spontaneous neurotransmitter release. Even neurons of the *munc13-1/2* double null mutant mouse sporadically released neurotransmitter quanta after α-latrotoxin application (Varoqueaux et al., 2002). Interestingly, neurotransmitter secretion is not completely abolished in *C. elegans* deficient for *unc18* (Weimer *et al*., 2003; Zhao & Nonet, 2000). One possible explanation for this discrepancy is that in nematodes, there is less biological need for the strict regulation of neurotransmitter secretion

that is observed in mammals. This might be reflected by the fact that mammals have three genes encoding munc18, *munc18-1* being specific for the brain, whereas nematodes only have one (Toonen & Verhage, 2003). Since in mammals, *munc18-1* is unique in that all synaptic vesicle secretion is blocked upon deletion, this implies that munc18-1 is currently the only protein identified to be essential in neuronal regulated secretion. This underlines the value of the *munc18-1* deficient mouse as a model to identify the role of regulated secretion in neurite outgrowth and synapse formation.

Spontaneous vesicular fusion and postsynaptic receptor organisation

In *Drosophila*, NMJ development was found to be normal in *synaptobrevin* mutants in which evoked but not spontaneous neurotransmitter release is absent (Saitoe et al., 2001). The same group found that synaptic glutamate receptor clusters were not formed in mutants for *dynamin* or *syntaxin* in which spontaneous secretion is also absent, and thus suggested that spontaneous neurotransmitter secretion is necessary for glutamate receptor clustering. This result has however been challenged by another group, who, using similar mutants of the same genes, found no defects in glutamate receptor clustering (Featherstone et al., 2002).
We found that AChR clusters were present in muscles from *munc18-1* deficient mice, supporting the hypothesis that spontaneous neurotransmitter release is not required for neurotransmitter receptor clustering. This is also confirmed by the finding that AChR clusters are present in developing muscles before synapse formation (Lin *et al*., 2001; Yang *et al*., 2001). However, we did find that AChR clusters in *munc18-1* deficient mouse diaphragms were distributed over a larger muscle area and often had a round shape, suggesting that less refinement of AChR cluster shape and localization had occurred. In *snap-25* deficient mice, no such aberrations were observed in the diaphragm (Washbourne et al., 2002). As *snap-25* deficient neurons are still capable of spontaneous release, the observed difference in AChR cluster refinement may be ascribed to spontaneous neurotransmitter secretion. Thus, in mice, spontaneous release is not required for the induction of AChR clusters but rather for their refinement and alignment with presynaptic terminals.

In hippocampal island cultures it was found that the presence of glutamatergic terminals is required for clustering of AMPA, but not NMDA or GABA receptors (Rao et al., 2000). The most likely explanation for this is that

glutamate secretion is required for AMPA receptors to cluster. When action potential dependent synaptic secretion is blocked using tetrodotoxin, AMPA receptor mediated mEPSPs actually increased in amplitude and frequency (Lauri et al., 2003), suggesting that action potential dependent secretion is not necessary for AMPA clustering/cluster stability.
We found that GABA, ACh, NMDA and AMPA receptors are present, and functional in the absence of *munc18-1*. In addition, AChRs cluster (chapter 2) and the kinetics of postsynaptic responses to GABA, AMPA and NMDA application suggest these receptors are clustered in the mutant as well (Keimpe Wierda, personal communication). This therefore suggests that neither evoked, nor spontaneous release is required for neurotransmitter receptors to cluster. With respect to the necessity for the presence of glutamatergic terminals in AMPA receptor clustering it might be that another compound from the presynaptic terminal is instrumental herein. In (glutamatergic) *Drosophila* NMJs, a vesicular factor other than neurotransmitter has been proposed to induce postsynaptic glutamate receptor clustering (Saitoe et al., 2001). Indeed, in mammalian NMJs presynaptic agrin was found to induce synthesis and clustering of AChRs (Gautam et al., 1996). Agrin was proposed to serve a similar function in central synapses. However, here induction of the postsynaptic apparatus appears to be more controversial (Smith & Hilgenberg, 2002).

Neurite outgrowth in munc18-1 deficient neurons

We found no differences in early embryonic gross brain morphology of *munc18-1* deficient mice (Verhage et al., 2000). In addition, mutant embryonic motor neuron outgrowth and cortical neuron outgrowth during the first 3DIV were unimpaired, suggesting that *munc18-1* is not essential for neurite outgrowth (chapter 2 and 4). After 3DIV however, *munc18-1* deficient growth cones showed less net elongation and after 7DIV mutant cortical neurons did have shorter neurites and fewer dendritic branches (chapter 4). Concordantly, overexpression of *munc18-1* in cultured hippocampal neurons led to increased branching and total neurite tree length (Steiner et al., 2002). Together, this suggests that especially the initial stages of neurite outgrowth are *munc18-1*-independent and possibly driven by TI-VAMP and/or exocyst (Hazuka *et al.*, 1999; Martinez-Arca *et al.*, 2001; Murthy *et al.*, 2003). Terminal neurite outgrowth, however, might very well be stimulated by *munc18-1*. This does not reveal, however, whether *munc18-1* functions in neurite outgrowth through regulation of membrane addition, or through

regulation of neuronal activity, which is also proposed to play a role (Brewer & Cotman, 1989; Lipton & Kater, 1989).

In this respect it was interesting to find that the absence of *munc18-1* had opposite effects on the final stages of neurite outgrowth, branching and synaptogenesis in central and peripheral neurons. In 7/8DIV cultured neocortical neurons neurite outgrowth and branching were reduced while synaptic density appeared normal (chapters 4 and 5). In E18 diaphragms and intercostal muscles, terminal branching and synapses were also reduced, but tertiary branches were longer in mutants (chapter 2). One possible explanation for this discrepancy is a difference in function. During development motor neurons search for synaptic partners in a pre-defined region (Lin *et al.*, 2001; Yang *et al.*, 2001), and upon failure to form functional synapses, resprout and grow further in search of new synaptic partners (Banks *et al.*, 2001; Brandon *et al.*, 2003; Heeroma *et al.*, 2003; Holland & Brown, 1980). Once a suitable synaptic partner is found this synapse remains. Central synapses on the other hand are predominantly plastic. Presumably, central synapses are also validated by functionality, but are continuously remodelled as brain activity patterns change throughout life. Thus, to maintain homeostasis in a functional network, neuronal activity might induce neurite outgrowth and branching without dramatically changing synaptic density (Burrone & Murthy, 2003; Lipton & Kater, 1989; Turrigiano & Nelson, 2000).

In conclusion, embryonic brain morphology and normal neurite outgrowth during the first 3DIV suggest that initial neurite outgrowth is independent of munc18-1 mediated processes, therefore independent on neurotransmitter secretion and probably innate at least until synapse formation occurs. Reduced neurite length, net elongation and branching after 3DIV indicate that later stages of neurite outgrowth are *munc18-1* dependent. As neurites are actually longer in *munc18-1* deficient peripheral nerves, *munc18-1* probably does not modulate neurite outgrowth by regulating synaptic vesicle mediated membrane addition to growth cones. The finding that munc18-1 deficient growth cones extend more and longer filopodia also argues strongly against the necessity of *munc18-1* for membrane expansion. Therefore, (*munc18-1* mediated) regulated secretion is likely to modulate neurite outgrowth by regulating neuronal activity, which may have different effects in different neuronal systems.

Synapse formation in munc18-1 deficient neurons

Similar to neurite outgrowth, basic synapse formation is possible in the absence of *munc18-1.* In the peripheral nervous system we found that *munc18-1* (mediated secretion) is not required for postsynaptic receptor clustering but rather for their organisation and refinement. Peripheral synapse formation was not absent but severely impaired in the absence of *munc18-1* (chapter 2). In developing neocortex, synapses are present but reduced in number (Bouwman *et al.*, 2004). Also in cultured neocortical neurons fewer synapses were formed, but this appears to be better correlated with a reduction in total dendritic tree length than synaptic density *per se* (chapters 4 and 5). Thus the reduced number of synapses found on *munc18-1* deficient neurons might be caused by reduced neurite outgrowth rather than a reduced synaptogenic potential *per se*.

Dynamic growth cone analysis of *munc18-1* deficient neurons revealed increased filopodial growth and motility (chapter 4). The increased number of outgrowing filopodia not only confirms that *munc18-1* is not essential for nerve terminal outgrowth but actually indicates a negative correlation between the presence of *munc18-1* and filopodium formation. Postsynaptic filopodia are regarded as postsynaptic precursors which transform into dendritic spines upon stabilization (Dailey & Smith, 1996; Prange & Murphy, 2001; Ziv & Smith, 1996). By analogy, presynaptic filopodia might represent synaptic terminal precursors that are yet to be stabilized. Thus, the higher occurrence and motility of filopodia in *munc18-1* deficient neurites might actually reflect a higher rate of attempted synapse formation combined with impaired, *munc18-1* dependent, synapse stabilization.

Early in development, dendritic filopodia are highly motile and in the process of synaptic network maturation (coinciding with filopodial transformation into spines) this motility decreases. This developmental decrease in motility is activity dependent, both in vitro and in vivo (Dailey & Smith, 1996; Dunaevsky *et al.*, 1999; Fischer *et al.*, 1998; Jontes & Smith, 2000; Lendvai *et al.*, 2000; Ziv & Smith, 1996). Presynaptically, similar rules apply as mossy fibre filopodial movement decreases during development and this can be mimicked by increasing kainate receptor activation (Tashiro *et al.*, 2003). Therefore, the observed increase in filopodial movement in *munc18-1* deficient neurons might indicate a lack of synaptic activity. Combined with the increased incidence of growth cone filopodia, this suggests that the lack of *munc18-1* mediated neurotransmitter secretion increases filopodial

growth and movement and impairs synapse stabilization.
It must be noted, however, that munc18-1 might also indirectly influence synaptic stability, for instance through its interaction with mint-1, a molecule that is involved in synapse formation and plasticity (Biederer & Sudhof, 2000; Scheiffele *et al*., 2000). Thus, although regulated secretion and synapse stabilization are correlated, it cannot be concluded from our experiments that this relationship is causal.

