Scuola Internazionale Superiore di Studi Avanzati-Trieste

International School for Advanced Studies

SISSA

Neuroscience

Trans-synaptic signaling at GABAergic connections: possible dysfunction in some forms of Autism Spectrum Disorders

Thesis submitted for the degree of
"Doctor Philosophiae"

14 December 2012

CANDIDATE: **Rocco Pizzarelli**

SUPERVISOR: **Prof. Enrico Cherubini**

Declaration

The original work presented in this thesis was carried out at the International School for Advanced Studies, Trieste, between November 2008 and November 2012. I performed and analyzed all the electrophysiological experiments and contributed to papers writing. Immunofluorescence and molecular biology experiments were performed by Zeynep Kasap, Roberta Antonelli and Paola Zacchi.

✓ Varley ZK*, **Pizzarelli R***, Antonelli R, Stancheva SH, Kneussel M, Cherubini E, Zacchi P. (2011) Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. *Journal of Biological Chemistry*. Jun 10; 286(23):20942-51.

* Equally contributed

✓ **Pizzarelli R** and CherubiniE (2012) Developmental regulation of GABAergic signalling in the hippocampus of neuroligin 3 R451C knock-in mice: an animal model of Autism, *submitted*

✓ **Pizzarelli R** and Cherubini E (2011) Alterations of GABAergic signaling in Autism Spectrum Disorders. *Neural Plasticity*. Epub 2011 Jun 23

The following publication, arising from a collaborative project in which I am co-author has not been included in the present thesis (see appendix).

✓ Martire M, Barrese V, D'Amico M, Iannotti FA, **Pizzarelli R**, Samengo I, Viggiano D, Ruth P, Cherubini E, Taglialatela M (2010) Pre-synaptic BK channels selectively control glutamate versus GABA release from cortical and hippocampal nerve terminals. *Journal of Neurochemistry*. 2010 Oct; 115(2):411-22.

TABLE OF CONTENTS

ABBREVIATIONS **5**

ABSTRACT **8**

INTRODUCTION **10**

Structural organization of synapses 10

Spikes are generated in the axon initial segment 11

Mechanisms of neurotransmitter release 12

Quantal nature of transmitter release 13

Short-term synaptic plasticity 15

The neurotransmitter concentration profile at active synapses 17

The postsynaptic organization of synapses 19

Excitatory synapses 19

- The postsynaptic density 19
- Glutamate Receptors 21
- Ionotropic glutamate receptors 22
- Metabotropic glutamate receptors 24

Inhibitory synapses 25

- Postsynaptic organization 25
- Ionotropic $GABA_A$ receptors 27
- Regulation of $GABA_A$ receptors assembly 28

Retrograde messenger 30

- Messenger derived from lipids 30
- Messenger derived from gases 31

Messenger derived from growth factors 31

Trans-synaptic signaling by cell-adhesion molecules 31

The neurexin-neuroligin complex 31

The hippocampus 33

Hippocampal anatomy 34

Local circuits of the hippocampus 36

Synaptic dysfunctions in neurodevelopmental disorders associated with autism 39

Disorders due to dysfunctions of neuroligins and neurexins 40

AIM OF THE WORK 41

MATERIAL AND METHODS 43

RESULTS 50

N.1 *Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures* 51

N.2 *Developmental regulation of GABAergic signaling in the hippocampus of neuroligin 3 R451C knock-in mice: an animal model of autism* 52

N.3 *Alterations of GABAergic signaling in Autism Spectrum Disorders* 53

CONCLUSIONS AND FUTURE PERSPECTIVES 54

REFERENCES 57

ACKNOWLEDGMENTS 83

Abbreviations

ACSF	Artificial Cerebrospinal Fluid
AIS	Axon Initial Segment
AMPA	(*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxadepropionate
AMPAR	AMPA receptor
ASDs	Autism Spectrum Disorders
BDNF	Brain Derived Neurotrophic Factor
CA	Cornus Ammonis
CB	Collybistin
CNS	Central Nervous System
D-AP5	D-2-amino-5-phosphonopentanoic acid
DCG-IV	(1R,2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropane-1,2-dicarboxylic acid
DG	Dentate Gyrus
DNQX	6,7-dinitroquinoxaline-2,3-dione
E/I	Excitatory/Inhibitory
EGFP	Enhanced Green Fluorescent Protein
EPSC	Excitatory Post Synaptic Current
GABA	Gamma-Aminobutyric Acid
$GABA_AR$	$GABA_A$ Receptor
GAD	Glutamic Acid Decarbosilase
GDPs	Giant Depolarizing Potentials
GKAP	Guanylate Kinase-Associated Protein
GPCR	$GABA_A$-mediated Postsynaptic Currents

IDs	Intellectual Disabilities
iGluR	ionotropic Glutamate Receptors
IPSC	Inhibitory Post Synaptic Current
KI	Knock-In
KO	Knock-Out
L-AP4	(2S)-2-Amino-4-Phosphonobutanoic Acid
LRRTM	Leucine-Rich Repeat Transmembrane
LTD	Long-Term-Depression
LTP	Long-Term- Potentiation
mGluRs	metabotropic Glutamate Receptors
NLs	Neuroligins
NMDA	N-Methyl-D-Aspartate
NMDAR	NMDA receptor
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
Nrxs	Neurexins
PPR	Paired-Pulse Ratio
PSC	Post Synaptic current
PSD	Post Synaptic Density
PTP	Post tetanic Potentiation
PV	Parvalbumin
SALM	Synaptic Adhesion Like Molecules
SAPs	Synapse associate Proteins
scFv	Single Chain Antibody Fragment
TPMPA	(1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid
TrK	Tyrosine Kynase Receptor

TTX	Tetrodotoxin
VGAT	Vesicular GABA Transporter
VGLUT	Vesicular Glutamate Transporter

ABSTRACT

Synapses are recognized as being highly plastic in structure and function, strongly influenced by their own histories of impulse traffic and by signals from nearby cells. Synaptic contacts are fundamental for the development, homeostasis and remodeling of complex neural circuits. Synapses are highly varied in their molecular composition. Understand this diversity is important because it sheds light on the way they function. In particular, this may be useful for understanding the mechanisms at the basis of synaptic dysfunctions associated with neurodevelopmental disorders, such as Autism Spectrum Disorders (ASD) in order to develop properly targeted therapeutic tools.

During the first part of my Phd course I characterized the functional role of gephyrin at inhibitory synapses (paper N. 1). Gephyrin is a scaffold protein essential for stabilizing glycine and $GABA_A$ receptors at inhibitory synapses. Using recombinant intrabodies against gephyrin (scFv-gephyrin) I tested the hypothesis that this protein exerts a trans-synaptic action on GABA and glutamate release. Pair recordings from interconnected hippocampal cells in culture revealed a reduced probability of GABA release in scFv-gephyrin-transfected neurons compared with controls. This effect was associated with a significant decrease in VGAT, the vesicular GABA transporter, and in neuroligin 2 (NL2), a protein that, interacting with the neurexins, ensures the cross-talk between the post- and presynaptic sites. I also found that, hampering gephyrin function produced a significant reduction in VGLUT, the vesicular glutamate transporter, an effect accompanied by a significant decrease in frequency of miniature excitatory postsynaptic currents. Over-expressing NLG2 in gephyrin-deprived neurons rescued GABAergic but not glutamatergic innervation, suggesting that the observed changes in the latter were not due to a homeostatic compensatory mechanism. These results suggest a key role of gephyrin in regulating trans-synaptic signaling at both inhibitory and excitatory synapses.

Several lines of evidence suggest that proteins involved in synaptic function are altered in ASDs. In particular, in a small percentage of cases, ASDs have been found to be associated with single mutations in genes encoding for cell adhesion molecules of the neuroligin-neurexin families. One of these involves the postsynaptic cell adhesion molecule neuroligin (NL) 3. In the second part of my PhD, I used transgenic mice carrying the human R451C mutation of *Nlgn3*, to study GABAergic and glutamatergic signaling in the hippocampus early in postnatal life (paper N. 2). I performed whole cell recordings from CA3 pyramidal neurons in hippocampal slices from NL3 ^{R451C} knock-in mice and I found an enhanced frequency of Giant Depolarizing Potentials, as compared to controls. This effect was probably dependent on an increased GABAergic drive to principal cells as demonstrated by the enhanced frequency of miniature $GABA_A$-mediated (GPSCs) postsynaptic currents, but not AMPA-mediated postsynaptic currents (EPSCs). The increase in frequency of mGPSCs suggest a presynaptic

type of action. This was further supported by the experiments with the fast-off $GABA_A$ receptor antagonist TPMPA that, as expected for an enhanced GABA transient in the cleft, showed a reduced blocking effect on miniature events.
Although an increased number of available postsynaptic $GABA_A$ receptors, if these are not saturated by the content of a single GABA containing vesicle may account for these results, this was not the case since a similar number of receptor channels was revealed with peak-scaled non-stationary fluctuation analysis in both WT and $NL3^{R451C}$ knock-in mice, indicating that the observed effects were not postsynaptic in origin. Presynaptic changes in GABA release can be attributed to modifications in the probability of GABA release, in the number of release sites or in the content of GABA in single synaptic vesicles. Changes in probability of GABA release seem unlikely considering that we examined miniature events generated by the release of a single quantum. Our data do not allow distinguishing between the other two possibilities (changes in the number of release sites or in vesicle GABA content). However, in agreement with previous data from Südhof group showing an enhancement of the presynaptic GABAergic marker VGAT (but not VGlut1) in the hippocampus of $NL3^{R451C}$ KI mice (Tabuchi et al., 2007), it is likely that an increased GABAergic innervation may contribute to the enhancement of GABA release.
In additional experiments I found that changes in frequency of miniature GABAergic events were associated with an acceleration of mGPSCs decay possibly of postsynaptic origin. The increased frequency of mEPSCs detected in adult, but not young $NL3^{R451C}$ mice may represent a late form of compensatory homeostatic correction to counter the excessive $GABA_A$-mediated inhibition. Therefore, it is reasonable to assume that alterations in the excitatory/inhibitory balance, crucial for the refinement of neuronal circuits early in postnatal development, accounts for the behavioral deficits observed in ASDs patients.
Although also in the present case, a modification of gephyrin expression in R451C NL 3 knock-in mice was associated with changes in GABAergic innervations suggesting the involvement of a trans-synaptic signal, the role of NL3 mutation in this effect remains to be elucidated.
Finally, I contribute in writing a review article (paper N. 3) that gives an up dated picture of alterations of GABAergic signaling present in different forms of Autism Spectrum Disorders.

—— *** ——

INTRODUCTION

Structural organization of synapses

Synapses are specialized intercellular junctions between neurons or between neurons and other excitable cells where signals are propagated from one cell to another with high spatial precision and speed. Synapses can be divided in electrical or chemicals depending whether transmission occurs through direct propagation of the electrical stimulus or *via* a chemical intermediate. A general feature of a synapse is a close apposition of specialized regions of the plasma membranes of the two participating cells to form the synaptic interface. On the presynaptic side a cluster of neurotransmitter-filled synaptic vesicles is associated with the presynaptic plasma membrane.