Degeneration of munc18-1 deficient neurons

Munc18-1 deficient neurons develop normally as far as synapse formation but subsequently degenerate (Verhage et al., 2000). DRGs, which do not depend on neurotransmitter secretion for their synaptic input, appear to be less prone to degenerate (Heeroma et al., 2003). Hence, we hypothesized that *munc18-1* deficient neurons degenerate due to a lack of synaptic input. Co-culturing with wildtype neurons did not prevent or even delay the degeneration of mutant neurons (chapter 3), but we could not determine whether *munc18-1* deficient neurons actually received functional synaptic input in these cultures. Still, mutant neurons express and cluster functional AMPA and NMDA receptors (chapter 3) and direct application of glutamate, mimicking synaptic input, did not prolong mutant neuronal survival (data not shown in this thesis, JH). Moreover, specific deletion of *munc18-1* in cerebellar purkinje cells, which presumably have completely normal synaptic input, did not prevent degeneration of *munc18-1* deficient neurons. Finally, neurons deficient for both *munc13-1* and *munc13-2*, which show an almost complete block of neurotransmitter secretion, show no signs of degeneration, neither *in vivo*, nor in culture (Varoqueaux et al., 2002). Thus, *munc18-1* deficient neurons most probably do not degenerate due to a lack of synaptic input.
One factor that cannot be excluded is the possibility that *munc18-1* deficient neurons degenerate due to a lack of retrograde signalling through their efferents. *Munc18-1* deficient neurons may have a normal synaptic density, but in the period when degeneration sets in, mutant neurons do have a shorter dendrite tree with reduced branching, which might imply a similar defect in axonal outgrowth. Indeed, we did observe reduced axonal outgrowth in 3DIV mutant organotypic cultures (chapter 4). In addition, we found that mutant peripheral neurons had a severely reduced number of terminal nerve branches and neuromuscular synapses. In fact, there were three consistent differences between *munc18-1* deficient mice and other

cases in which the effect of impaired neurotransmitter section on NMJ was studied (de Paiva *et al.*, 1999;Houenou *et al.*, 1990;Washbourne *et al.*, 2002); 1) *munc18-1* deficient mice are the only mice in which both spontaneous and evoked neurotransmitter release are completely absent, 2) *munc18-1* motor neurons form fewer synapses whereas motor neurons in the other studies formed more synapses and 3) *munc18-1* deficient neurons degenerated while motor neurons in the other studies showed increased survival. Thus, when *munc18-1* deficient neurons do not degenerate due to the absence of neurotransmitter secretion, a lack of retrograde signals through efferent synapses might be involved. Still, this does not explain why DRGs are less susceptible to degeneration than other *munc18-1* deficient neurons.

Munc18-1 deletion results in a 90% reduction in LDCV release in adrenal chromaffin cells (Voets et al., 2001). Neurosecretory cells in the pituitary show a similar reduction in vesicular secretion (Korteweg *et al.*, in preparation), implying that LDCV secretion might be affected in central synapses as well. As these vesicles are known to contain neurotrophic substances (Berg *et al.*, 2000; Wang *et al.*, 2003), lack of trophic support was an alternative explanation for the degeneration of *munc18-1* mutant neurons. In chapter 4 we found that adding neurotrophic substances, such as BDNF or insulin, delayed, but did not prevent mutant neuronal degeneration, as did culturing mutant neurons on a glial feeder layer, or in (co-culture with wildtype) organotypic slices. One explanation for the observed delays in degeneration is that many trophic substances can promote temporal survival of neurons under conditions that would normally cause rapid cell death (Hamabe *et al.*, 2003; Yamada *et al.*, 2001). All attempts to increase trophic support to *munc18-1* deficient neurons were *in vitro* experiments and thus an incomplete representation of physiological conditions. The failure to prevent mutant neuron degeneration therefore does not imply that trophic support cannot rescue *munc18-1* deficient neurons in principle. However, it must be noted that growing on a glial feeder layer significantly delayed degeneration of *munc18-1* deficient neurons. As glia are not known to express *munc18-1*, this delay in degeneration is not likely related to compensation of a *munc18-1* mediated neuronal function. Moreover, *munc18-1* deficient purkinje cells also degenerated in an otherwise wildtype cerebellum where environmental conditions presumably were physiological.
To recapitulate, if degeneration of *munc18-1* deficient neurons can be ascribed to a lack of regulated secretion, this can involve a number of schemes. First although regulated secretion implies focused secretion both

in a temporal and spatial sense, *munc18-1* could also be involved in less regulated, more general secretion. Second, focused secretion is likely to occur at synaptic loci, this being at either afferent or efferent synapses. Third, regulated secretion can be divided into neurotransmitter secretion and the secretion of neurotrophic/modulatory substances.
General application of both neurotransmitter and neurotrophic substances failed to prevent degeneration of mutant neurons thus arguing against a defect in general secretion. Our finding that *munc18-1* deficient neurons still degenerate in a Purkinje cell specific null mutant brain strongly argues against afferent defects being directly involved. The normal viability of *munc13-1/2* deficient neurons in mono-culture supports this and reduces the likelihood that *munc18-1* mutant neuronal degeneration is due to a lack of synaptic efferent secretion of neurotransmitters. What cannot be excluded is the possible requirement autocrine secretion of neurotrophins (*i.e.*, neurotrophins being both secreted and received by either a presynaptic terminal or a postsynaptic compartment) that cannot be mimicked by general application. Further, it cannot be excluded that *munc18-1* is required for the secretion of a substance that in turn elicits an essential response, either anterogradely or retrogradely and might even be *munc18-1* dependent itself. Finally, it is possible that *munc18-1* has another function, distinct from regulated secretion that is indispensable for long term neuronal survival.

Interestingly the only other secretion mutant that shows neuronal degeneration is the syntaxin null mutant fly (Schulze & Bellen, 1996). Syntaxins are involved in a variety of cellular functions and as munc18-1 is a high-affinity binding partner of syntaxin-1, the lack of munc18-1 might allow syntaxin-1 to have promiscuous interactions (Toonen & Verhage, 2003). This could enable undesired fusion events which in turn might cause neurons to degenerate. However, syntaxin-1 does not appear to be mis-targeted in *munc18-1* deficient neurons, thus reducing the likelihood that munc18-1 is required to prevent syntaxin-1 from having promiscuous interactions (data not shown in this thesis, JH). *Munc18-1* has also been shown to regulate ion channel function through interaction with syntaxin-1 (Naren et al., 1997). However, *munc18-1* deficient neurons displayed normal biophysical properties, suggesting no defect in ion channel function. In addition, we did not observe any effect on survival in *munc18-1* null mutant neurons that expressed mutant forms of *munc18-1* with severely reduced affinity for syntaxin-1 (chapter 5). Thus, the absence of interaction with syntaxin-1 is not likely to underlie degeneration of *munc18-1* deficient neurons.

Munc18-1 might also be involved in synaptic recognition through interactions with mint-1 which binds to CASK and neurexin (Biederer & Sudhof, 2000). With regard to possible retrograde signalling (see above) this might be an interesting function of *munc18-1*. However, as many of the syntaxin-1 binding mutations described in chapter 5 also affect mint-1 binding, it is unlikely that a defect in mint-1 binding underlies *munc18-1* deficient neuronal degeneration. Interestingly, mint-1 also regulates synaptic strength through modulation of beta amyloid protein metabolism. This function of mint-1 is dependent on the presence of *munc18-1*, but does not require direct binding to munc18-1 (Ho *et al*., 2002; Kamenetz *et al*., 2003). Therefore, defective beta amyloid protein metabolism, which also underlies neuronal degeneration in Alzheimers disease, might also be involved in the degeneration of *munc18-1* deficient neurons.

Finally, munc18-1 binds cytoskeletal elements such as microtubules and neurofilaments, suggesting a role for munc18-1 in cytoskeletal dynamics (Bhaskar et al., 2004). In support of this, transduction experiments with *munc18-1*-EGFP often visualized cytoskeleton-like structures in cultured glial cells (data not shown in this thesis, JH). This offers interesting new possibilities for how *munc18-1* might function in regulated secretion, neurite outgrowth and neuronal survival. First, in addition to munc18-1, munc18-2 has been found to bind microtubules, through which it regulates exocytosis in non-neuronal cells (Martin-Verdeaux et al., 2003). Second, spectrin is a protein that binds to synapsin and actin, and injection of peptides interfering with spectrin binding completely blocked neurotransmitter secretion (Sikorski *et al*., 2000; Zimmer *et al*., 2000) presumably by physically shielding fusion sites. Correspondingly, in pancreatic acinar cells a negative correlation was found between the thickness of the actin shield and the level of exocytosis (Muallem et al., 1995). Third, apart from a direct interaction of munc18-1 with microtubules, mint-1 interacts with an ADP ribosylation factor (ARF), which in turn is involved in axonal elongation and branching (Hernandez-Deviez *et al*., 2004; Hill *et al*., 2003). Fourth, cypin, another microtubule binding protein, was recently found to increase neurite branching and elongation after stimulation of neuronal activity (Akum et al., 2004). Should munc18-1 have a similar function then it could regulate neurite outgrowth and branching not only by mediating neurotransmitter release but also by rearrangement of structural proteins. Finally, disruption of cytoskeletal rearrangements might directly cause a cell to enter apoptosis. For instance, it has been found that a defect in actin dynamics induces apoptosis in non-neuronal cells, interestingly by becoming unresponsive to trophic support

(Morley & Bierer, 2001). Thus, the association of munc18-1 with the neuronal cytoskeleton might prove to be important, not only for neuronal survival but also, through yet unsuspected involvement, in regulated secretion and neurite outgrowth. However, according to our experiments, (the lack of) regulated secretion itself does not appear to be a crucial determinant of neuronal viability.

General conclusions

In this thesis *munc18-1* deficient neurons were used as a model to investigate the role of regulated synaptic secretion in neuronal survival, neurite outgrowth and synapse formation.
Our finding that cultured *munc18-1* deficient neurons show absolutely no neurotransmitter secretion but display otherwise normal biophysical behaviour during the first 10DIV justifies the use of this model. As degeneration of *munc18-1* deficient neurons could not be prevented by simulation of neurotransmitter secretion and/or neurotrophic support, *munc18-1* can be assumed to be essential for long-term neuronal survival, but not necessarily through its role in regulated secretion.
Basic neurite outgrowth and some branching is observed in *munc18-1* deficient neurons and thus, regulated secretion is not essential for these processes. Later stages of neurite outgrowth and branching are aberrant in mutant neurons. Our finding that the absence of *munc18-1* has an inverse effect on neurite length in peripheral and central neurons, combined with the increased formation and length of growth cone filopodia, minimizes the chance that *munc18-1* mediated vesicular fusion actually regulates neurite outgrowth by providing membrane to growth cones. Regulated secretion might direct neurite outgrowth and branching through regulation of neuronal activity which might have different effects in central and peripheral neurons. Alternatively *munc18-1* might regulate outgrowth and branching by directly binding to cytoskeletal proteins such as microtubules. Thus, there is a clear correlation between *munc18-1* and neurite outgrowth and branching, but it is unclear to what extend regulated secretion is necessary for these processes.
Synapse formation does occur in the absence of *munc18-1* and is therefore in principle not dependent on regulated secretion. Although preliminary, our data suggest that central synaptic density is not affected by the lack of *munc18-1* and the reduced number of synapses observed in mutant

neurons is more likely to reduced dendrite outgrowth rather than a defect in synapse formation *per se*. The higher rate of new filopodium formation, but not filopodial number actually suggests an increase in synapse formation combined with a lack of synapse stabilization. Also peripherally, the lack of refinement of postsynaptic receptor clusters suggests a lack of synapse stabilization rather that formation. A lack of regulated secretion is likely to underlie this effect of *munc18-1* on synapse stabilization, but alternatively other functions of munc18-1, such as binding to mint-1, might be involved. Thus, despite a high correlation between *munc18-1*, regulated secretion and synapse stabilization, it cannot be concluded that there is a causal relationship between regulated secretion and synapse formation or stabilization.