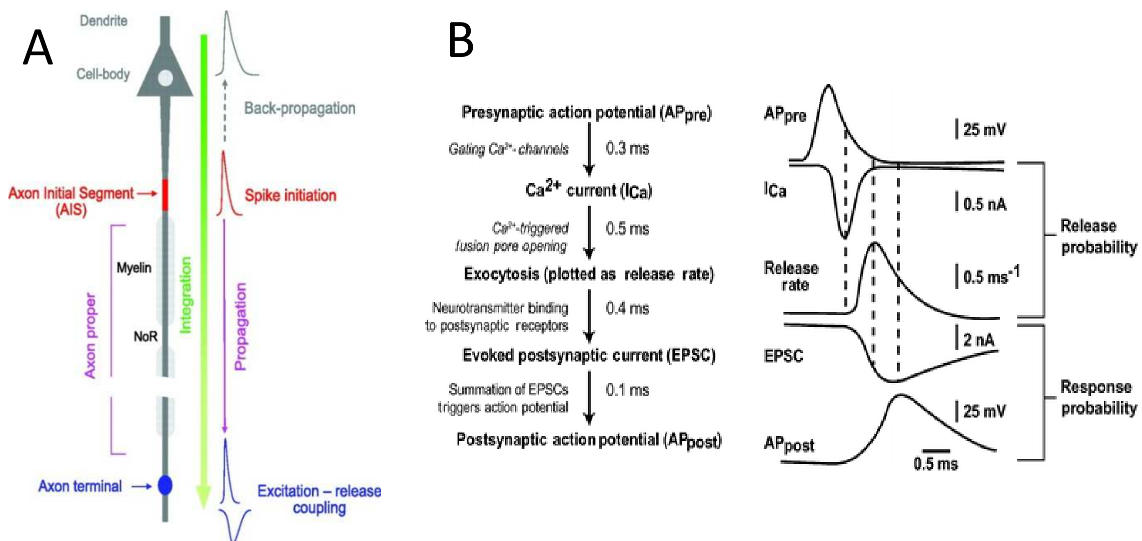


Fig. 1 Summary of axonal function and reaction sequence and timing of synaptic transmission. (A) A pyramidal neuron is schematized with its different compartments. Four major functions of the axon are illustrated (i.e., spike initiation, spike propagation, excitation-release coupling, and integration). A spike initiates in the axon initial segment (AIS) and propagate towards the terminal where the neurotransmitter is released. In addition, electrical signals generated in the somatodendritic compartment are integrated along the axon to influence spike duration and neurotransmitter release**. (B)** Time constants of reaction sequences taken from the calyx of Held (left) and corresponding traces (right). (A, *modified* from Debanne 2011; B, *modified* from Sudhof, 2004)

On the postsynaptic membrane an accumulation of neurotransmitter receptors is marked by a thickening of the membrane and by the presence of a sub-membraneous electron-dense scaffold. In the majority of the cases, the presynaptic compartment is localized in the out-pocketing of an axonal branch

and the postsynaptic compartment is localized at the surface of the cell body or dendrite. Morphological studies of central nervous system (CNS) synapses had led to their classification into two major groups, type I and type II (Grey, 1959) or asymmetric and symmetric respectively (Colonnier, 1968). Type I synapses, which involve dendritic spines and shafts, are formed by axon terminals that contain round vesicles. These synapses have a synaptic cleft of ~ 20nm and a prominent density on the cytoplasmic face of the postsynaptic membrane. Type II synapses involve neuronal perikarya and dendritic shafts. Respect to type I, type II synapses have a narrower synaptic cleft (~ 12nm), and a less prominent density beneath the postsynaptic membrane. In addition, the vesicles are smaller. It is widely accepted that terminals with larger vesicles (belonging to asymmetric synapses) are excitatory while with smaller vesicles (belonging to symmetric synapses) are inhibitory.

Spikes are generated in the axon initial segment

Communication in neurons occurs mainly through action potentials. Variations in potential arising from somato-dendritic integration of multiple inputs, culminate into the axon initial segment (AIS) where a supra-threshold resultant will trigger the action potential. Relative to their somato-dendritic compartment, the AIS bears a higher density of sodium (Na^+) channels (20- to 1.000-fold higher). These comprise three different isoforms: Nav1.1, Nav1.2 and Nav1.6, which are differently expressed in various neurons. For instance, Nav1.1 channels prevail in GABAergic interneurons (Ogiwara et al., 2007). Interestingly, sodium channels localized in the distal part of AIS display the lowest threshold for action potential generation and this account for spike initiation and back propagation.
The duration of the action potentials is determined by potassium channels which are highly expressed in the AIS. Among these, Kv1.1 and Kv1.2 are frequently associated with excitatory and inhibitory synapses and carry currents that are 10-fold larger than those measured at the soma (Kole et al., 2007). Kv2.2 channels have been involved in regulation of interspike intervals during repetitive firing (Johnston et al., 2008). Kv7 channels (7.2 and 7.3), responsible for the M-current are involved in the control of the resting membrane potential and action potential threshold (Pan et al., 2001; Shah et al., 2008; Yue and Yaari, 2006). Calcium channels are also present in the AIS where they regulate spike-timing, burst-firing, and action potential threshold (Bender and Trussell, 2009). Using calcium imaging, pharmacological tools, and immunochemistry, a recent study has reported the presence of P/Q-type (Cav2.1) and N-type (Cav2.2) Ca^{2+} channels in the AIS of L5 neocortical pyramidal neurons (Yu et al., 2010). These channels determine pyramidal cell excitability through the activation of calcium-activated BK channels.

After initiation the spike will propagate towards the axons terminal where the neurotransmitter is released. Action potentials invade also the soma and dendrites (Fig. 1, left), providing a retrograde signal that would affect synapses impinging on the soma and dendrites. The efficacy of this back-propagation depends on the presence of active conductances along the dendritic tree. Back-propagating action potentials which occur also *in vivo* (Svoboda et al., 1999; Buzsaki and Kandel, 1998) act as coincident detectors for enhancing synaptic efficacy. The depolarization provided by the spike can relieve the Mg^{2+} block of NMDA channels leading to calcium influx and second messenger activation.

Mechanisms of neurotransmitter release

Synaptic transmission involves a sequence of complex and coordinated events; once the action potential invades the presynaptic terminal, Ca^{2+}enters through voltage-gated Ca^{2+} channels, thus increasing intracellular Ca^{2+} concentration. Ca^{2+} binds to presynaptic Ca^{2+} sensors, which will result in the exocytosis of synaptic vesicles and finally neurotransmitter release. Once released, the neurotransmitter diffuses across the synaptic cleft and binds to postsynaptic receptors. In this way, a voltage change in the presynaptic neuron is converted into a chemical signal (which gives rise to an electrical response in the postsynaptic cell). Remarkably, these biophysical and biochemical events are extremely fast as the whole process occurs in less than one millisecond (Borst & Sakmann, 1996).
Ca^{2+} entering in mammalian central neurons is mediated mainly by $Ca_V2.1$ and $Ca_V2.2$ channels (Dunlap et al., 1995; Olivera et al., 1994). $Ca_V2.2$ channels (responsible for the N-type Ca^{2+} currents), are most important at synapses formed by neurons of the peripheral nervous system. However, in some central synapses, including a subset of inhibitory interneurons of the hippocampus (Poncer et al., 1997), $Ca_V2.2$ channels have been found to be involved in neurotransmitter release. $Ca_V2.1$ channels, which mediate P/Q-type Ca^{2+}currents, have a major role at most synapses formed by neurons of the mammalian central nervous system. Ca^{2+} binds to the Ca^{2+} sensor, synaptotagmin, inducing a conformational change of the SNARE complex from a *trans* to a *cis* state (Sudhof, 2004), resulting in vesicles exocytosis and neurotransmitter release. Neurotransmitter release is proportional to the third or fourth power of Ca^{2+} entry (Augustine et al., 1987; Dodge and Rahamimoff, 1967; Katz and Miledi, 1970; Zucker and Regehr, 2002). Thus, a 2-fold change in presynaptic Ca^{2+} current will cause an 8- to 16-fold change in transmitter release. In the majority of synapses, neurotransmitter is released in a fast and synchronous (phasic) manner resulting in large postsynaptic currents (Llinas et al., 1981; Sabatini and Regehr, 1996). However, examples of slow and asynchronous (tonic) neurotransmitter release resulting from residual Ca^{2+}

remaining in the terminal after an action potential, have been described in the literature (Hagler and Goda, 2001; Lu and Trussell, 2000; Atluri and Regehr, 1998; Barrett and Stevens, 1972; Goda and Stevens, 1994; Hubbard, 1963; Rahamimoff and Yaari, 1973; Heft and Jonas, 2005).

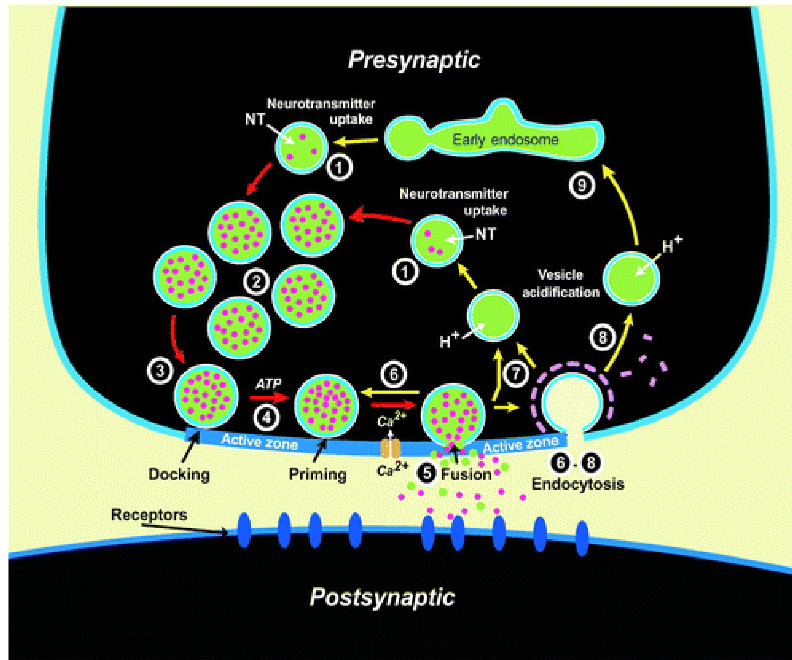


Fig. 2 The synaptic vesicle cycle. Synaptic vesicles are filled with neurotransmitters by mechanisms involving active transport (step1) and thus form the clusters of vesicles that represent the reserve pool (step2). Filled vesicles are docked at the active zone (step3) where they undergo a priming reaction (step4) that makes them competent for Ca^{2+} triggered fusion-pore opening (step5). Synaptic vesicles following fusion-pore opening undergo endocytosis and recycle via several routes: local reuse (step 6), fast recycling without an endosomal intermediate (step 7) or clathrin-mediated endocytosis (step8) with recycling via endosomes (step 9). Red arrows indicated steps in exocytosis while yellow arrows indicate step in endocytosis and recycling. (*modified* from Sudhof 2004)

Phasic and tonic transmission depend both on Ca^{2+}. Phasic transmission play a key role in the synchronization of the activity of principal cell ensembles and in the generation of high-frequency oscillatory activity in interneuron networks (Cobb et al., 1995; Buzsáki & Draguhn, 2004); tonic transmission may set the gain or offset of the input-output relations of postsynaptic target cells (Mitchell and Silver, 2003).

Quantal synaptic transmission

Pioneering studies from Katz and colleagues have shown that, at the frog neuromuscular junction, synaptic transmission is quantized, *i.e.* presynaptic action potentials give rise to postsynaptic signals that, from trial to trial display

fluctuations in amplitude among discrete levels (Katz, 1969). These discrete levels occur at integral multiples (0, 1, 2…) of an underlying unit, the "*quantum*". The quantal hypothesis first enunciated by del Castillo and Katz (1954) was a major conceptual advance in the understanding of synaptic mechanisms. Thus according to the quantal theory, neurotransmitter is packaged in discrete quantities (*quanta*) that are released in an all-or-none manner. As release occurs at specific sites, synaptic efficacy E can be described using three quantal parameters:

$$\boldsymbol{E} = N*Pr*Q.$$

where N is the number of functional release sites, Pr the probability of release of a single site and (Q) is the quantal size, i.e. the amplitude of the response to a single quantum. Changes in one or more of these parameters are responsible for modifications in synaptic strength. Assuming that each site acts independently, binomial and Poisson statistics can be used to model the stochastic behavior of synaptic transmission, to estimate quantal parameters, and to identify the site of changes in synaptic efficacy.

Quantal analysis was applied first to central synapses by Kuno (1964). However, it should be stressed that this model of synaptic transmission has received only partial support from studies in the CNS (for review see Redman and Faber, 2003). The more general finding is that, although synaptic transmission is stochastic and can even fail on occasions (i.e. a presynaptic action potential does not generate a postsynaptic response) postsynaptic responses tend to cluster at preferred amplitudes (Raastad, 1995). Quantal analysis resulted particularly useful to extract information on the mechanisms regulating changes in synaptic strength. Application of quantal analysis to central neurons relies on few assumptions, some of them found to be reasonably robust. For example if transmission failures reflect cases where the presynaptic action potential did not trigger exocytosis, it may be possible to use failures frequency as an indirect measure of the state of presynaptic terminals. Despite the differences in quantal neurotransmission between the CNS and neuromuscular junction, measuring the trial to trial fluctuations in the amplitudes of postsynaptic currents or potentials yields a powerful insight in the *loci* of alterations in synaptic strength.

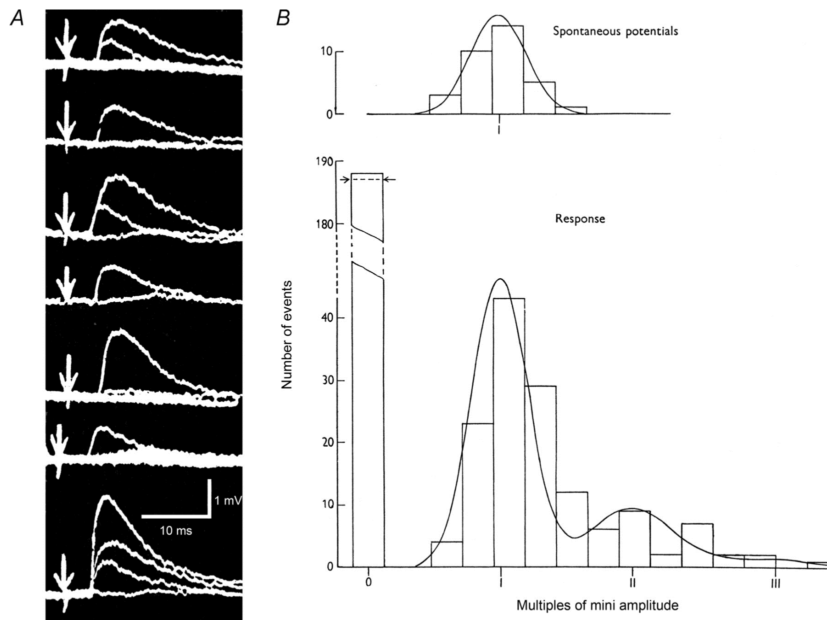


Fig. 3 Quantal transmitter release. *A*, recordings of EPP from a neuromuscular junction superfused with low calcium Ringer containing solution. Superimposed traces represent responses to stimulation of a presynaptic motor neuron (arrows). Note the step-like fluctuations in EPP amplitude recorded from the same junction. *B*, quantitative analysis of EPP amplitude fluctuations. The upper graph plots the distribution of amplitudes of minis, which can be described by a Gaussian function. The lower graph illustrates the pronounced trial-to-trial fluctuations in EPP amplitude, including 'failures" (leftmost histobar) where no EPPs were elicited. Smooth curve represents the predictions of a Poisson series, assuming that EPPs represent multiple release of mini-like quanta. The number of quanta in each EPP is indicated by the roman numbers on the abscissa. (*modified* from Fatt & Katz, 1952)

Short-term synaptic plasticity

Synapses are not static transmitter devices, on the contrary they display a plastic behavior. Short-term synaptic plasticity is referred to changes in the synaptic efficacy that usually occurs on a timescale of milliseconds to minutes. Short-term plasticity is important for information processing and plays a key role in a multitude of computational processes in the brain (Abbott and Regehr, 2004; Katz and Miledi, 1968; Zucker and Regehr, 2002). Short-term plasticity typically reflects a presynaptic change in neurotransmitter release (Del Castillo and Katz, 1954; Katz and Miledi, 1968; Zucker and Regehr, 2002) however it is important to note that also postsynaptic mechanisms may contribute to short-term plasticity (see below). Short-term plasticity can result in synaptic enhancement (through three processes: *facilitation*, *augmentation* and *post-tetanic potentiation*) or in synaptic depression (Zucker and Regehr, 2002). Although the molecular mechanisms responsible for the various types of short-term plasticity

are not yet fully understood, it is well established that they are all Ca^{2+} dependent (Katz and Miledi, 1968; Zucker and Regehr, 2002).
According to Katz and Miledi (1968), the residual Ca^{2+} remaining in the synapse after an action potential acts to enhance synaptic transmission. This hypothesis has been supported by a series of more recent experiments in which introducing the slow Ca^{2+} chelator EGTA into the presynaptic terminal reduces synaptic facilitation (Atluri and Regehr, 1996; Habets and Borst, 2005; Hochner et al., 1991; Korogod et al., 2005; Regehr et al., 1994; Van der Kloot and Molgo, 1993). This effect can be partially explained assuming that, the residual Ca^{2+} binds to the Ca^{2+} sensor synaptotagmin (Sudhof, 2004) to increases exocytosis and neurotransmitter release (Katz and Miledi, 1968). However, the scenario seems to be more complicated as more recent data indicate that residual Ca^{2+}acts not only on calcium sensors but also on Ca^{2+} -binding proteins (Blatow et al., 2003; Felmy et al., 2003; Muller et al., 2007; Sippy et al., 2003; Tsujimoto et al., 2002). Augmentation and PTP require longer trains of stimuli than facilitation and are defined by their longer decay time constants (augmentation: from 5 to 10 s; PTP: from 30 s to minutes) (Zucker and Regehr, 2002), but they may represent overlapping physiological processes. Augmentation is caused by an increase in the probability of vesicle release rather than an increase in the size of the readily releasable pool of vesicles (Stevens and Wesseling, 1999). The rate of Ca^{2+} clearance from the synapse can determine whether augmentation or PTP occurs (Korogod et al., 2005; Zucker and Regehr, 2002). Residual Ca^{2+} that accumulates during the long stimuli that induce augmentation and PTP is eliminated from the synapse by the Ca^{2+}-ATPase and by the Na^+/Ca^{2+}exchanger. Long trains of action potentials increase intracellular $Ca^{2+}$and Na^+ concentrations and slow the rate of Ca^{2+} clearance by the Na^+/Ca^{2+} exchanger or even drive it in reverse. Residual Ca^{2+}driving PTP can also result from the slow efflux of Ca^{2+} from mitochondrial or endoplasmic reticulum where it accumulates during tetanic stimulation (Lin et al., 1998; Narita et al., 2000; Tang and Zucker, 1997).
Synaptic depression reduces the synaptic strength during repeated stimuli, delivered as closely paired stimuli (paired-pulse depression) or trains of stimuli. Depletion of the pool of readily releasable vesicles seem the main explanation for synaptic depression (Zucker and Regehr, 2002). However, in some circumstances vesicle depletion does not fully account for rapid synaptic depression (Sullivan, 2007; Xu and Wu, 2005). Decreased release probability caused by a reduced Ca^{2+} entry (Forsythe et al., 1998; Xu and Wu, 2005), or changes occurring downstream to Ca^{2+} entry (Wu and Borst, 1999) have been proposed. Therefore, like facilitation, augmentation, and PTP, multiple mechanisms contribute to synaptic depression, and their relative roles are still under debate.
Postsynaptic mechanisms also contribute to short-term plasticity. In fact, postsynaptic receptors saturations can prevent an increase in synaptic strength also if the neurotransmitter release is enhanced (Foster et al., 2000; 2005). A

similar situation is represented by receptor desensitization following repetitive activation of presynaptic terminals (Jones and Westbrook, 1996; Overstreet et al., 2000). On the contrary, relief of the polyamine block in Ca^{2+} permeable AMPARs induces facilitation in some cortical synapses (Rozov and Burnashev, 1999).

The neurotransmitter concentration profile at active synapses

Once released from the presynaptic terminal, the neurotransmitter reaches a peak concentration and then decays rapidly. The synaptic agonist transient (1-5 mM) is thought to undergo extremely fast clearance (time constant of ~ 100 μs) (Clements, 1996). Such a brief transmitter transient is even faster than receptor opening, implying that synaptic receptor activation occurs in conditions of non-equilibrium.

Understanding the temporal and concentration profile of neurotransmitters released during synaptic transmission, is a fundamental aspect towards the comprehension of neuronal communication. In the brain, the spatial and temporal properties of synaptic inputs are important determinants for specific neuronal functions including signal integration, network oscillations and selection of neuronal ensembles (Freund and Katona, 2007; Klausberger and Somogyi, 2008). Generally, it is thought that the major determinants of kinetic properties of post-synaptic currents (PSCs) commonly rely on the gating properties of post-synaptic receptors as exemplified for glutamatergic NMDAR- and AMPAR-mediated EPSCs with slow and fast decay, respectively (Lester and Jahr, 1992). Similarly, GABAergic synapses are characterized by a marked kinetic diversity that is commonly ascribed to the variety of post-synaptic $GABA_A$ receptors ($GABA_AR$) isoforms (Cherubini and Conti, 2001; Eyre et al., 2012). For instance, $GABA_A$ receptors containing α1 or α3 subunits (along with β and γ2 subunits) have been demonstrated to underlie fast and slow IPSCs kinetics, respectively (Gingrich et al., 1995; Mozrzymas et al., 2003). However it is important to note that the kinetics of synaptic currents can be in some cases, different from predictions made exclusively on the basis of the gating properties of post-synaptic receptors. Several experimental works have demonstrated that at excitatory and inhibitory synapses the profile of neurotransmitter concentration in the synaptic cleft may exert a significant role in determining the amplitude and kinetics of synaptic currents.

During the last decade, many efforts have been dedicated to estimate the neurotransmitter profile at active synapses. However, several difficulties (synapses size and geometry, experimental inaccessibility of the synaptic cleft, quantal properties of neurotransmitter release etc.) lead only to a rough approximation of neurotransmitter behavior in the synaptic cleft. In this section I will provide a short description of the main methods employed for the

estimation of synaptic agonist time course (for an extensive review see Clements, 1996; Scimemi and Beato, 2009; Barberis et al., 2011).

Low affinity competitive agonist

Clements and colleagues (1992) introduced the method of low affinity competitive antagonist to estimate the time course of neurotransmitter in the cleft. In the presence of a quickly dissociate competitive antagonist, the amount of synaptic currents block, strongly depends on the strength of the pre-synaptic neurotransmitter release, because, antagonists and agonist compete for the same binding site. For an efficient displacement of competitive antagonist by synaptic agonist a fundamental prerequisite is that the dissociation time constant of the antagonist is comparable with the duration time of the neurotransmitter transient in synaptic cleft. Different low affinity antagonists have been used to estimate the time course of the main neurotransmitters in the CNS (Overstreet et al., 2003; Clements et al., 1992; Beato, 2008). Despite the low affinity antagonist method is widely used, it is important to remember that it has some limitations; the most notable is that for a given concentration of antagonist, the same amount of inhibition of the control response can be observed both with a short pulse of high agonist concentration or a long pulse of low agonist concentration (Diamond, 2001).

Mathematical simulation

Mathematical models of a typical synaptic connection incorporating the geometry of the synaptic cleft, the extra-synaptic space, the binding of transmitter to receptors and uptake sites allow to obtain prediction on the profile of the neurotransmitter in the synaptic cleft. In particular, complex 3D reconstruction of synaptic structures together with Monte Carlo simulations have been used to explore the role of morphological and kinetics variable on synaptic currents (Wahl et al., 1996; Kruk et al., 1997). Despite the utility of these approaches, their predictive power is affected by some uncertainties associated with the parameters that are used (difference in synapse structure, size, number and distributions of receptors within a cell population).

Modifiers of gating

Compounds affecting the agonist binding to post-synaptic receptors resulted particularly useful in providing information about the agonist transient (Barberis et al., 2011). This method is based on the consideration that even minimal up or down regulation of the agonist binding rate has a marked effect on the amplitude and time course of currents obtained after a short exposure to synaptically released neurotransmitter. To this purpose several compounds have been used (dextran, benzodiazepine, zinc, chlorpromazine). This method seems to provide

more qualitative than quantitative details especially when it is used to analyze synaptic currents (Barberis et al., 2011).

The postsynaptic organization of synapses

The presence of ion channels and neurotransmitter receptors stabilized by scaffold proteins, allow the postsynaptic side to receive the neurotransmitter signal released from the presynaptic terminal and transduce it into electrical and biochemical changes. Excitatory and inhibitory postsynaptic specializations are quite different both in the structural and molecular organization. Excitatory synapses occur mainly on tiny protrusion called dendritic spine (Bourne & Harris, 2008) while inhibitory synapses are formed mainly on the shaft of dendrites or, on cell bodies and axon initial segment (Wierengart, 2008).
The molecular and structural diversity of the synapses contribute in a fundamental way to the development of specific connectivity patterns and to the functional properties of neuronal networks. In this section I will discuss the main structural and molecular features of both excitatory and inhibitory synapses.