In conclusion, studying *munc18-1* deficient neurons has not confirmed the suspected causal relationship between regulated secretion, neuronal survival, neurite outgrowth and synapse formation. Interestingly, *munc18-1* is not only essential for regulated secretion, but also performs several other cellular functions that might influence these parameters. Thus, *munc18-1* itself might regulate neuronal survival, neurite outgrowth and synaptic plasticity but not necessarily through its effect on secretion.

Future perspectives

As pointed out in the general introduction, conventional pharmacology is insufficient to explain the role of regulated secretion in neurite outgrowth, synapse formation and neuronal survival. Although confirming that these parameters are correlated, the model of *munc18-1* deficient neurons has not provided a definitive solution. Thus, regulated secretion appears to be less directly involved in neuronal morphology and survival than anticipated. However, *munc18-1* deficient neurons might still be a valuable tool to address this issue. Especially the system described in chapter 5 in which mutant forms of *munc18-1* can be analyzed on a null mutant background might solve whether these parameters are interrelated or not.

One of the major obstacles in this project was to keep *munc18-1* deficient neurons in culture long enough to analyze synapse formation and turnover. With the development of glial islands and organotypic culturing methods and microscopes to monitor these cultures in real time, these points can now finally be addressed.

An alternative approach to test the relation between *munc18-1*, regulated

secretion and neurite outgrowth would be to compare wildtype neurons with *munc18-1* overexpressing neurons. Normal pharmacology can now be used to stimulate or block secretion and/or the reception of neurotransmitters. In this way *munc18-1* content, regulated secretion and neuronal activity can be isolated and their involvement in neurite outgrowth and branching addressed. In addition, new mutants such as vesicular neurotransmitter transporter deficient mice could be analysed and compared with existing models.

Finally, it is intriguing that *munc13-1/2* deficient neurons that are similar, but not identical to *munc18-1* mutants in their secretion phenotype, are apparently unaffected in neurite outgrowth, synapse formation and neuronal survival. Closer examination of the differences and common features of these two mutants might therefore shine new light on the mysterious role of regulated secretion.

Reference List

Akum, B. F., Chen, M., Gunderson, S. I., Riefler, G. M., Scerri-Hansen, M. M., & Firestein, B. L. (2004). Cypin regulates dendrite patterning in hippocampal neurons by promoting microtubule assembly. *Nat.Neurosci.* 7, 145-152.

Banks, G. B., Chau, T. N., Bartlett, S. E., & Noakes, P. G. (2001). Promotion of motoneuron survival and branching in rapsyn-deficient mice. *J.Comp Neurol.* 429, 156-165.

Berg, E. A., Johnson, R. J., Leeman, S. E., Boyd, N., Kimerer, L., & Fine, R. E. (2000). Isolation and characterization of substance P-containing dense core vesicles from rabbit optic nerve and termini. *J Neurosci Res* 62, 830-9.

Bhaskar, K., Shareef, M. M., Sharma, V. M., Shetty, A. P., Ramamohan, Y., Pant, H. C., Raju, T. R., & Shetty, K. T. (2004). Co-purification and localization of Munc18-1 (p67) and Cdk5 with neuronal cytoskeletal proteins. *Neurochem Int* 44, 35-44.

Biederer, T. & Sudhof, T. C. (2000). Mints as adaptors. Direct binding to neurexins and recruitment of munc18. *J Biol Chem* 275, 39803-6.

Bouwman, J., Maia, A. S., Camoletto, P. G., Posthuma, G., Roubos, E. W., Oorschot, V. M. J., Klumperman, J., & Verhage, M. (2004). Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. *Neuroscience* 126, 115-126.

Brandon, E. P., Lin, W., D'Amour, K. A., Pizzo, D. P., Dominguez, B., Sugiura, Y., Thode, S., Ko, C. P., Thal, L. J., Gage, F. H., & Lee, K. F. (2003). Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. *J Neurosci* 23, 539-49.

Brewer, G. J. & Cotman, C. W. (1989). NMDA receptor regulation of neuronal morphology in cultured hippocampal neurons. *Neurosci Lett* 99, 268-73.

Burrone, J. & Murthy, V. N. (2003). Synaptic gain control and homeostasis. *Curr.Opin.Neurobiol.* 13, 560-567.

Dailey, M. E. & Smith, S. J. (1996). The dynamics of dendritic structure in developing hippocampal slices. *J Neurosci* 16, 2983-94.

de Paiva, A., Meunier, F. A., Molgo, J., Aoki, K. R., & Dolly, J. O. (1999). Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. *Proc.Natl.Acad.Sci.U.S.A* 96, 3200-3205.

Dunaevsky, A., Tashiro, A., Majewska, A., Mason, C., & Yuste, R. (1999). Developmental regulation of spine motility in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 96, 13438-43.

Featherstone, D. E., Rushton, E., & Broadie, K. (2002). Developmental regulation of glutamate

receptor field size by nonvesicular glutamate release. *Nat Neurosci* 5, 141-6.
Fischer, M., Kaech, S., Knutti, D., & Matus, A. (1998). Rapid actin-based plasticity in dendritic spines. *Neuron* 20, 847-54.
Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P., & Sanes, J. R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85, 525-535.
Hamabe, W., Fujita, R., & Ueda, H. (2003). Neuronal necrosis inhibition by insulin through protein kinase C-activation. *J Pharmacol Exp Ther*.
Hazuka, C. D., Foletti, D. L., Hsu, S. C., Kee, Y., Hopf, F. W., & Scheller, R. H. (1999). The sec6/8 complex is located at neurite outgrowth and axonal synapse- assembly domains. *J Neurosci* 19, 1324-34.
Heeroma, J. H., Plomp, J. J., Roubos, E. W., & Verhage, M. (2003). Development of the mouse neuromuscular junction in the absence of regulated secretion. *Neuroscience* 120, 733-44.
Hernandez-Deviez, D. J., Roth, M. G., Casanova, J. E., & Wilson, J. M. (2004). ARNO and ARF6 Regulate Axonal Elongation and Branching through Downstream Activation of Phosphatidylinositol 4-Phosphate 5-Kinase {alpha}. *Molecular Biology of the Cell* 15, 111-120.
Hill, K., Li, Y., Bennett, M., McKay, M., Zhu, X., Shern, J., Torre, E., Lah, J. J., Levey, A. I., & Kahn, R. A. (2003). Munc18 Interacting Proteins: ADP-RIBOSYLATION FACTOR-DEPENDENT COAT PROTEINS THAT REGULATE THE TRAFFIC OF {beta}-ALZHEIMER'S PRECURSOR PROTEIN. *Journal of Biological Chemistry* 278, 36032-36040.
Ho, C. S., Marinescu, V., Steinhilb, M. L., Gaut, J. R., Turner, R. S., & Stuenkel, E. L. (2002). Synergistic effects of Munc18a and X11 proteins on amyloid precursor protein metabolism. *J Biol Chem* 277, 27021-8.
Holland, R. L. & Brown, M. C. (1980). Postsynaptic transmission block can cause terminal sprouting of a motor nerve. *Science* 207, 649-651.
Houenou, L. J., Pincon-Raymond, M., Garcia, L., Harris, A. J., & Rieger, F. (1990). Neuromuscular development following tetrodotoxin-induced inactivity in mouse embryos. *J.Neurobiol.* 21, 1249-1261.
Jontes, J. D. & Smith, S. J. (2000). Filopodia, spines, and the generation of synaptic diversity. *Neuron* 27, 11-4.
Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S., & Malinow, R. (2003). APP Processing and Synaptic Function. *Neuron* 37, 925-37.
Lauri, S. E., Lamsa, K., Pavlov, I., Riekki, R., Johnson, B. E., Molnar, E., Rauvala, H., & Taira, T. (2003). Activity blockade increases the number of functional synapses in the hippocampus of newborn rats. *Mol Cell Neurosci* 22, 107-17.
Lendvai, B., Stern, E. A., Chen, B., & Svoboda, K. (2000). Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature* 404, 876-81.
Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R., & Lee, K. F. (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410, 1057-64.
Lipton, S. A. & Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* 12, 265-70.
Martin-Verdeaux, S., Pombo, I., Iannascoli, B., Roa, M., Varin-Blank, N., Rivera, J., & Blank, U. (2003). Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. *J Cell Sci* 116, 325-334.
Martinez-Arca, S., Coco, S., Mainguy, G., Schenk, U., Alberts, P., Bouille, P., Mezzina, M., Prochiantz, A., Matteoli, M., Louvard, D., & Galli, T. (2001). A common exocytotic mechanism mediates axonal and dendritic outgrowth. *J.Neurosci.* 21, 3830-3838.
Morley, S. C. & Bierer, B. E. (2001). The actin cytoskeleton, membrane lipid microdomains, and T cell signal transduction. *Adv.Immunol.* 77, 1-43.
Muallem, S., Kwiatkowska, K., Xu, X., & Yin, H. L. (1995). Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J Cell Biol.* 128, 589-598.
Murthy, M., Garza, D., Scheller, R. H., & Schwarz, T. L. (2003). Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. *Neuron* 37, 433-47.

Naren, A. P., Nelson, D. J., Xie, W., Jovov, B., Pevsner, J., Bennett, M. K., Benos, D. J., Quick, M. W., & Kirk, K. L. (1997). Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature* 390, 302-305.

Prange, O. & Murphy, T. H. (2001). Modular transport of postsynaptic density-95 clusters and association with stable spine precursors during early development of cortical neurons. *J Neurosci* 21, 9325-33.

Rao, A., Cha, E. M., & Craig, A. M. (2000). Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. *J Neurosci* 20, 8344-53.

Rosenmund, C. & Stevens, C. F. (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron* 16, 1197-207.

Saitoe, M., Schwarz, T. L., Umbach, J. A., Gundersen, C. B., & Kidokoro, Y. (2001). Absence of junctional glutamate receptor clusters in Drosophila mutants lacking spontaneous transmitter release. *Science* 293, 514-7.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., & Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101, 657-69.

Schulze, K. L. & Bellen, H. J. (1996). Drosophila syntaxin is required for cell viability and may function in membrane formation and stabilization. *Genetics* 144, 1713-24.