Excitatory synapses

The postsynaptic density

Excitatory synapses are characterized by morphological and functional specialization of the postsynaptic membrane called postsynaptic density (PSD). The PSD contains the glutamate receptors and several associated signaling and structural molecules (Sheng, 2011). It is now widely accepted that the PSD exerts a fundamental role allowing the correct apposition of pre- and postsynaptic membranes to cluster postsynaptic receptors and to link active postsynaptic receptors to biochemical events in the postsynaptic neuron. The PSD was first observed in electron microscopy as an electron-dense thick portion of the postsynaptic membrane (Siekevitz, 1985). The PSD is highly heterogeneous with a diameter ranging from 200-800 nm (mean 300-400 nm) and thickness of 30-60 nm (Carlin et al., 1980). The structure and composition of PSDs is developmentally regulated (Petralia et al., 2005; Swulius et al. 2010) and generally, the expression level of many PSD proteins increase during development, reaching their peaks at ~ 2-4 week after birth and correlates with the formation and maturation of synapses within the brain.
As already stated, the PSD is endowed with a large number of scaffolding proteins that can be present in different splice isoforms. Synaptic scaffolding proteins contain multiple domains for protein-protein interaction. A hallmark of PSD scaffold proteins is a ~ 90 aminoacid motif, the PDZ domain that is fundamental for the interaction with binding partners (Funke et al., 2004).

Probably, the best studied protein of the excitatory synapses is PSD-95. PSD-95 together with its family members (PSD-93, SAP97 and SAP 102) is a very versatile protein exerting a major role in several synaptic functions.

• *Interaction and localization of synaptic proteins:* PSD-95 stabilizes interacting membrane proteins at synapses by suppressing their lateral diffusion and/or internalization (Bats et al., 2007; Roche et al., 2001). PSD-95 binds to the C-terminal tail of many postsynaptic adhesion molecules including neuroligins (NLs), synaptic adhesion like molecules (SALMs) and leucine-rich repeat trans-membrane neuronal proteins (LRRTM) (Irie et al., 1997; Craig and Kang, 2007; Sudhof, 2008; Brose, 2009.) In addition PSD-95 participates in the assembly of NMDA receptor-associated protein complex and in the recruitment of signaling proteins able to modulate the function of glutamate receptors (Bhattacharyya et al., 2009). PSD-95 recruits AMPA receptors to the synapse thus participating to receptor trafficking (Chen et al., 200; Schnell et al., 2002; Nicoll et al., 2006; Bats et al., 2007). PSD-95 binds directly to the carboxy-terminal tails of the NR2 subunits of NMDA receptors (Kornau et al., 1997). The simultaneous interaction with adhesion molecules, glutamate receptors and signaling proteins confer to the PSD-95 family proteins a central role in the morphological and functional maturation of synapses and their maintenance.

• *Regulation of synaptic strength and plasticity:* overexpression of PSD-95 in cell cultures enhances AMPA receptor mediated excitatory synaptic transmission (El-Husseini et al., 2000); in brain slices, the overexpression of PSD-95 increases the frequency of mEPSCs, enhances AMPA but not NMDA receptor mediated EPSCs (Stein et al., 2003; Ehrlich and Malinow, 2004). On the contrary, knocking down PSD-95 with RNAi has a relatively modest effect on NMDA receptor-mediated transmission (Elias et al., 2006; Futai et al., 2007). Transgenic mice with targeted truncation of PSD-95 exhibit normal excitatory synaptic structure and function (Migaud et al., 1998), while mice deficient of both PSD-95 and PSD-93 show a strong reduction in the frequency of AMPA receptor –mediated synaptic currents (Elias et al., 2006). In PSD-95 null mice and in mice with targeted PSD-95 truncation LTP is enhanced (Beique et al., 2006; Migaud et al., 1998). Beyond PSD-95 others important proteins of the PSD are represented by the Shank family, the Homer family and the GKAP family.

The Shank family is composed by large proteins (~ 200 kDa) with multiple interacting domains that undergo differential splicing. Shank proteins (Shank 1-3) exert a crucial role in dendritic spine differentiation and synaptic transmission (Sala et al., 2001). The Homer family of scaffolding proteins (Homer

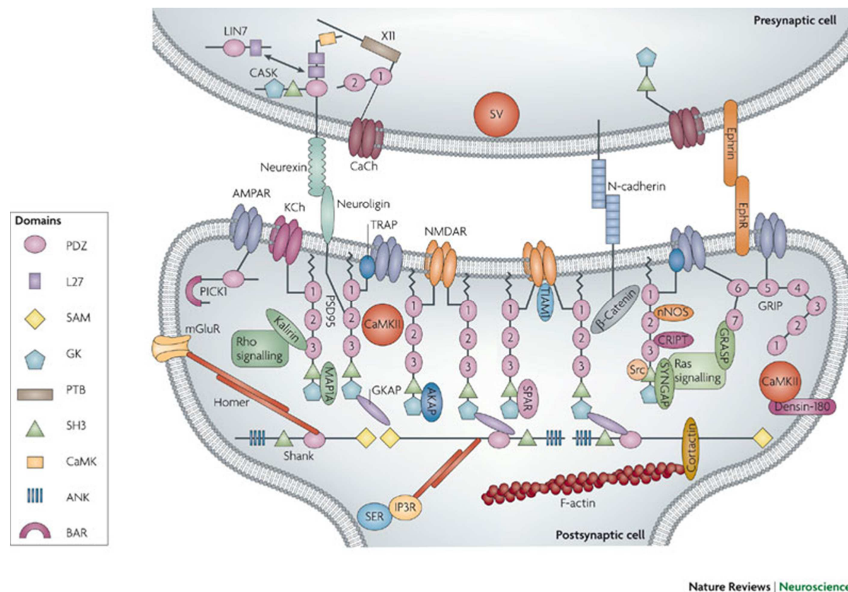


Fig. 4 Structural organization of excitatory synapse. The postsynaptic density is endowed of membrane receptors and ion channels, scaffold and adaptor proteins, signaling proteins, cell-adhesion molecules and components of the cytoskeleton. Glutamate receptors, such as NMDARs and AMPARs are located at the postsynaptic membrane, with the NMDARs at the centre and the AMPARs more peripheral. The PDZ-domain-containing scaffold proteins PSD95 and the Src-homology domain 3 (SH3) and multiple ankyrin repeat domains (Shank) family form a two-layer protein network below the postsynaptic membrane, which is bridged by guanylate kinase-associated protein (GKAP). The presynaptic and postsynaptic membranes are connected by cell-adhesion molecules. (*modified* from Feng & Zhang, 2009)

1-3) are associated with Shank and group I metabotropic glutamate receptors (mGluR1 and mGluR5).

The GKAP family of scaffolding proteins (GKAP/SAPAP1-4) interacts with the C-terminal GK-like domain of PSD-95 family proteins (Kim et al., 2004).The carboxyl terminus of GKAP, interacts with the PDZ domain of Shank, which binds to Homer forming an interacting scaffold in the deeper part of PSD. In addition AMPA receptor binding scaffolds GRIP and PICK1 (present in the PSD) participate in AMPA receptor trafficking (Shepherd and Huganir, 2007).

In addition, the PSD contains a huge variety of cytoplasmic signaling molecules such as kinases (CaMKIIα) and phosphatases (e.g. serine/threonine protein phosphatase I and protein tyrosine phosphatases) (Sheng and Hoogenraad 2007).

Glutamate receptors

The vast majority of excitation in the central nervous system is mediated by glutamate, a ubiquitous amino acid (Watkins and Jane 2006). In neurons, glutamate is packed within synaptic vesicles by dedicated vesicular transporters (VGluTs) and following presynaptic activity is released in the synaptic cleft. Once

released, glutamate acts on several structurally and pharmacologically different types of receptors: the ionotropic and the metabotropic receptors.

Ionotropic glutamate receptors (iGluRs)

Because iGluRs form ion channels permeable to cations, and the equilibrium potential for glutamate is close to 0 mV, positive to the resting membrane potential, activation of iGluRs results in membrane depolarization. Besides mediating the basic excitatory synaptic responses, iGluRs are also central in the induction and maintenance of synaptic plasticity. Mammalian iGluRs are encoded by a total of 18 genes that assemble into four major families: AMPA, kainate, NMDA, and delta receptors (Hollmann et al. 1989; Seeburg, 1993; Nakanishi and Masu, 1994; Dingledine et al., 1999). **iGluRs** are tetrameric protein complexes made up of four subunits surrounding an ion channel pore. Each iGluR subunit shows a characteristic modular organization consisting in: (1) a large extracellular domain(~ 380 aminoacids); (2) an agonist binding domain (~300 aminoacids); (3) a transmembrane domain formed by three membrane spanning-segment (M1, M2, M4) and a membrane reentrant loop (M2); (4) a cytoplasmic carboxy-terminal domain whose residues interact with numerous intracellular scaffolding and trafficking proteins.

AMPA receptors are encoded by four different genes (GluA1-4, also known as GluR1-4); AMPAR subunits can form both homo- and heteromers. The latter constitute the majority of native AMPARs (Jonas and Burnashev, 1995; Rossmann et al., 2011). Each AMPA receptor subunit may exist in the *flip* or *flop* version, dependent on alternative splicing of two exons encoding a 38-aa segment of the extracellular loop between M3 and M4.Flip forms desensitize with a slower kinetics compared to flop forms. *Flip* subunits are mainly expressed early in development. In addition, the mRNA encoding for the GluR2 subunit can undergo editing mechanisms. Post-transcriptional modifications cause the substitution of glutamine (Q), located in position 586 on the transmembrane segment M2, with arginine (R) on the same position. The presence of an arginine is responsible for the electrostatic interaction that makes the receptor Ca^{2+} impermeable. This editing mechanism seems to be developmentally regulated. Infact, the edited and non- edited forms of the GluR2 subunit coexist during the first postnatal days while in the adult the edited form is predominant. Calcium impermeable $AMPAR_S$ receptors show a strong rectification at depolarizing potentials; this phenomenon is due to the presence in the cytoplasma of polyamines such as spermine and spermidine that, at positive potentials, enter through the channel pore causing its block. The majority of AMPA receptors expressed by excitatory neurons include one or two GluR2 subunits: in this case the receptors are Ca^{2+} impermeable; However, AMPA

receptors expressed by astrocytes and several interneurons lack the GluR2 subunits and therefore are calcium permeable.

Ionotropic Glutamate receptors (iGluRs)

AMPARs	KainateRs	NMDARs	DeltaRs
GluA1	GluK1	GluN1	GluD1
GluA2	GluK2	GluN2 (A,B,C,D)	GluD2
GluA3	GluK3	GluN3 (A,B)	
GluA4	GluK4		
	GluK5		

Table 1. The four families of iGluR subunits. Each subunit is encoded by a distinct gene. There is no known mixing of subunits between families.

NMDA receptors are encoded by seven genes (GluN1; GluN2A-D; GluN3A-B, also known as NR1; NR2A-D; NR3A-B). NMDARs are obligatory heteromers associating two GluN1 subunits with either two GluN2 subunits (which can be of different types) or a combination of GluN2 and GluN3 subunits (Cull-Candy and Leszkiewicz, 2004; Ulbrich and Isacoff, 2007; Paoletti, 2011). The NMDA sub-unit composition is developmentally regulated as NR2B subunit expressed in the cortex and in the hippocampus during the first postnatal week is substituted with NR2A in the adult ; this substitution results in faster decay currents.
NMDARsare characterized by a high affinity for glutamate, Ca^{2+}permeabilityanda slow gating kinetics. At resting potentials, physiological concentrations of Mg^{2+} (Mcbain and Mayer, 1994) block the NMDA receptors. The receptors are activated only when glutamate release is paired with postsynaptic depolarization (i.e. activation of AMPA receptors). These properties make them ideally suited for their role as a coincidence detectors underlying Hebbian processes in synaptic plasticity such as learning, chronic pain, drug tolerance and dependence (Collingridge and Singer, 1990; Bear and Malenka, 1994; Trujillo and Akil, 1995; Collingridge and Bliss, 1995; Dickenson, 1997).