Sikorski, A. F., Sangerman, J., Goodman, S. R., & Critz, S. D. (2000). Spectrin (betaSpIIsigma1) is an essential component of synaptic transmission. *Brain Res* 852, 161-6.

Smith, M. A. & Hilgenberg, L. G. (2002). Agrin in the CNS: a protein in search of a function? *Neuroreport* 13, 1485-1495.

Steiner, P., Sarria, J. C., Huni, B., Marsault, R., Catsicas, S., & Hirling, H. (2002). Overexpression of neuronal Sec1 enhances axonal branching in hippocampal neurons. *Neuroscience* 113, 893-905.

Tashiro, A., Dunaevsky, A., Blazeski, R., Mason, C. A., & Yuste, R. (2003). Bidirectional regulation of hippocampal mossy fiber filopodial motility by kainate receptors: a two-step model of synaptogenesis. *Neuron* 38, 773-84.

Toonen, R. F. & Verhage, M. (2003). Vesicle trafficking: pleasure and pain from SM genes. *Trends Cell Biol* 13, 177-86.

Turrigiano, G. G. & Nelson, S. B. (2000). Hebb and homeostasis in neuronal plasticity. *Curr.Opin.Neurobiol.* 10, 358-364.

Varoqueaux, F., Sigler, A., Rhee, J. S., Brose, N., Enk, C., Reim, K., & Rosenmund, C. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc Natl Acad Sci U S A* 99, 9037-42.

Verhage, M., Maia, A. S., Plomp, J. J., Brussaard, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., van den Berg, T. K., Missler, M., Geuze, H. J., & Sudhof, T. C. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287, 864-869.

Voets, T., Toonen, R. F., Brian, E. C., de Wit, H., Moser, T., Rettig, J., Sudhof, T. C., Neher, E., & Verhage, M. (2001). Munc18-1 promotes large dense-core vesicle docking. *Neuron* 31, 581-91.

Wang, X., Butowt, R., & von Bartheld, C. S. (2003). Presynaptic neurotrophin-3 increases the number of tectal synapses, vesicle density, and number of docked vesicles in chick embryos. *J Comp Neurol* 458, 62-77.

Washbourne, P., Thompson, P. M., Carta, M., Costa, E. T., Mathews, J. R., Lopez-Bendito, G., Molnar, Z., Becher, M. W., Valenzuela, C. F., Partridge, L. D., & Wilson, M. C. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci* 5, 19-26.

Weimer, R. M., Richmond, J. E., Davis, W. S., Hadwiger, G., Nonet, M. L., & Jorgensen, E. M. (2003). Defects in synaptic vesicle docking in unc-18 mutants. *Nat.Neurosci.* 6, 1023-1030.

Yamada, M., Tanabe, K., Wada, K., Shimoke, K., Ishikawa, Y., Ikeuchi, T., Koizumi, S., & Hatanaka, H. (2001). Differences in survival-promoting effects and intracellular signaling properties of BDNF and IGF-1 in cultured cerebral cortical neurons. *J Neurochem* 78, 940-51.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C., & Burden, S. J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* 30, 399-410.

Zhao, H. & Nonet, M. L. (2000). A retrograde signal is involved in activity-dependent remodeling at a C. elegans neuromuscular junction. *Development* 127, 1253-1266.
Zimmer, W. E., Zhao, Y., Sikorski, A. F., Critz, S. D., Sangerman, J., Elferink, L. A., Xu, X. S., & Goodman, S. R. (2000). The domain of brain beta-spectrin responsible for synaptic vesicle association is essential for synaptic transmission. *Brain Res.* 881, 18-27.
Ziv, N. E. & Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17, 91-102.

Nederlandse samenvatting

De rol van gereguleerde secretie in neurietuitgroei, synapsformatie en neuronale overleving

Munc18-1 als een spin in het web

Zenuwcellen, ook wel neuronen genoemd, communiceren met elkaar door de afgifte van neurotransmitters en andere stoffen, zogeheten neurotrofe substanties. Die communicatie vindt plaats in de contactpunten tussen neuronen, de synapsen, en geschiedt door middel van de fusie van kleine, neurotransmittergevulde blaasjes met het membraan van de cel. Om het brein te laten werken is het essentieel dat de communicatie tussen neuronen betrouwbaar is en dus dat de secretie (afgifte) van transmitters goed gereguleerd wordt. Er zijn talloze factoren geïdentificeerd die in meer of mindere mate neurotransmittersecretie beïnvloeden en daarmee dus neuronale communicatie en vermoedelijk de werking van de hersenen beïnvloeden. Een van deze factoren, munc18-1, onderscheidt zich van de rest in die zin dat het absoluut noodzakelijk is voor neurotransmitter-secretie; zonder munc18-1 geen secretie.

In tegenstelling tot wat lange tijd is aangenomen is het brein niet statisch maar bijzonder dynamisch. Voortdurend sterven er neuronen en komen er nieuwe bij. Er bestaan zelfs schattingen dat tot 20% van de ruwweg 150 miljoen miljard synapsen in onze hersenen niet langer dan 1 dag bestaan. Het ligt voor de hand om aan te nemen dat deze plasticiteit in de hersenen verbonden is aan de functie van de hersenen, namelijk het opslaan van informatie. Hieruit volgt de aanname dat de neurotransmittersecretie die ten grondslag ligt aan hersenactiviteit een rol speelt bij het vormen en veranderen van de verbindingen tussen neuronen en daarmee de structuur van de hersenen beïnvloedt.

Het doel van mijn promotieonderzoek was om te kijken in hoeverre gereguleerde secretie, en dan in het bijzonder neurotransmitter secretie, bepalend is voor de uitgroei van neurieten (verbindingen tussen neuronen) en voor de levensloop van neuronen en synapsen. Hiertoe heb ik de ontwikkeling van neuronen bestudeerd in een muis die genetisch zo gemanipuleerd is dat er geen munc18-1 meer wordt aangemaakt en er dus ook totaal geen gereguleerde secretie in deze neuronen plaatsvindt.

In hoofdstuk 2 hebben we gekeken naar de embryonale ontwikkeling van de middenrifspier en de zenuw die deze aanstuurt. De relatief grote afmetingen en karakteristieke structuur van deze zenuw maken een nauwkeurige analyse mogelijk van wat er wel en niet goed gaat met de vorming van dit systeem (de interface tussen spier en zenuw) in de afwezigheid van neurotransmit tersecretie. In munc18-1-loze muizen groeide de middenrifzenuw volledig uit naar de spier, vertakte deze in eerste instantie normaal en vormde zelfs synapsen met de spier. Ook leken de receptoren in de spier, die normaal gesproken het neurotransmittersignaal zouden omzetten in samentrekking van de spier, in normale hoeveelheden aanwezig en functioneel. Los van de initiële vertakking bleken de laatste fasen van zenuwvertakking abnormaal te zijn en er waren dan ook veel minder synapsen dan normaal aanwezig. Bovendien waren de receptoren in de spier, die dus vaak niet tegen een zenuwuiteinde aanlagen, abnormaal geclusterd en gedistribueerd door de spier. Dus, hoewel neurotransmittersecretie niet strikt noodzakelijk is voor de uitgroei van neurieten en de vorming van basale synapsen, lijkt het wel essentieel te zijn voor de bulkfase van synapsvorming alsmede de afwerking van deze synapsen. Tot slot stierven de middenrifzenuwen die de spier aansturen massaal af terwijl vreemd genoeg de sensorische zenuwen, die informatie uit de spier terugkoppelen naar de hersenen, niet aangedaan leken te zijn. Aangezien deze laatste groep zijn input niet krijgt via neurotransmittersecretie leidde dat tot de hypothese dat wellicht het ontvangen van input belangrijker is voor het in leven blijven van neuronen dan het genereren van output (de secretie van neurotransmitters).
In hoofdstuk 3 zijn we dieper op deze input-output kwestie ingegaan en hebben we geprobeerd gekweekte munc18-1 deficiënte neuronen in leven te houden door ze op allerlei manieren van input te voorzien. Het gemixed kweken van munc18-1-loze en normale neuronen, waarbij wordt aangenomen dat de normale neuronen de mutante neuronen van input voorzien, had geen effect op de levensvatbaarheid. Verrassend genoeg, leken mutante neuronen het langer uit te houden wanneer ze niet volledig gedissocieerd maar in hersenweefsel, omgeven door gliacellen, gekweekt werden. Dit effect werd versterkt door mutant weefsel in combinatie met normaal weefsel te kweken. Dit leidde tot de aanname dat mutante neuronen wellicht niet eens zozeer afhankelijk waren van neurotransmitter- secretie, maar meer van de secretie van andere stoffen, de zogeheten neurotrofines. Het direct toevoegen van neurotrofe stoffen aan het kweekmedium, of het kweken op een voedende laag gliacellen kon inderdaad het afsterven uitstellen maar nooit voorkomen. Om de input op mutante cellen zo natuurgetrouw mogelijk te maken hebben we zelfs een mutante muis gemaakt waarin pas na de ontwikkeling van de

hersenen in een specifieke groep neuronen munc18-1 werd uitgeschakeld, maar ook deze mutante neuronen gingen dood. Op basis hiervan hebben we geconcludeerd dat neuronen zonder munc18-1 weliswaar dood gaan, maar dat dit niet noodzakelijk komt door het ontbreken van gereguleerde secretie maar door een ander, onbekend, effect.
In hoofdstuk 4 hebben we gebruik gemaakt van het levensverlengende effect van neurotrofe stoffen en gliacellen om langer naar de ontwikkeling van de neurieten en synapsen van munc18-1-loze neuronen te kunnen kijken. We vonden dat de uitgroei van neurieten van munc18-1-loze neuronen de eerste drie dagen in kweek volkomen normaal was, en dus niet afhankelijk van gereguleerde secretie. Echter, na drie dagen was de uitgroeisnelheid van deze neurieten wel minder terwijl er meer kleine uitlopers op deze neurieten zaten. Verder waren deze uitlopers, die ook wel gezien worden als voorlopers van synapsen, langer dan normaal. Na zeven dagen in kweek waren de neurieten in mutante neuronen korter dan normaal en hadden deze veel minder vertakkingen, terwijl op het eerste gezicht de dichtheid van synapsen op deze neurieten normaal was. Samen leidde dit tot het model dat de initiële uitgroei van neurieten onafhankelijk is van munc18-1/neurotransmittersecretie tot het begin van synapsformatie. Vanaf dit punt lijken functionele synapsen zichzelf te stabiliseren en een beginpunt voor verdere neurietvertakking en uitgroei te worden. Munc18-1-loze neuronen lijken daarentegen continu nieuwe pogingen te wagen om synapsen te vormen die echter niet gestabiliseerd kunnen worden en verdere neurietuitgroei in de weg staan.
In hoofdstuk 5 wilden we uitsluiten dat munc18-1-loze neuronen in kweek sterven door een latent defect dat al tijdens de embryonale ontwikkeling was ontstaan en tegelijkertijd een systeem ontwikkelen om mutante vormen van munc18-1 te testen waarmee we uiteindelijk de verschillende functies van munc18-1 kunnen ontrafelen. We hebben virussen ontworpen om na de embryonale ontwikkeling alsnog munc18-1 in mutante neuronen aan te maken. Deze neuronen ontwikkelden zich volledig normaal, maakten normale uitlopers, vertakkingen en synapsen die normaal neurotransmitters konden secreteren. Dit betekende dus dat de mutante neuronen op het moment van kweken in principe gezond waren en dat we op vergelijkbare manier de werking van mutante vormen van munc18-1 konden analyseren. Uiterst preliminaire resultaten lijken aan te geven dat de meest voor de hand liggende interacties van munc18-1 niet zo strikt vereist zijn voor neuronale levensvatbaarheid, neurietuitgroei en gereguleerde secretie als wij van tevoren hadden aangenomen.
Kort samengevat heb ik in dit promotieonderzoek getracht causale

verbanden te vinden tussen de functie (gereguleerde secretie/activiteit), de vorm (uitgroei en contacten) en de levensvatbaarheid van zenuwen. Hiertoe heb ik een genetisch gemanipuleerde muis geanalyseerd die geen munc18-1 maakt, hierdoor geen neurotransmitters kan uitscheiden en als gevolg volledig hersendood is. De resultaten van mijn studie geven aan dat de factoren “gereguleerde secretie”, “neurietuitgroei”, “synapsformatie” en “neuronale overleving” allemaal direct met munc18-1 verbonden zijn en op die manier met elkaar correleren. Echter, directe causale verbanden tussen deze factoren zelf heb ik niet kunnen aantonen. Vandaar dat ik via de titel en subtitel van dit proefschrift stel dat het niet zozeer hersenactiviteit, maar munc18-1 is dat als een spin in het neuronale web zit.

Dankwoord

Voor diegenen die het tot hier gered hebben als bonus een dankwoord ;-) Heel kort samengevat wil ik bij dezen iedereen danken die mij heeft geholpen deze “survival of the fittest” te doorstaan. Meer specifiek wil ik als eerste de leescommissie bedanken en dan speciaal Eric Boddeke die mij enorm heeft gestimuleerd en de charme van de wetenschap heeft laten zien. Daarnaast wil ik uiteraard mijn promotor Matthijs bedanken die het stokje heeft overgenomen en mij naar de eindstreep heeft geleid. Al heb je het mij de afgelopen 6 jaar beslist niet gemakkelijk gemaakt, je tomeloze energie, inzicht en doelgerichtheid zijn een bron van inspiratie voor me geweest en ik heb ontzettend veel van je geleerd. Je hebt me de realiteit van de wetenschap bijgebracht. Naast de leiding van Matthijs heb ik veel gehad aan de begeleiding van Ruud en Sander. De honderden peukje-pauzes en mensa-maaltijden vormen de basis van menig experiment en model in dit proefschrift en waren vooral bijzonder aangenaam. Daarnaast wil ik alle collega’s bedanken met wie ik al deze jaren, eerst op het RMI, toen op de VU en nu bij het UCL, werk, lief, leed, lol en katers heb gedeeld. Onderzoek doe je niet alleen. Anita, Bert, Chris, Danielle, Désirée, Jaap, Johan, Joost, Karlijn, Keimpe, Martijn, Matthijs, Robert, Robbie, Ruud en Tatjana, ontzettend bedankt voor de onmisbare bijdrage die jullie aan dit boekje hebben geleverd. Speciale dank gaat uit naar mijn lotgenoten Jildau en Elisabeth en andere kamergenoten, Chris, K.J., Linda en op de valreep Roland. Het aantal uren dat wij hebben doorgebracht met geroddel, geleuter, gelach of gekanker al naar gelang onze situatie is ontelbaar en van grote therapeutische waarde geweest. Tina en Jildau waren onvermoeibaar en vergezelden mij vaak in de avonduren voor nog meer geklep, maar ook de broodnodige etentjes en filmpjes. En, Tina, zonder jou zou dit boekje er niet geweest zijn. Bedankt! Speciaal wil ik mijn paranimfen Daniëlle en Robbie bedanken, niet alleen omdat jullie deze ondankbare baan hebben aangenomen, maar vooral omdat jullie altijd de perfecte combinatie van “business” maar vooral “pleasure” zijn geweest.
Ook al heeft het er af en toe wel de schijn van gehad, mijn leven heeft de afgelopen jaren niet alleen uit werk bestaan. Weliswaar fysiek weg uit Groningen worden de goede herinneringen aan die tijd springlevend gehouden door jaarclub en NWP-clan. Andere relikwieën van die goede ouwe tijd zijn Dylan&Wietske, Martin&Inge, Richard en Annelies, altijd gewillig om te borrelen, filosoferen of mij op een andere manier te “entertainen”. Uit een nog ouder tijdperk zijn er de mensen van de “banaan” met wie ik geloof ik zo’n beetje mijn halve leven tót het begin van mijn OIO-schap heb

doorgebracht. Ik ben jullie allen niet alleen dank verschuldigd maar ook mijn excuses omdat ik dit proefschrift maar al te vaak voor heb laten gaan.
Bas, Daan, Jochem, Joel en Willem, jullie vormen nu al zo'n 20 jaar mijn surrogaatfamilie en hopelijk zijn we nog lang niet klaar. De beste manier om met de frustraties van het promovendusschap om te gaan is af en toe even stevig gal te spuwen en jullie hebben mij daar, letterlijk en figuurlijk, een zee van mogelijkheden voor gegeven...en bedankt.
Tot slot, Papa, Mama, Eva, Hugo & Tom: van nul tot nu zijn jullie er altijd onvoorwaardelijk voor me geweest. Meer dan wie ook hebben jullie het mogelijk gemaakt dat ik hier gekomen ben. Ook al was ik druk, druk, druk en had ik vaak geen tijd, jullie zijn me altijd blijven steunen en stimuleren. Helaas leidt mijn zucht naar kennis mij telkens verder weg. Gelukkig blijven jullie dichtbij middels email, telefoon en vliegtuig. Ik hou van jullie en beloof beterschap (ooit ;-))!

Curriculum vitae

Joost Heeroma werd op 3 juni 1975 in Zutphen geboren. In 1987 begon hij met het gymnasium aan het Stedelijk Lyceum waar hij in 1993 eindexamen deed. In datzelfde jaar begon hij aan de studie Biologie aan de Rijksuniversiteit Groningen. Tijdens de specialisatiefase deed hij twee onderzoekstages bij professor Luiten. In 1996 bestudeerde hij interacties tussen PKC_ en 14-3-3 eiwitten tijdens leertaken in ratten. In 1997 onderzocht hij de effecten van corticosteroïden en serotonine agonisten op neurodegeneratie na een beroerte. In 1998 schreef hij onder begeleiding van professor Boddeke een afstudeerscriptie over genetische factoren die de proliferatieve status van neuronen bepalen, hoe deze factoren betrokken zijn bij hersentumoren enerzijds en neurodegeneratieve ziekten anderzijds en hoe deze factoren mogelijk therapeutisch gebruikt kunnen worden. Ook kloneerde hij tijdens een laatste onderzoeksstage bij professor Boddeke een aantal chemokine receptoren en analyseerde hij hun regulatie tijdens neuro-inflammatoire processen. In 1999 studeerde Joost af in de richtingen Neurobiologie en Medische fysiologie. Van 1999 tot 2004 deed hij zijn promotieonderzoek naar het effect van gereguleerde secretie op neurietuitgroei, synapsformatie en neuronale viabiliteit. Dit gebeurde onder begeleiding van professor Verhage, eerst aan het Rudolf Magnus Instituut voor Neurowetenschappen in Utrecht en daarna aan de Vrije Universiteit in Amsterdam. De resultaten van dat onderzoek zijn in dit proefschrift beschreven. Inmiddels werkt Joost bij de vakgroep Experimentele Epilepsie van professor Kullmann aan het University College London waar hij onder andere de fijnregulatie van synaptische transmissie bestudeert en de rol hiervan in leren en geheugen.

English version

Joost Heeroma was born on June 3rd 1975 in Zutphen, the Netherlands. He started grammar school in 1987 at the “Stedelijk Lyceum” where he graduated in 1993. That year he started to study Biology at the “Rijksuniversiteit Groningen”. During his specialisation he did two projects with professor Luiten. In 1996 he studied the interactions between PKC_ and 14-3-3 proteins during learning tasks in rats. In 1997 he did research on the effects of corticosteroids and serotonin agonists on neurodegeneration after stroke. In 1998 he wrote a thesis under the supervision of professor Boddeke about genetic factors that are of influence on the proliferative status of neurons, how these factors are involved in both brain tumours and neurodegenerative diseases and how these factors could be used therapeutically. In a last

research project with professor Boddeke, he cloned a number of chemokine receptors and analysed their regulation during neuro-inflammatory processes. In 1999 Joost graduated in Neurobiology and Medical physiology. From 1999 to 2004 he did his PhD research on the effects of regulated secretion on neurite outgrowth, synapse formation and neuronal viability. This was supervised by professor Verhage, first at the Rudolf Magnus Institute of Neuroscience in Utrecht, then at the "Vrije Universiteit" in Amsterdam. The results of that study are described in this dissertation. Joost is currently working at the department of Experimental Epilepsy, headed by professor Kullmann, at University College London, where, among other things, he studies mechanisms underlying subtle regulation of neurotransmitter secretion and their relationship to learning and memory.