Kainate receptors are encoded by five different genes (GluK1-5, also known as GluR5-7, KA1-2). They are distributed throughout the brain but, unlike AMPA receptors (AMPARs) and NMDA receptors (NMDARs), they are mainly expressed on nerve terminals (both excitatory and inhibitory) where they regulate

transmitter release. More peculiarly, they link to metabotropic signaling pathways in addition to operating as conventional ionotropic receptors (Contractor et al., 2011).
In KAR, only GluK1–GluK3 subunits can function as homomers; GluK4 and GluK5 form functional receptors only when co-expressed with GluK1–GluK3 (Contractor et al., 2011).

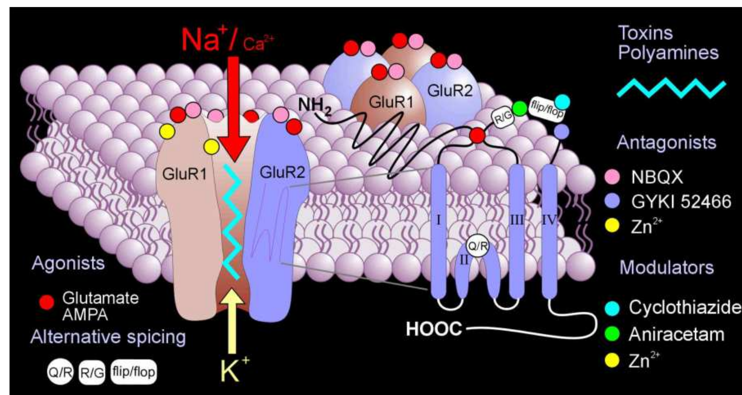


Fig. 5 Structural organization of AMPARs. AMPARs are displayed as heteromers composed by four subunits. The site of binding of agonist and antagonist are illustrated (*modified* from http://chrisparsons.de/Chris/ampa.htm)

Metabotropic glutamate receptors

mGluRs are members of the G-protein-coupled receptor (GPCR) superfamily, the most abundant receptor gene family in the human genome. GPCRs are membrane-bound proteins that are activated by extracellular ligands such as light, peptides, and neurotransmitters, and transduce intracellular signals via interactions with G proteins. The resulting change in conformation of the GPCR induced by ligand binding activates the G protein, which is composed of a heterotrimeric complex of α, β, and γ subunits. In their inactive state, G proteins are bound to guanosine 5- diphosphate (GDP); activation of the G protein causes the exchange of guanosine 5-triphosphate (GTP) for GDP within the α subunit. Activated G-protein subunits then modulate the function of various effector molecules such as enzymes, ion channels, and transcription factors. Inactivation of the G protein occurs when the bound GTP is hydrolyzed to GDP, resulting in reassembly of the heterotrimer.
GPCRs consist of an extracellular N-terminal domain, seven transmembrane-spanning domains, and an intracellular C-terminus. mGluRs are classified into three groups based on sequences homology, G-protein coupling, and ligand selectivity.

Group I includes mGluRs 1 and 5. These receptors are generally localized on the postsynaptic membranes outside the synaptic cleft (Baude et al., 1993). They are coupled via G proteins to phospholipase C and, they are active during high-frequency activation of afferent fibers when, glutamate overflows the synapse; their activation leads to an increase in both inositol triphosphate and diacilglycerol (Fagni et al., 2000).
Group II (which includes mGluRs 2 and 3) are localized on presynaptic nerve terminals where they exert a key role in down regulating glutamate release. Group II receptors are activated only when glutamate overflows the synapse.
Group III (which includes mGluRs, 4, 6, 7, 8) are localized on presynaptic terminals, very close or even within the active zones (Shigemoto et al., 1996, 1997). The distinction between group II and III receptors can be done pharmacologically: while group III are blocked by L-AP4, group II by DCG IV.

Inhibitory synapses

Postsynaptic organization

The major scaffold protein of the inhibitory PSD (both GABAergic and glycinergic) is the multi-domain, 93 kDa protein gephyrin (Kneussel and Betz, 2000; Fritschy et al., 2008). Originally co-purified with glycine receptors (GlyR; Pfeiffer et al., 1982), gephyrin has been later found to be associated also with GABAergic synapses (Sassoè-Pognetto et al., 1995; Sassoè-Pognetto and Fritschy, 2000). This molecule is composed by three domains: the E and G domains connected by a linker region. Gephyrin lacks the PDZ domains but forms aggregates (dimers of trimers) by spontaneous oligomerization giving rise to an hexagonal structure known as "*lattice*". The precise mechanisms by which gephyrin forms postsynaptic scaffolds is not yet known in the molecular details (Fritschy et al., 2008; Tretter et al., 2012). Gephyrin binds a cytoplasmic loop of the GlyR β subunit (Meyer et al., 1995) and is essential for postsynaptic clustering of GlyRs (Kirsch et al., 1993; Feng et al., 1998) and $GABA_A$Rs (Essrich et al., 1998; Yu et al., 2007).
The clustering of gephyrin and $GABA_A$Rs is to some extent mutually dependent, since deletion of $GABA_A$Rs disrupts gephyrin clusters (Essrich et al., 1998; Schweizer et al., 2003; Li et al., 2005; Kralic et al., 2006; Studer et al., 2006) and removal of gephyrin by knocking down its gene results in a massive loss of post-synaptic $GABA_A$R aggregates (Kneussel et al., 1999, 2001; Fischer et al., 2000; Lévi et al., 2004). The mechanisms by which gephyrin contributes to $GABA_A$Rs clusterization are not fully elucidated. However, there is a general *consensus* that this molecule decreases the lateral mobility of $GABA_A$ receptors in the plasma membrane (Jacob et al., 2005; Thomas et al., 2005).

Recently, several interactions have been demonstrated to occur between gephyrin and $GABA_A$Rs subunits. Alldred et collegues (2005) have identified the fourth trans-membrane domain of the $GABA_A$R γ2 subunit as essential for mediating postsynaptic $GABA_A$Rs clustering , whereas the major γ2 cytoplasmic loop is required for the recruitment of gephyrin into $GABA_A$R clusters. Direct interactions between gephyrin and the $GABA_A$R α2 and α3 subunits have emerged only recently (Tretter and Moss, 2008; Saiepour et al., 2010). The relevance of the numerous gephyrin isoforms generated by alternative splicing is not completely understood (Paarmann et al., 2006; Fritschy et al., 2008). Gephyrin function may depend on post-translational modifications. To this purpose recent studies indicate that phosphorylation of gephyrin at specific residues (particularly enriched in the linker region) participate in anchoring GlyRs and $GABA_A$Rs at postsynaptic sites (Zita et al., 2007; Charrier et al., 2010; Tyagarajan et al., 2011). A still unresolved issue is whether gephyrin equally contributes to the clustering of all major subtypes of synaptic $GABA_A$Rs. The analysis of spinal cord sections, retina organotypic cultures, and cultured hippocampal neurons derived from gephyrin null mice lead to the idea that this protein mediates the postsynaptic accumulation of $GABA_A$Rs containing the α2 or the α3 subunits, and suggested the existence of additional clustering mechanisms (Fischer et al., 2000; Kneussel et al., 2001; Lévi et al., 2004). However, in the brain gephyrin colocalizes with all major types of postsynaptic $GABA_A$Rs containing either the α1, α2, or α3 subunits (Sassoè-Pognetto et al., 2000), indicating that its function is not restricted to α2 and α3-containingsynapses. Despite the enormous progress made in the understanding gephyrin function, this protein still remains an "enigmatic" organizer of the inhibitor synapse (Tretter et al., 2012).

Another central molecule of the inhibitory synapse is collybistin (CB). Three main CB variants, CB 1–3, differing in the C-terminal region have been found in the rat. CB is formed by three major functional domains: an N-terminal SH3 domain, encoded by a spliced exon (SH3), a DH (or GEF) domain and a PH domain. CB seems to have a strong role in inhibitory synapses maintenance as several loss-of-function mutations in the collybistin gene ARGEF9 are associated with severe forms of X-linked mental retardation and hyperekplexia (Kalscheuer et al., 2009). In recombinant expression systems, CB is essential for cell-surface translocation of gephyrin (Kins et al., 2000). Based on data from recombinant systems, this interaction was suggested to be responsible for a conformational change relieving the auto-inhibition of the DH domain, thus facilitating cell-surface translocation of gephyrin and $GABA_A$Rs.

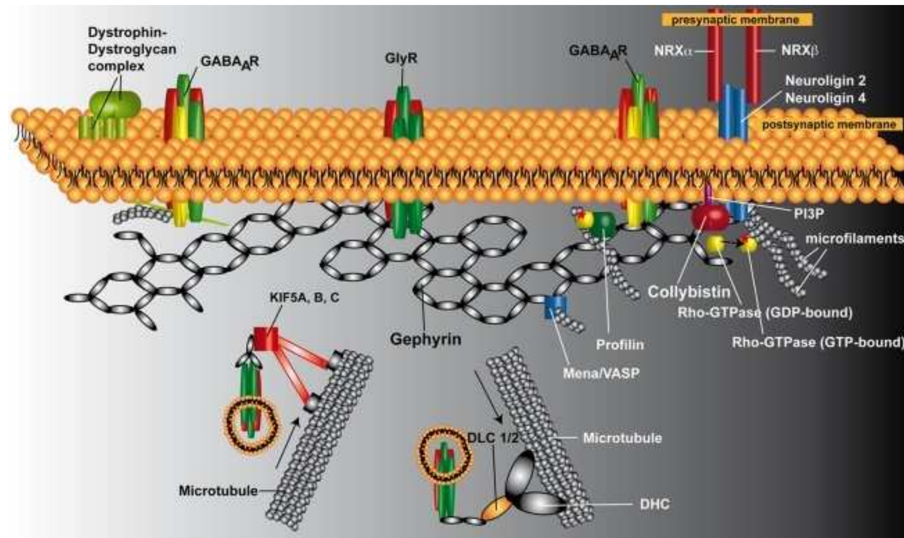


Fig. 6 Structural organizationof the inhibitory synape. Gephyrin the major scaffold molecule of the inhibitory synapse, forms an hexagonal structure known as "lattice" that is needed to anchor GABA and Gly receptors. Collybistin is required for the post-synaptic clustering of gephyrin at GABAergic synapses while at glycinergic synapses gephyrin and Glyrs are targeted to the postsynapses independently of collybistin. Neuroligin2 binds to collybistin on the postsynaptic site and with neurexins on the presynaptic site ensuring a correct cross-talk between the two synaptic elements. Interactions of gephyrin with elements of the cytoskeleton is essential for its trafficking toward the membrane. Abbreviation: DHL, dynein heavy chains; DLC ½, dynein light chain1/2; KIF 5A, B, C, kinesin family protein 5 A, B, C; Mena, microfilament adaptors of the mammalian enabled; NRX, neurexins; PI3P, phosphatidylinositol-3-phosphate; VASP, vasodilator stimulated phosphoprotein (*modified* from Poulopoulos et al., 2009)

Ionotropic $GABA_A$ receptors

Ionotropic $GABA_A$ receptors ($GABA_A$Rs) are members of the superfamily of hetero pentameric ligand-gated ion channels that also include the nicotinic acetylcholine receptors, glycine receptors, and 5-HT3 receptors (reviewed in Unwin, 1989). In the adult $GABA_A$Rs mediate fast inhibitory neurotransmission thus exerting a fundamental role in regulating neuronal excitability. The receptor subunits share a common ancestral structure that includes an extracellular N-terminal domain, four trans-membrane domains (TM1-4), and an extended cytoplasmic loop region between TM3 and TM4 that mediates interactions with trafficking and signaling factors (Figure 6). $GABA_A$R subunits are encoded by 19 different genes that have been grouped into eight subclasses based on sequence homology (α 1-6, β 1-3, γ 1-3, δ, ε, θ, π, ρ 1-3). Alternative splicing contributes to additional receptor diversity. This molecular heterogeneity give rise to different type of $GABA_A$Rs. Receptors with different subunit compositions exhibit different pharmacology and channel gating properties, are differentially expressed during development and in the adult brain, accumulate at different neuronal cell surfaces, and are subject to differential regulation by extracellular cues.