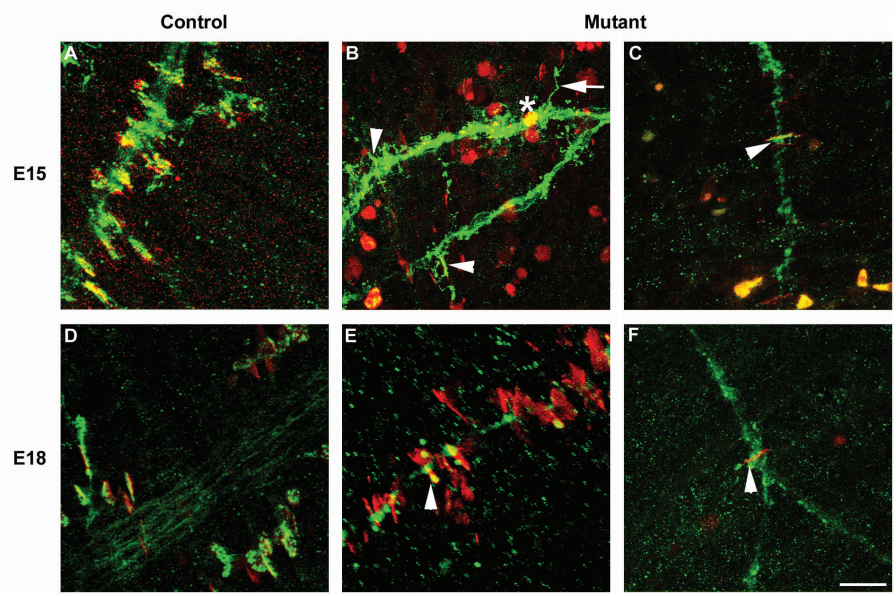

Figure 4. *Synapses are formed in the mutant. At E15 and E18 there is a high degree of co-localization (yellow) of GAP-43/synapsin staining (green) and α-btx (red) in control diaphragms (A, D), indicating the presence of synapses. In E15 mutant diaphragms many AChR clusters are close to presynaptic staining but not in direct contact (B). There are some terminal branches in the mutant (arrow in B) and a few clusters co-localize with these terminal branches (arrowheads in B and C). Co-localization of clusters with higher order (i.e. not terminal) branches (asterisk in B) was considered to be coincidental and was not counted as a synapse. The bright yellow spots in C are autofluorescent erythrocytes. At E18 there are still some spots of co-localization in the mutant diaphragm (arrowheads in E, F). The scale bar is 30 µm (A, B, C, E, F) and 50 µm (D) respectively.*

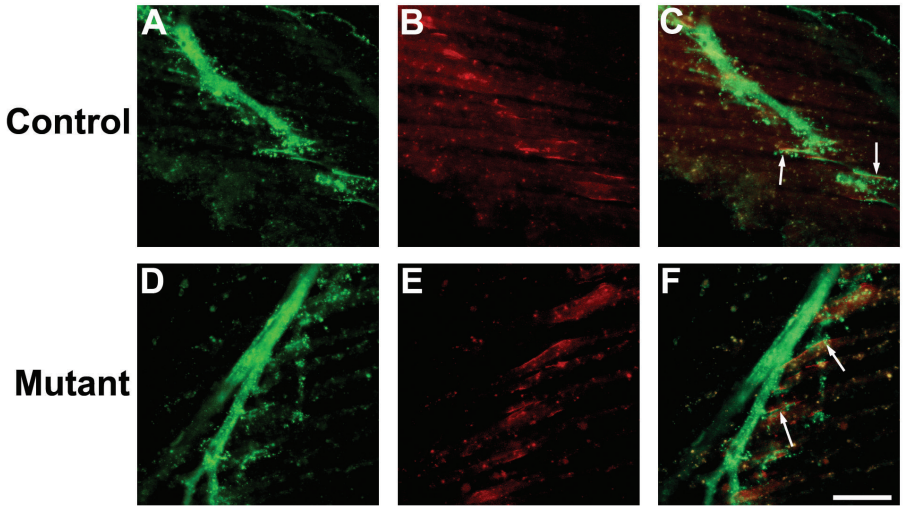

Figure 6. *Synapses are formed in mutant intercostal muscles. At E15 there is co-localization (C) of GAP-43/synapsin stained nerve terminal branches (A) and AChR clusters (B), indicative of synapses. Mutant intercostal muscles also show co-localization (F) of nerve terminal branches (D) and AChR clusters (E) at E15 suggesting that synapses (examples are marked by arrows in C and F) are also formed in mutant intercostal muscles. Scalebar is 50 µm.*

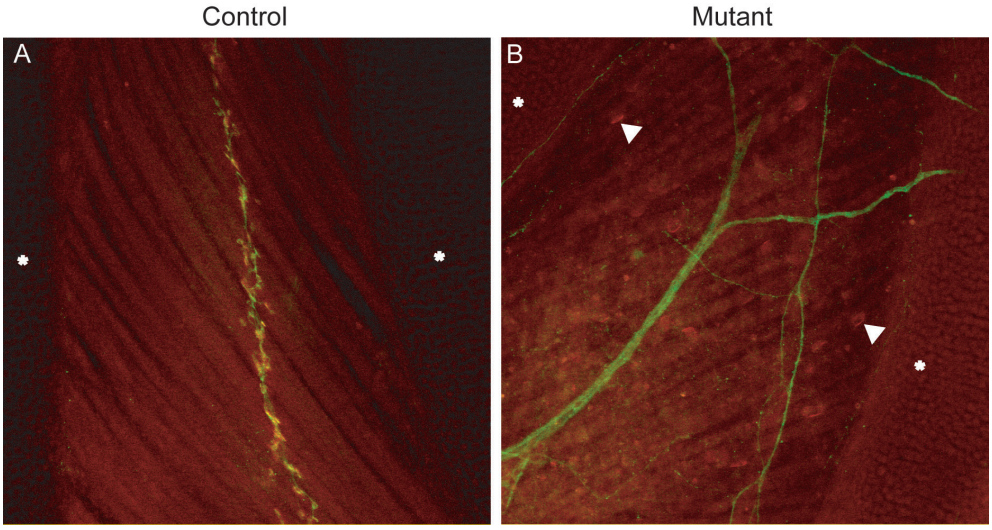

Figure 7. *Combined staining of GAP-43 (green) and α-btx (red) in control E18 intercostal muscles (A) show a highly organized innervation pattern in perfect co-localization (yellow) with all AChR clusters. In mutant muscle (B) co-localization of terminal branches with AChR clusters is absent. As in the diaphragm, the majority of AChR clusters is present in the central half of the muscle, but clearly some clusters are present in ectopic places (arrowheads). White lines demarcate the edges of the muscles. Scalebar is 100 µm.*

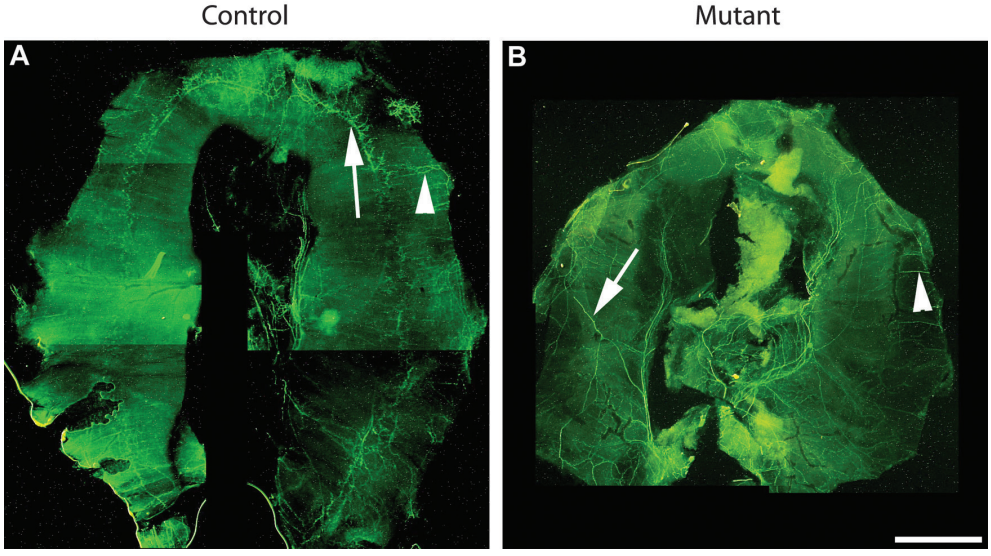

Figure 8. *Motor neuronal innervation is lost. GAP-43 staining in E18 diaphragms shows the characteristic motor neuronal innervation pattern (arrow in A) is lost in the mutant. Most neuronal staining in the mutant diaphragm has no apparent organization or orientation (arrow in B). The presumably sensory neuronal staining at the margin of the diaphragm (arrowheads in A, B) appears to be normal. The scale bar is 1000 µm.*

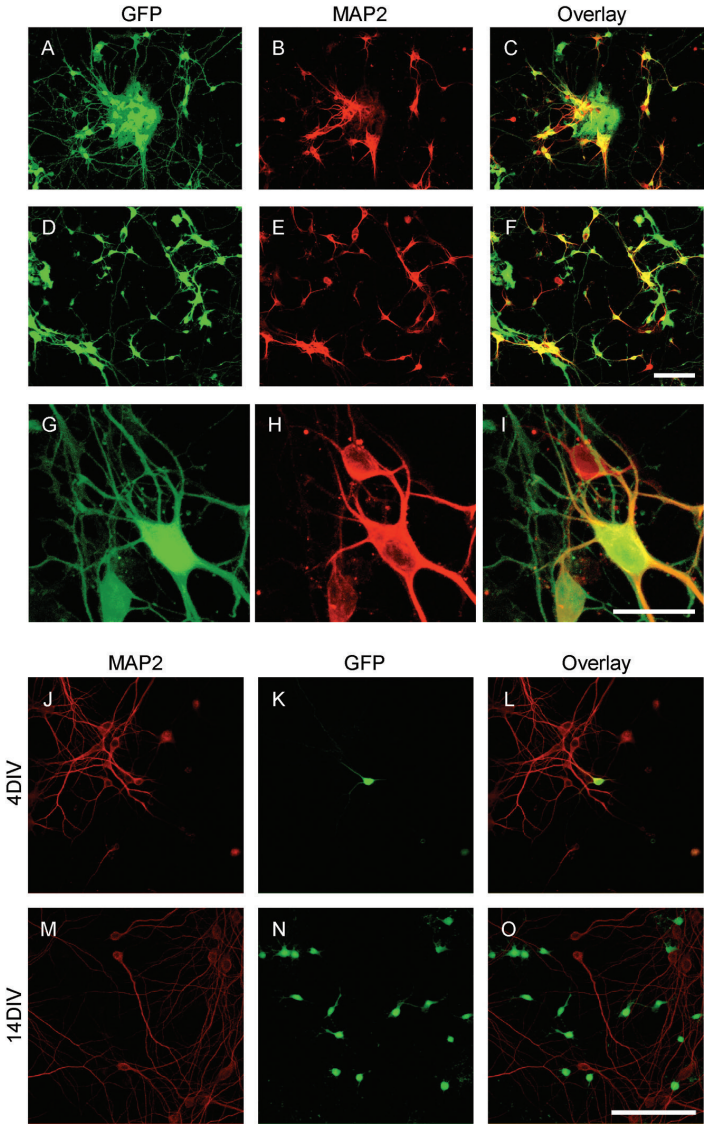

Figure 3: *Co-culturing gfp expressing wild-type neurons and munc18-1 deficient neurons resulted in the formation of neural networks of mixed origin (A – I). Left panels show only the gfp expressing neurons, middle panels show MAP2 counterstaining to identify neurons and right panels show an overlay of both. The higher magnifications (G – I) clearly underlined the fact that wild-type and munc18-1 deficient neurons intermingle. Together they formed dense neuronal networks suggestive of wild-type afferent innervation onto munc18 deficient neurons. Co-cultures of gfp expressing, munc18-1 deficient and wild-type neurons show a rapid decline in the numbers of munc18-1 deficient neurons (J - O). At 4DIV, very few MAP2 positive green cells were present in culture (J - L), suggesting few neurons had survived up to 4DIV. At 14DIV, none of the gfp expressing cells was MAP2 positive (M - O), indicating that all the munc18-1 deficient neurons degenerated. Scale bar is 50 μm (A - F), 25 μm (G - I) and 100 μm (J - O).*