$GABA_A$ receptors are localized at both synaptic and extrasynaptic sites. The first mediate fast synaptic inhibition while the second tonic inhibition. $GABA_A$ receptors facing the presynaptic releasing sites have a low affinity for GABA (they are exposed for a very short period of time to high GABA concentrations) while $GABA_A$ receptors localized away from the synapses have a high affinity for GABA (they are exposed for a long period of time to very low GABA concentrations) and in comparison with synaptic receptors desensitize less. Because of their high affinity and low desensitization rate, extrasynaptic receptors are persistently able to sense low concentrations of GABA.
Activation of extrasynaptic $GABA_A$ receptors by GABA escaping from the cleft generates a persistent (tonic) $GABA_A$-mediated conductance (Farrant and Nusser, 2005). Tonic GABAergic signaling in mature neurons was first identified in granule cells of the cerebellar cortex (Kaneda et al., 1995; Brickley et al., 1996) where, application of $GABA_A$Rs antagonists was found to reduce the current required to keep the cell at a fixed potential (the 'holding current'). This was accompanied by a decrease in the background noise, consistent with the block of stochastic ion channels openings. To date, similar GABA-mediated tonic conductance has been demonstrated in several brain structures including the hippocampus and the cortex. Tonic inhibition regulates a numbers of physiological processes such as signals integration, network excitability and information processing. Therefore, it is not surprisingly that alterations in tonic $GABA_A$R-mediated conductance are implicated in different pathological states (Brickley and Mody, 2012).

Regulation of $GABA_A$R assembly

Despite the high number of $GABA_A$Rs would predict a large heterogeneity of receptor subtypes, basic rules of assembly (Luscher et al., 2011) and the differential distribution of subunit types among brain region and neuronal population restricts the overall number of receptor subtypes.
At synapses, $GABA_A$Rs are composed mainly of two α1, α2, or α3 subunits together with two β2 or β3 subunits and a single γ2 subunit. Compared to other $GABA_A$R subtypes discussed below these receptors exhibit low affinity for GABA and thus are optimized to respond selectively to relatively high concentrations of GABA released into the synaptic cleft (300 μM, Perrais and Ropert, 1999). The γ2 subunit is essential for postsynaptic clustering of $GABA_A$Rs (Essrich et al., 1998). However, the γ3 subunit can substitute for the γ2 subunit and contribute to postsynaptic $GABA_A$Rs in the developing postnatal brain (Baer et al., 1999). Freeze-fracture replica immunogold labeling indicates that α2, α3 and β3 subunit-containing receptors are 50–130 times more concentrated at synapses than at extrasynaptic sites (Kasugai et al., 2010). Not all γ2-containing receptors are concentrated at synapses. In particular, α5βγ2 receptors are found almost

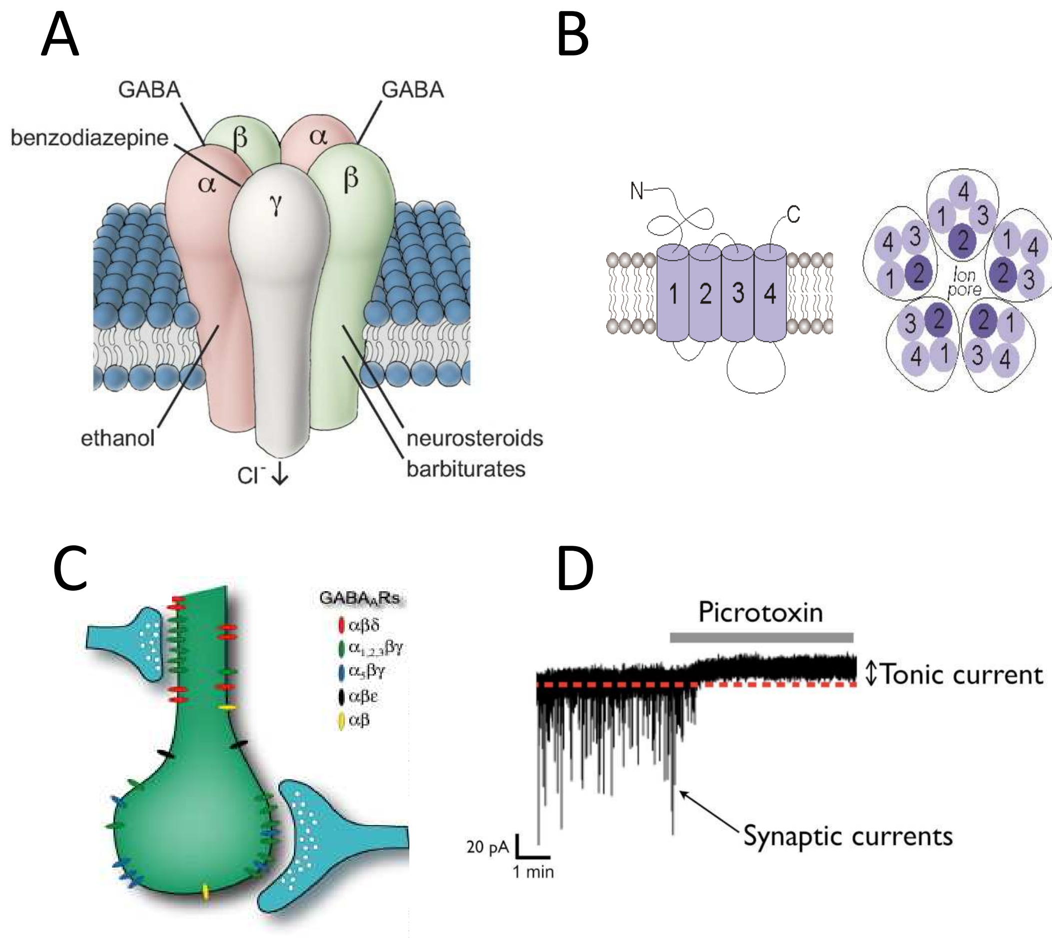


Fig. 7 Structural organization of the GABA$_A$R. (A) The binding of GABA ito GABA binding sites at the interface between α and β subunits open the receptor associated chloride (Cl^-) channel. The benzodiazepine binding site is located at the interface between α and Y_2 subunits. Barbiturates, ethanol and neurosteroids bind to sites in the membrane-spanning regions of the subunits. (B) Topography of GABA$_A$ R subunit ; The extracellular N-terminal domain is responsible for the binding of the neurotransmitter; four transmembrane domains forming the ion channel; the intracellular loop between transmembrane domain 3 and 4 forms the motif for regulatory phosphorylation sites and for intracellular factors anchoring the receptor in the right position (*modified* from Uusi-Oukari& Korpi, 2010). (C) Several types of receptors are involved in generating tonic conductance; different sub-units are denoted by different color (*modified* from Glykys & Mody, 2007). (D) The shift of the holding current following the application of GABAAR antagonist picrotoxin reveals the magnitude of tonic conductance (*modified* from Walker & Kullmann, 2012)

exclusively at extrasynaptic sites (Brunig et al., 2002; Crestani et al., 2002; Serwanski et al., 2006) where they contribute to tonic GABA$_A$-mediated inhibition (Caraiscos et al., 2004; Glykys et al., 2008).The most prominent population of non-synaptic GABA$_A$Rs mediating tonic inhibition consists in α4βδ receptors in the forebrain and α6βδ receptors in the cerebellum. In addition,

α1βδ receptors underlie tonic inhibition of hippocampal interneurons (Glykys et al., 2007). Lastly, $GABA_A$Rs also are present on axons, including the axon initial segment of pyramidal cells (Nusser et al., 1996; Brunig et al., 2002; Szabadics et al., 2006), mossy fiber terminals of hippocampal granule cells (Ruiz et al., 2003; Jang et al., 2006; Alle and Geiger, 2007), axon terminals of retinal bipolar neurons (Shields et al., 2000) and cerebellar parallel fibers (Stell et al., 2007). Axonal $GABA_A$Rs are thought to modulate action potential conductance and neurotransmitter release (Kullmann et al., 2005).

Retrograde messengers and trans-synaptic signaling

It is well known that under physiological conditions, postsynaptic neurons can release from their cell bodies and/or dendrites substances that act as trans-synaptic retrograde messengers to modulate transmitter release. A retrograde messenger can be defined as a substance released "on request" from the postsynaptic neuron and acting on presynaptic terminals, where is able to modify synaptic transmission.
To be defined as a retrograde messenger, a given molecule should fulfill the following criteria:

✓ The synthesis and release of the retrograde messenger must be in the postsynaptic neuron.

✓ Abolishing the synthesis and/or release of the messenger from the postsynaptic neuron must abolish the retrograde signaling.

✓ Disrupting presynaptic targets must abolish retrograde signaling

As outlined in the examples below, several molecules with completely different chemical properties and different mechanisms of synthesis, release and way of action have been proposed as retrograde messengers.

Messengers derived from lipids

Some signaling molecules are synthesized from lipid precursors. Usually the synthesis and release of these lipophilic molecules are dependent on postsynaptic calcium rise, although the complete pathway is formed by different regulatory steps. The release of such molecules does not require vesicle fusion. After leaving the postsynaptic cell, these molecules activate receptors on the surface of presynaptic terminals. The endocannabinoid signaling system

provides an example of lipophilic retrograde messengers (Freund et al., 2003; Llano et al., 1991; Pitler and Alger, 1992).

Messengers derived from gases

Different types of gases have been classified as retrograde messengers in the central nervous system. Probably the best characterized is the nitric oxide (NO); NO is synthesized on request, as for its particular nature cannot be stored. Calcium influx through NMDA receptors activates nitric oxide synthase (NOS), which synthesizes NO from L-arginine (Boehningand, 2003; Garthwaite, 2008).

Messenger derived from growth factors

Different growth factors can be synthesized by neurons especially during period of intense synaptogenesis; growth factors can act also as retrograde messenger. For example, BDNF, released from the postsynaptic neuron during depolarization can acts on presynaptic TrkB receptors to regulate synaptic strength. (Du and Poo, 2004; Zhang and Poo, 2002). Other secreted proteins that may act as retrograde messengers include growth factors such as NGF, NT-3, and NT-4.

Trans-synaptic modulation by cell-adhesion molecules

Every synapse shows unique features about its structure and function; this diversity is present even among synapses formed onto a single neuron. Several evidences have shown that, despite the huge heterogeneity, postsynaptic and presynaptic structures and functions are well coordinated at the level of the individual synapse. This specificity emerges from the assembly of specific molecules on both sides of the synaptic cleft; however, what kinds of molecules are involved in these processes, which side of the synapse 'instructs' the other and which kind of mechanisms are involved, are questions only partially addressed.
In this section I will discuss the evidence for trans-synaptic modulation of the neurotransmitter release by cell-adhesion molecules of the neuroligins family.

The neurexin-neuroligin complex

Neurexins (Nrxs) and neuroligins (NLs) are transmembrane proteins localized on the pre- and post- synaptic sites, respectively. Their structural organization consists of an extracellular domain necessary for trans-synaptic interactions, a trans-membrane domain and an intracellular domain that allow neurexin and neuroligin to interact with different proteins present on their respective synaptic

sites. The extracellular sequences of NLs are composed of a single domain that is homologous to acetylcholinesterase; this domain allows neuroligins to form dimers. Mammals express four NLs genes (NL1-4). In humans the *Nlgn3* and *Nlgn4* genes are localized in the X-chromosome. The *Nlgn4* gene is complemented on the Y-chromosome by a similar *Nlgn5* gene. The different NL isoforms show a conserved aminoacidic sequence, but different patterns of cellular localization. NL1 seems to be localized exclusively at excitatory synapse, NL2 and NL4 at inhibitory synapses and NL3 both at excitatory and inhibitory synapses.

The NRXs protein family consists of three known members, Nrxs1–3. Nrxs are localized on the presynaptic site (but see Zhang et al., 2010 for a potential postsynaptic role). Their distribution to excitatory and inhibitory synapses appears to be regulated by complex alternative splicing mechanisms.

Initial evidence that neuroligins had a role in synapses function and maturation was provided by Scheiffele et al., (2000) who demonstrated that NLs expressed in non-neuronal cells can induce in co-cultured neurons presynaptic specializations. In similar experiments it was demonstrated that also NRXs, when expressed in a non-neuronal cell, can induce formation of postsynaptic specializations in co-cultured neurons (Graf et al., 2004). In addition, overexpression of NLs in neurons causes an increase in synapse numbers (Chih et al., 2006). Altogether, these studies suggest that, at least in cell cultures, NLs and Nrxns have a powerful synaptogenic effect. However, this *scenario* has been complicated by the analysis of knockout (KO) mice showing that NLs and α-Nrxns are essentials for synaptic function but not synapse formation (Missler et al., 2003; Varoqueaux et al., 2006).

Different papers have highlighted the role of NLs as trans-synaptic signals. Thus, affecting postsynaptic NLs function may alters neurotransmitter release from presynaptic terminals (this effect has been described also for other synaptic proteins; see Gottmann 2006 for a review). In agreement with an increase in presynaptic release probability, most studies found an increase in frequency but not amplitude of miniature events (Prange et al., 2004; Levinson et al., 2005). Transfecting cultured neurons with a dominant-negative form NL1, lacking the C-terminal sequence for PSD-95 binding led to a reduction in the frequency of AMPAR-mediated mEPSCs, again supporting a trans-synaptic regulation of presynaptic release probability (Nam and Chen, 2005). In contrast, by over-expressing NL1 an increase of mEPSCs frequency and glutamate release was found (Stan et al., 2006). In 2007 Futai *and colleagues* provided evidence that NL1 regulates neurotransmitter release. They convincingly demonstrated in organotypic hippocampal slices that the scaffolding protein PSD-95 and neuroligin1 are able to modulate the release probability in a retrograde way, resulting in altered presynaptic short-term plasticity. This effect seems to be mediated by the presynaptic β-neurexin. These results indicate that trans-synaptic protein-protein interactions can functionally link postsynaptic and

presynaptic function. In a recent study from our laboratory, that will be the object of this thesis, we demonstrated that hampering gephyrin function with selective intrabodies alters the probability of GABA release at inhibitory synapses, an effect mediated by NL2 since it can be rescued by over expressing this protein in gephyrin-deprived neurons (Varley et al., 2011). Thus, at GABAergic synapses, NL2 appears to act in a way similar to that of NL 1 at excitatory synapses (Graf et al., 2004; Varoqueaux et al., 2004). Deletion of NL2 decreases the frequency and amplitude of miniature GABAergic currents in CA1 hippocampal pyramidal cells and in the spinal cord. This reduction was accompanied by an increase in failures rate, again suggesting a presynaptic effect (Poulopoulos et al., 2009). Based on these evidences, it is plausible to assume that NLs regulate *via* trans-synaptic signaling the probability of neurotransmitter release at both excitatory and inhibitory synapses. However, how the NL-NRX complex controls vesicle exocytosis and recycling is not completely understood.

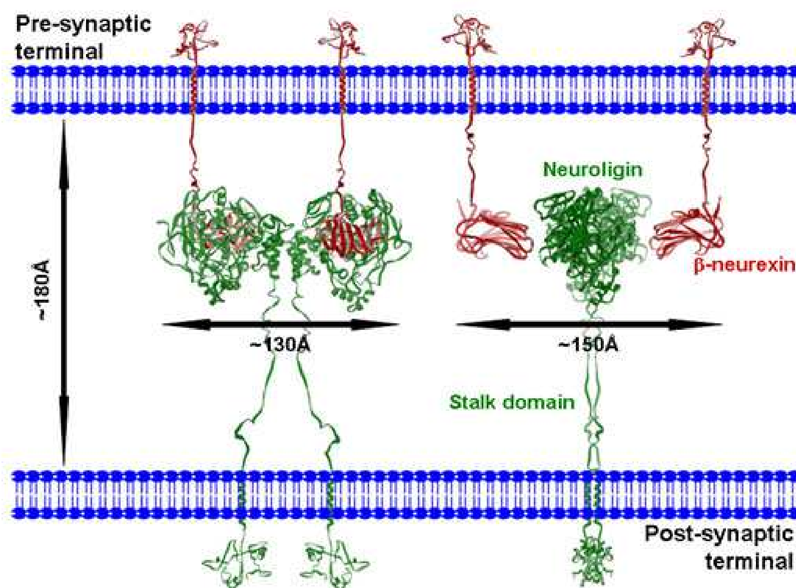


Fig. 8 model of the neuroligin1/β-neurexin assembly at central synapse. The neurexin/neuroligin complex is tethered to pre- and post-synaptic membranes. The protein-protein interactions span and maintain the physical space of the synaptic cleft ensuring a functional and reliable synaptic transmission (*modified* from Comoletti et al., 2007)

The hippocampus

One of the reasons for the interest in the hippocampus is that since the early 1950s, this structure was recognized to play a fundamental role in some forms of learning and memory. In 1957, Scoville and Milner described the case of a patient known by his initials as H.M., who underwent bilateral removal of the hippocampus for the treatment of an intractable form of epilepsy. HM, probably

the most studied neuropsychological subject in memory research suffered a permanent loss of the ability to encode new information into long term memories. This anterograde form of memory impairment has been seen in other patients with bilateral damage of the hippocampus. This and subsequent studies have suggested that the deep structures of the temporal lobe, including the hippocampus (Amaral and Witter, 1989), are involved in the storage of long-term memory traces (Milner et al., 1998; Eichenbaum et al., 1999; Kim and Baxter, 2001; Burgess et al., 2002). Evidence has been provided that the hippocampus is involved in storing and processing spatial information. Studies on rodents have demonstrated that some neurons in the hippocampus possess spatial firing fields. These cells, called "*place cells*" (Muller, 1996), fire when the animal finds itself in a particular location in the space regardless the direction of navigation. Place cells have been also seen in humans involved in finding their way around a virtual reality town (Ekstrom et al., 2003). The discovery of place cells led to the idea that the hippocampus may represent a "*cognitive map*". Recent evidence has cast doubt on this perspective, indicating that the hippocampus may be crucial for more fundamental processes within navigation. Thus, studies on animals have shown that the intact hippocampus is required for simple spatial memory tasks (for instance, finding the way back to a hidden goal) (Kwok and Buckley, 2006).

Hippocampal anatomy

The hippocampus is an elongated structure located on the medial wall of the lateral ventricle, whose longitudinal axis forms a semicircle around the thalamus. The hippocampus *proper* and its neighbouring cortical regions, the dentate gyrus (DG), subiculum and enthorinal cortex, are collectively termed "hippocampal formation". The hippocampus *proper* is divided into *stratum oriens*, *stratum pyramidale*, *stratum radiatum* and *stratum lacunosum-moleculare*. In their classical works, Ramon y Cajal and Lorente de No' grouped excitatory neurons in four regions called CA1-CA4. In general, CA4 is considered the part of the CA3 closed to the dentate gyrus. The CA2 region represents the small portion between the CA3 and the CA1. This part is often ignored but it could have an important role in epilepsy because of the large amount of recurrent collaterals (Shepherd, 1998; Whitner et al., 2009). All pyramidal neurons are endowed with basal dendrites that arborise and form the *stratum oriens* and apical dendrites that are radially oriented in the *stratum radiatum* and *lacunosum-moleculare*. In the DG, granule cells represent the principal neurons, while the area between DG and the CA3 region is called the *hilus* (Figure 9). The main inputs to the hippocampus come from the enthorinal cortex, the septum and the contralateral hippocampus, whereas a unique unidirectional progression of excitatory pathways links each region of the hippocampus, creating a sort of trisynaptic circuit. The perforant path, originating from the enthorinal cortex passes through

the subicular complex and terminates mainly in the dentate gyrus, making synapses on granule cells. Then the distinctive unmyelinated axons of the granule cells (the mossy fibers) project to the *hilus* and to the *stratum lucidum* of the CA3 region. Here they make synapses *en passant* on CA3 pyramidal neurons showing the large, presynaptic varicosities typical of mossy fibres-CA3 contacts. These presynaptic expansions form a unique synaptic complex with equally intricate postsynaptic processes called *thorny excrescences* and may contain tens of releasing sites (Jonas et al., 1993). Information is therefore transferred, through Schaffer collaterals, from CA3 to CA1 pyramidal neurons, which send their axons to the subiculum and the deep layers of the enthorinal cortex. Thus, information entering the enthorinal cortex from a particular cortical area, crosses the entire hippocampus and returns to the cortical area from which it was originated. The transformations that take place during this process are presumably essential for information storage (Shepherd, 1998).

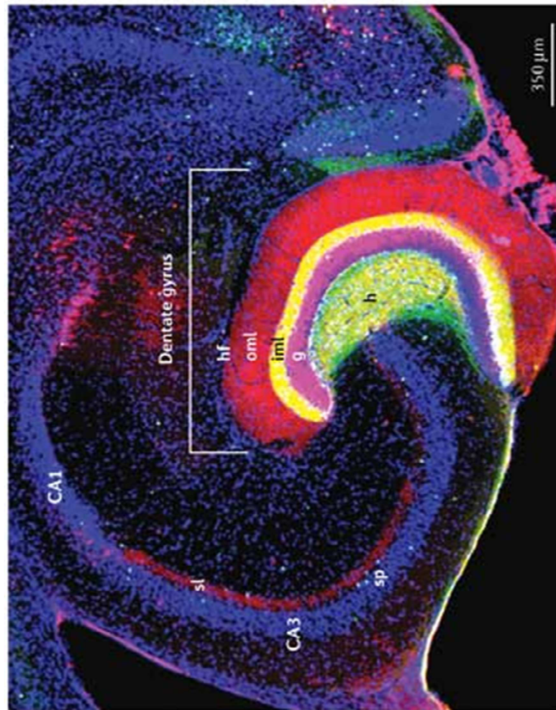


Fig. 9 The hippocampus. The mossy cells in the hilar region (h) of the hippocampus are immunostained green for the calcium binding protein calretinin. Principal cell bodies are stained in blue labeling the pyramidal layer (sp) in areas CA1 and CA3 and the granule cell layer (g) of the dentate gyrus.. (*modified* from Foster et al., 2006).

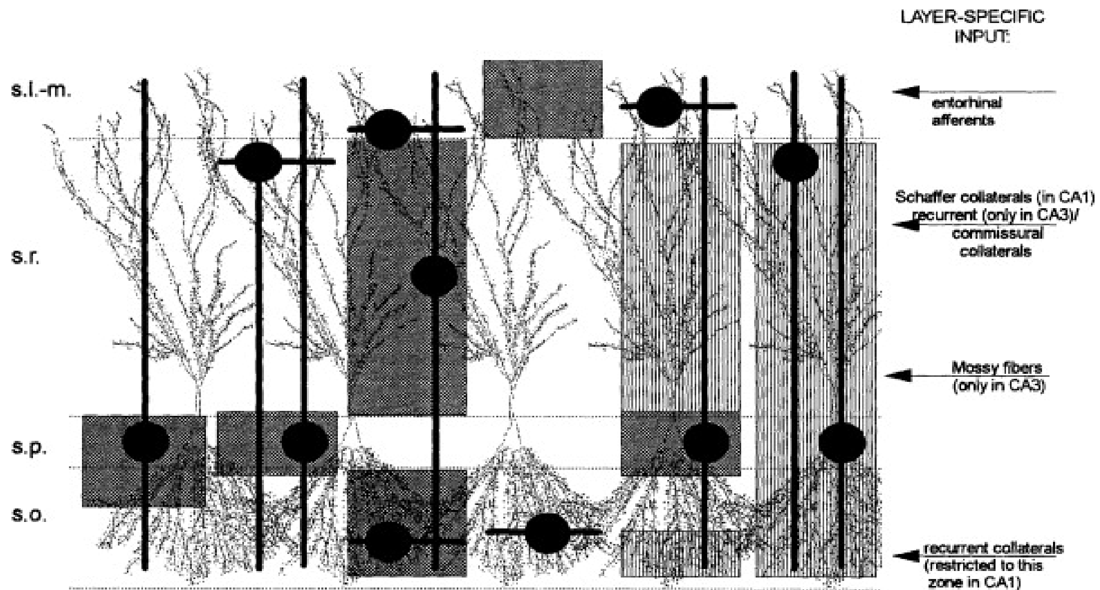


Fig. 10 laminar structure of the hippocampus. The hippocampus *proper* is divided into *stratum oriens (s.o.)*, *stratum pyramidale (s.p.)*, *stratum radiatum (s.r.)* and *stratum lacunosum-moleculare (s.l.-m)*. filled circles represent interneurons. The major inputs to the hippocampus are represented on the right (*modified* from Freund and Buzsaki, 1996).