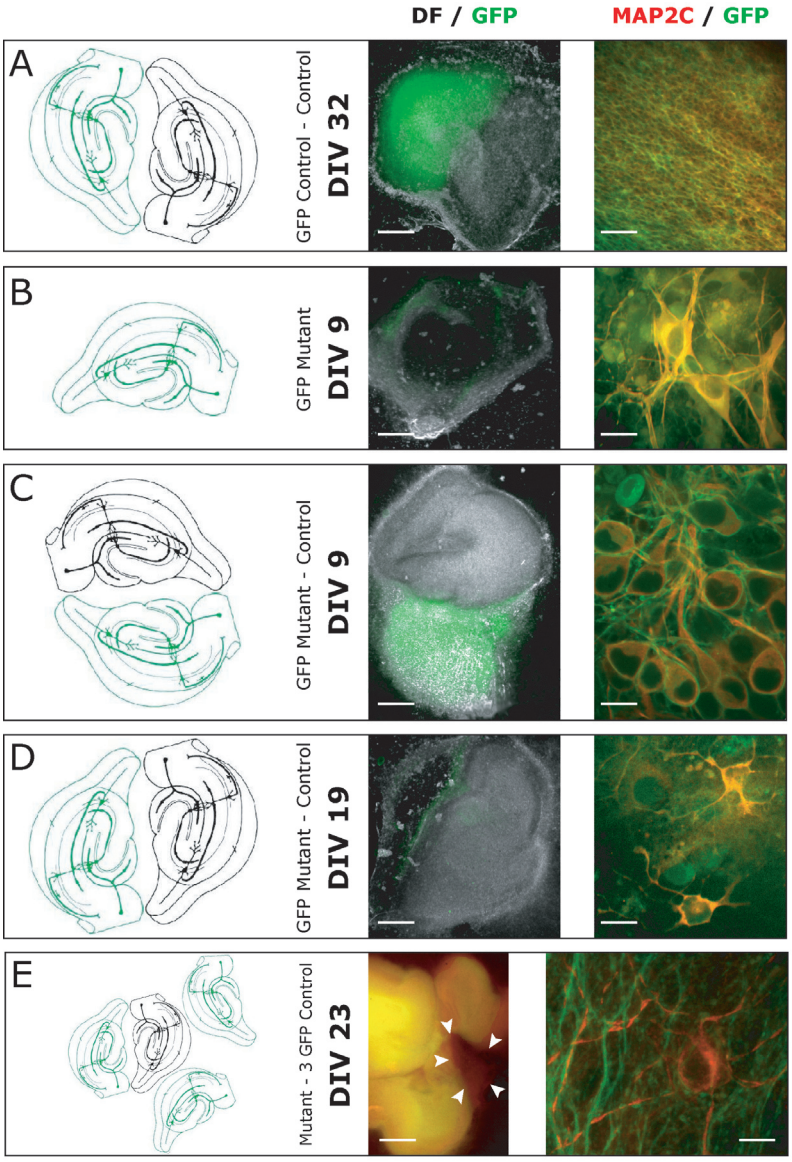

Figure 5: Survival of hippocampal organotypic slice cultures of gfp expressing, munc18-1 deficient neurons with and without co-cultured wild-type slices. At 32DIV, the gross morphology of co-cultures of fluorescent and non-fluorescent (Dark Field) control slices was normal (A, mid panel). In a control hippocampal slice of 9DIV, neurons were densely packed (A, right panel). In a munc18-1 deficient slice of 9DIV (B, mid panel), few MAP2 positive neurons were present (B, right panel). Co-culturing munc18-1 deficient slices with control slices prevented massive degeneration before 9DIV (C, mid panel) and many MAP2 positive, munc18-1 deficient neurons were present (C, right panel). At 19DIV in co-culture, the munc18-1 deficient slice had deteriorated (D, mid panel) and few munc18-1 deficient neurons were left (D, right panel). To maximize the innervation of wild-type axons we also made quadra-cultures of 3 gfp expressing wild-type slice co-cultured with 1 munc18-1 deficient slice. In these cultures, counterstained with MAP2C (red) at 23 DIV, still most of the munc18-1 deficient slice had deteriorated as indicated by the arrowheads (E, mid panel). However some neurons were nonetheless present and gfp expressing wild-type axons had innervated the munc18-1 deficient tissue. The scale bar is 300 µm (A-E, mid panels), 100 (A, left panel) and 40 µm (B-E, left panels), respectively.

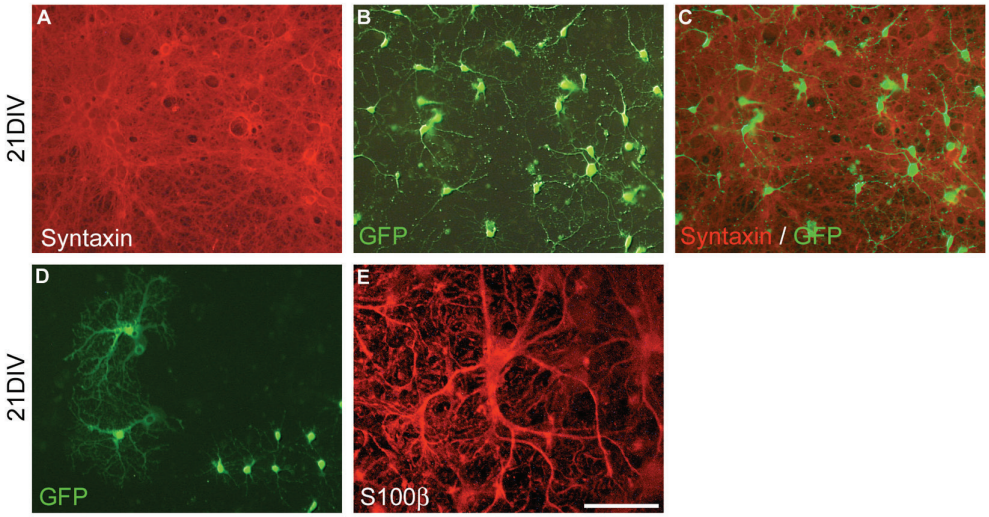

Figure 6: *Munc18-1 deficient glial cells remain in culture. When control and munc18-1 deficient – gfp expressing cortical cells were co-cultured for 21DIV, no munc18-1 deficient neurons were left in culture. There was extensive syntaxin staining, showing the presence of a dense neuronal network (A). There were a number of gfp expressing cells indicating that some munc18-1 deficient cells were still present (B). However, the gfp expressing cells showed no structural overlap with the syntaxin staining (C) and therefore were not syntaxin positive/of neuronal origin. When munc18-1 deficient cortical cells were plated in mono-culture, two cell types survived. Some of these remaining cells had the morphological appearance of oligodendrocytes (D) whereas others were S100β positive and resembled type 2 astrocytes (E). The scale bar is 50 μm.*

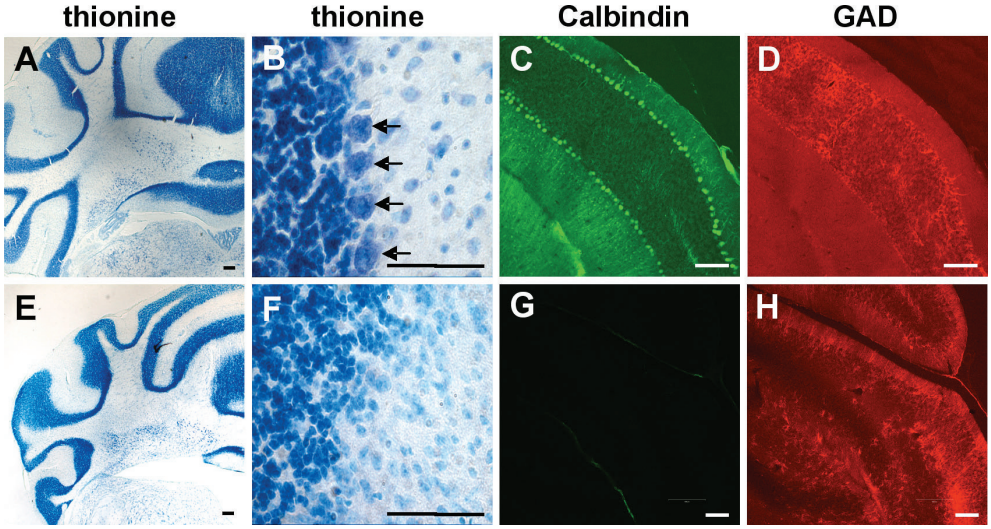

Figure 7: *Loss of munc18-1 deficient neurons in the cerebellum in vivo. The Cre-LoxP system was used to specifically delete munc18-1 in postnatal cerebellar Purkinje cells (E-H). Nissl staining showed normal gross cerebellar anatomy in adult mutant mice (E) compared to wild-types (A). Purkinje cells were present in wild-types (B, arrows) but absent in mutants (F). Staining for Purkinje cell marker calbindin showed a normal pattern in wild-types (C), but no positive cells in mutants (G). Staining for GAD showed GABAergic interneurons and Purkinje cells in wild-types (D) but only interneurons in mutants (H). The scale bar is 100 μm.*

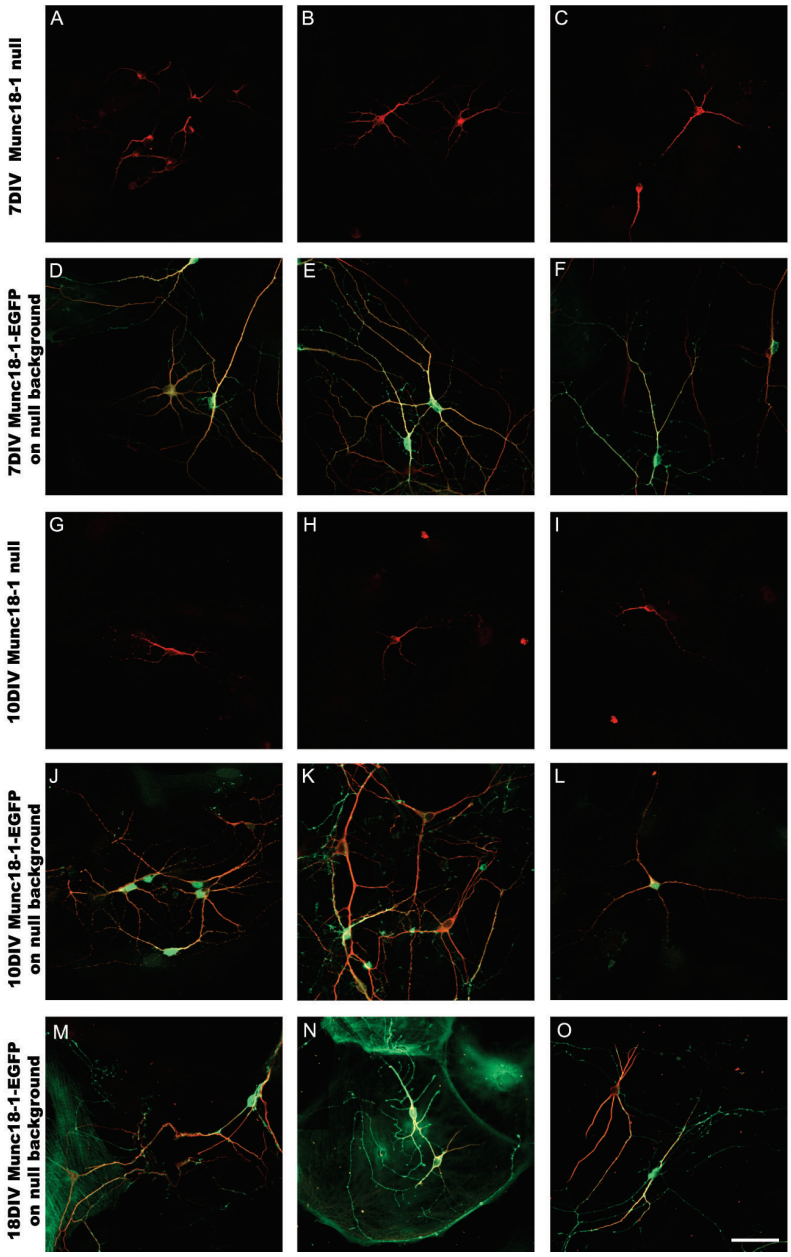

Figure 1: *Transduction of munc18-1 prevents degeneration of munc18-1 deficient neurons. Neurons were stained for dendrite marker MAP2 (red). Panels A-C depict munc18-1 null mutant neurons after 7DIV. Munc18-1-egfp is transduced on a null background have a more pronounced neurite tree (D-F). After 10DIV, only few munc18-1 null mutant neurons are left in culture (G-I), but munc18-1-egfp transduced neurons are still abundant and extending large neurites (J-L). After 18DIV, only munc18-1-egfp expressing neurons survive (M-O). Scale bar is 50 µm.*

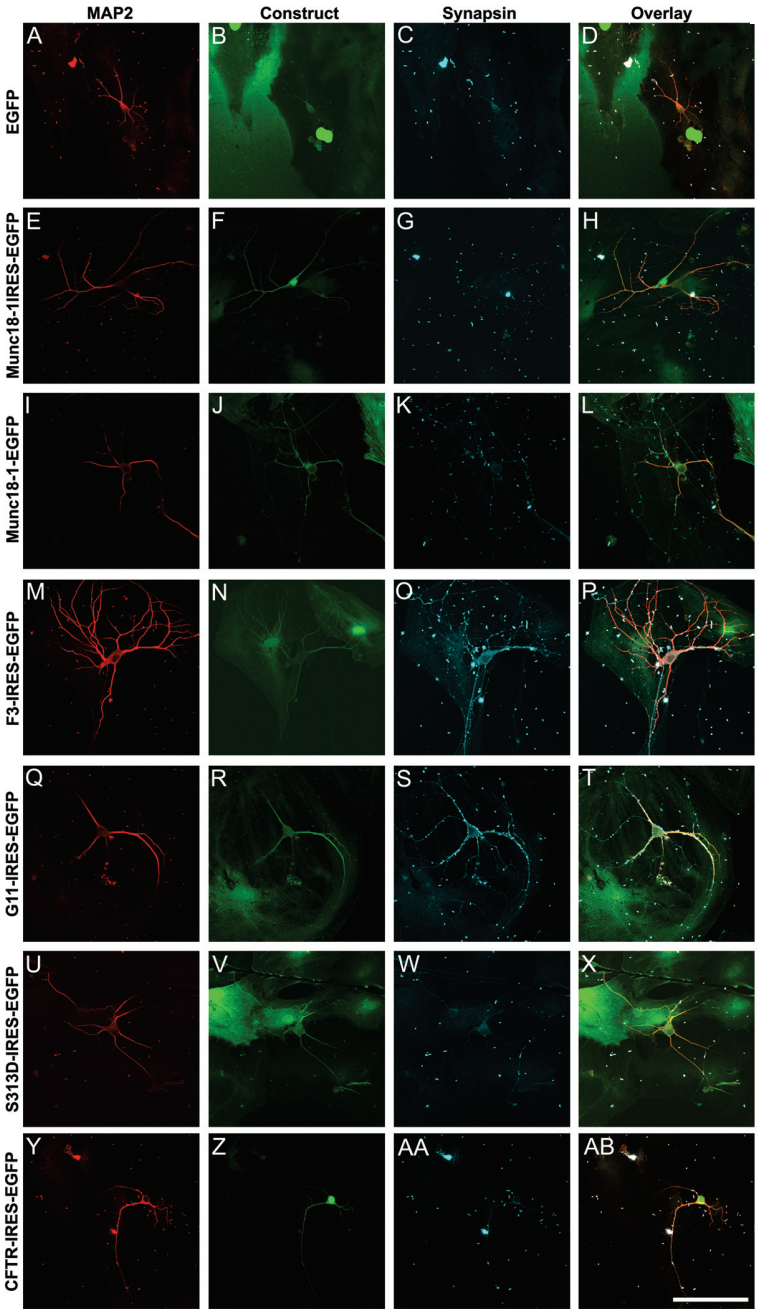

Figure 6: *Cytochemical characterization of 8DIV munc18-1 deficient neurons transgenically expressing munc18-1 mutant proteins. Cortical neurons, deficient for munc18-1, were transduced with egfp, munc18-1-ires-egfp, munc18-1-egfp, r39c-ires-egfp, d34n-ires-egfp, s313d-ires-egfp and d34n;m38v-ires-egfp (green in B, F, J, N, R, V and Z resp.) at div1. At 8DIV the neurons were stained for MAP2 (red in A, E, I, M, Q, T and Y resp.) and synapsin (blue in C, G, K, O, S, W and AA resp.). The overlays are in D, H, L, P, T, X and AB resp. The scale bar is 100 µm.*

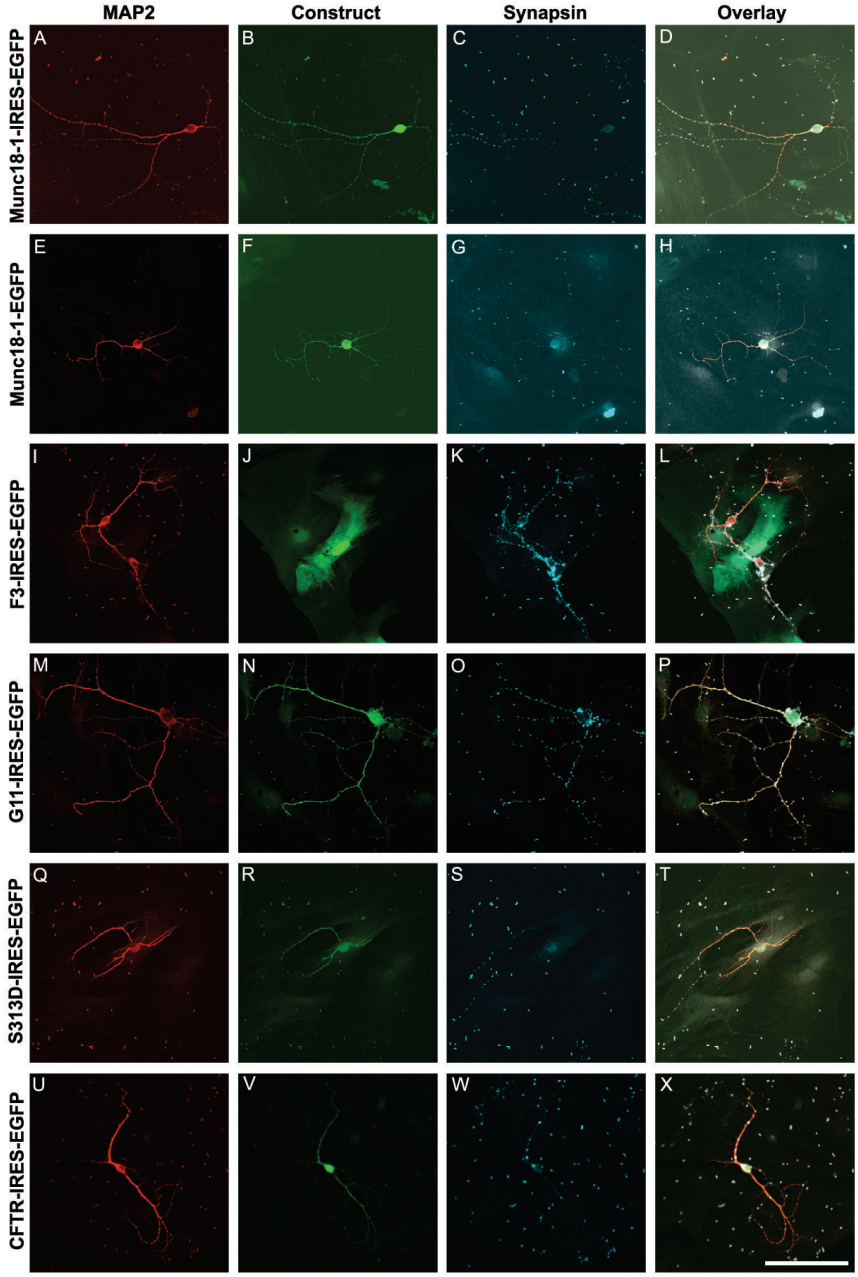

Figure 7: *Cytochemical characterization of 21DIV munc18-1 deficient neurons transgenically expressing munc18-1 mutant proteins. Cortical neurons, deficient for munc18-1, were transduced with, munc18-1-ires-egfp, munc18-1-egfp, r39c-ires-egfp, d34n-ires-egfp, s313d-ires-egfp and d34n;m38v-ires-egfp (green in B, F, J, N, R, and V resp.) at div1. At 21DIV the neurons were stained for MAP2 (red in A, E, I, M, Q, and T resp.) and synapsin (blue in C, G, K, O, S, and W resp.). The overlays are in D, H, L, P, T, and X resp. The scale bar is 100 µm.*