Local Circuits of the hippocampus

Hippocampal neurons are divided into two major classes: principal cells and interneurons. Although considerably less abundant (< 10%) than principal neurons, recent technical and conceptual progress have indicated an astonishing variety of interneuron classes endowed with different morphological and functional properties (Freund and Buzsaki, 1996; PING 2008). The interaction between GABAergic interneurons and glutamatergic principal cells are reciprocal: interneurons inhibit principal cells and are excited by them. The connectivity between these two neuronal classes is quite high: individual interneurons can inhibit > 50% of principal cells located within ~ 100 μm and receive excitatory input from a large fraction of them (Ali et al., 1999; Fino and Yuste, 2011; Glichfeld et al., 2008)

All hippocampal subfields receive an abundance of extrinsic afferentsthat, can be grouped into three broad classes:

1) Glutamatergic inputs originating from the enthorinal cortex and/or other ipsilateral and controlateral hippocampal subregions.

2) The septo-hippocampal GABAergic projection.

3) Different pathways from the brainstem and forebrain nuclei releasing neurotransmitters often referred to as "neuromodulators" such as acethylcoline, dopamine, noradrenaline etc…

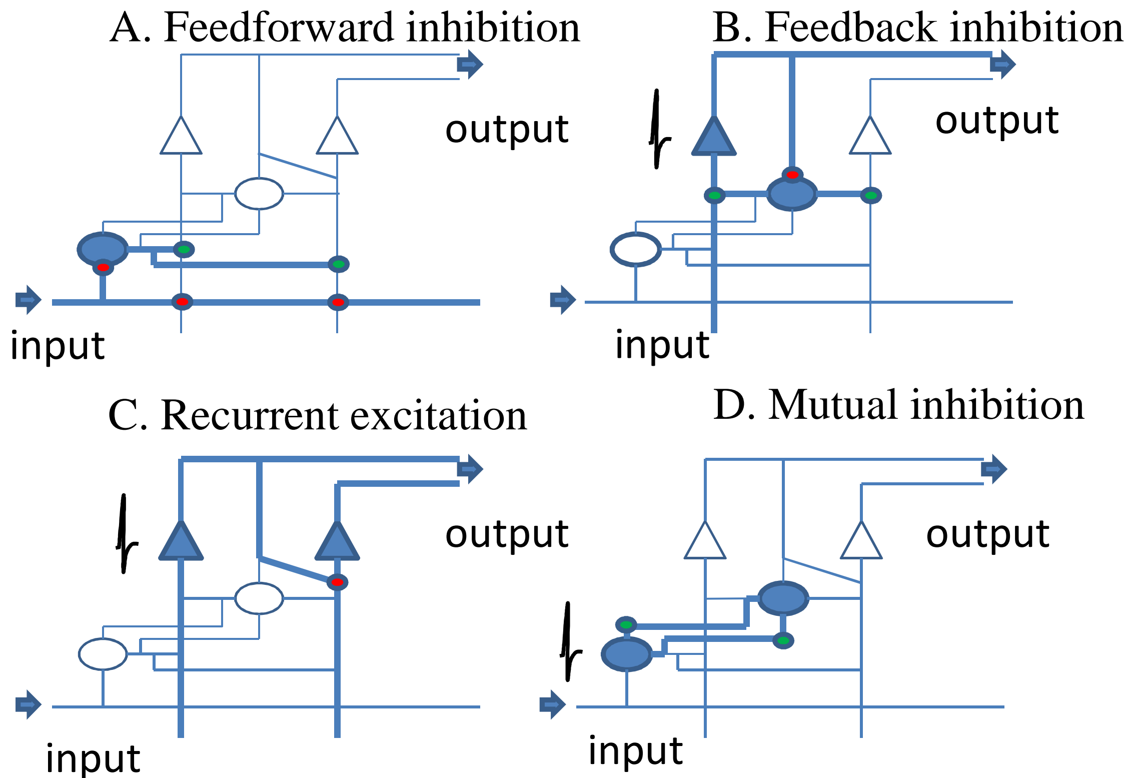


Fig. 11 Local circuit of the hippocampus.*A*. Feedforward inhibition. Axon collateral from excitatory afferent fibers contact local interneurons. *B*. feedback inhibition. Axon collaterals from local principal cells contact local interneurons, interneuron populations in *A* and *B* are not always mutually exclusive. *C*. Recurrent excitation. Axon collaterals from local principal cells also contact other local principal cells. *D*. mutual inhibition. Some interneurons subtypes contact other interneurons as well as principal cells and some interneurons contact other interneurons exclusively. Red circles, excitatory synapses; green circles, inhibitory synapses (*modified* from Buhl and Whittington, 2007)

Glutamatergic afferent pathways show a high degree of laminar selectivity. Enthorinal afferents target the distal apical dendrites of granule and pyramidal cells, whereas commissural/associational fibers innervate the proximal dendrites of granule cells and the apical and basal dendrites of pyramidal neurons in the strata radiatum and oriens of the CA3 and CA1 subfields. This laminar specificity of inputs can have powerful effects on shaping any consequent output from the target population.

These excitatory afferent inputs diverge on both principal cells and interneurons (Buzsaki, 1984).When cortical principal cells make excitatory synaptic contacts on local interneuron that in turn form inhibitory synaptic contacts on the principal cell population feed-back inhibition arises. On the contrary, when long-range afferent inputs diverge onto both principal cells and local interneurons feed-forward inhibition is generated. Feed-forward inhibition serves to impose a temporal framework on a target area on the basis of the input received. The extra delay associated with an additional synapse in the circuit ensures that the feed-forward inhibitory synaptic event does not impinge on the initial, direct extrinsic synaptic event in principal neurons. Some interneurons receive input only from extrinsic afferents and can therefore participate only in feed-forward inhibition as for example cells associated with the perforant path in the molecular layer of the dentate gurus and neurogliaform cells in area CA1 (Han et al., 1993; Vida et al., 1998). Feed-forward inhibition can account for most of the input from extrinsic sources. For example, the mossy fiber axons in area CA3 make about 10 times as many contact with CA3 interneurons as with the far more numerous CA3 principal cells (Acsady et al., 1998).

If principal neurons fire, a number of interneurons receive synaptic excitation. The strength of excitatory synaptic innervation is such that there is a high probability that the target interneuron will fire an action potential. As consequence, interneurons target local principal cells, thus providing a feedback inhibitory circuit. In area CA1, excitatory neurons have a strong preference for interneurons as targets (Gulyas et al., 1998). Many interneurons can be involved in both forms of inhibition thus providing a functional link between afferent input patterns and any resulting output from the target area.

In addition to circuits involving both principal excitatory neurons and inhibitory interneurons, there are many examples of interneuron-interneuron synaptic interactions. There is evidence of a broad heterogeneity of interneuron-interneuron connections, with single presynaptic interneurons innervating other interneurons with different postsynaptic target (dendritic and perisomatic compartments of pyramidal cells) (Tamas et al., 1998). On the contrary of feed-forward and feed-back inhibition, this mutual inhibition can be relatively weak. For example, CA1 basket cells have a higher probability to form synapses with principal cells than with other basket cells; some classes of interneurons appear to target exclusively other interneurons (Freund and Buzsaky, 1996). This recurrent pathway provides a mechanism by which rhythmic patterns of activity can be generated in interneuron circuits and projected onto principal cells, generating in such a way an oscillatory activity (Whittington et al., 1995).

Recurrent connections are not limited to interneurons. In fact, also recurrent excitation is present in hippocampal local circuits. In the hippocampus there are probably few, if any, neurons without local axon collaterals synapsing on neighboring cells. In general, principal neurons recurrently excite other local (or neighboring) principal neurons as well as interneurons. The recurrent

connections between pyramidal neurons are particularly abundant in the CA3 region and are responsible for making this region quite unstable. These connections are responsible for generating in particular conditions epileptiform activity, characterized by spontaneous, synchronized and rhythmic firing in a large number of neurons (Miles and Wong, 1986; Traub and Miles, 1991)
The hippocampus is characterized by a high degree of neuronal interconnectivity. This is due to a multitude of converging inputs and diverging outputs.

Synaptic dysfunctions in neurodevelopmental disorders associated with Autism

Combined genetic and neurobiological approaches have provided evidence that synaptic mutations increase the risk of Autism Spectrum Disorders (ASDs). ASDs comprise a complex and heterogeneous group of pathological conditions including autism, Rett and Asperger syndromes, and pervasive developmental disorders-otherwise non specified, characterized by impaired social interactions, deficits in verbal and non-verbal communication, and a limited interest in the surrounding environment associated with stereotyped and repetitive behaviors (APA, 2000). The incidence of these disorders varies between 10 and 20 per 10000 children. In approximately 70% of the cases, ASDs are associated with intellectual disability and in 25% of the cases with seizures (Tuchman and Rapin, 2002). Clinical signs start appearing at the age of 3 years, but prospective studies of infants at risk have demonstrated that deficits in social responsiveness, communication, and play could be present already at the age of 6–12 months. Genetic factors are thought to account for 80% of autism cases. This hypothesis is strengthen by the fact that ASDs are the most heritable neurodevelopmental disorders of early childhood. Since autism is a spectrum of disorders, it is conceivable that in most cases different genes act in combination in different individuals (Garber, 2007). Despite the etiopathology of autism is still not yet very well understood, in recent years, several important discoveries have pointed out that alterations of synaptic functions could be implicated in the etiopathogenesis of autism, leading to the concept that ASDs (but also others brain disorders such as schizophrenia,) can be considered "synaptopathies".
The most common single-gene mutations associated to ASDs occur in the fragile X syndrome (FMR1), in the tuberous sclerosis (TSC1, TSC2), in the Angelman syndrome (UBE3A), in the Rett syndrome (MECP2), and in Phelan-McDermid syndrome SHANK3 (for review see Pizzarelli and Cherubini, 2011). Rare mutations in the neuroligins (NL2, 3, 4) and neurexins (Nrxs1) genes have also been found to be associated with autism (Jamain et al., 2003). Some of these mutations have been introduced in mice in order to create mice model of ASDs. Despite the difficulties to re-create autistic features in mice (Nestler and Hyman, 2010), these models are particularly useful because allow to study cellular

function along with synaptic activity and ultimately with behavior, Moreover it is possible to use these models to implement pharmacological strategies to rescue behavioral phenotypes reminiscent of those found in autistic patients.
In the following paragraph I will dicuss evidences that link neuroligins to ASDs.

Disorders due to dysfunction of neuroligins and neurexins

Single mutations of the X-linked *Nlgn 3* and *Nlgn 4* genes encoding for the adhesion molecules NL3 and NL4 were among the first to be identified in ASD patients (Jamain et al., 2003). One of these, a missense mutation causing the R451C substitution (arg451→cys451) within the extracellular domain of NL3 was detected in two male siblings, One exhibited an autistic behavior associated with severe intellectual disabilities (ID) and seizures; his brother resulted affected by the Asperger syndrome. Typical of many X-linked disorders, their mother, a carrier, did not manifest any autistic symptom (Jamain et al., 2003). Similarly to NL3, the first identified NL4 mutation occurred in a male with autism, in his brother with Asperger syndrome, and in the asymptomatic carrier mother (Jamain et al., 2003). Other truncating mutations were identified in all affected members of a large family with X-linked ID (Laumonnier et al., 2004) and in two male siblings; one with autism, ID, and a motor tics, and another with Tourette syndrome exhibiting attention deficit, hyperactivity, cognitive defects, and (Lawson Yuen et al., 2008). A de novo single-base substitution near the promoter region of NL4 has beenfound in one male with autism and ID (Daoud et al., 2009). Thus, mutations in NL3 and NL4 cause a wide range of social and cognitive abnormalities ranging from Asperger syndrome to severe cognitive impairement and tics. Also mutations in the neuroligin binding partners neurexins have been associated with autism; the first involving neurexin1 was identified in two individuals with ASD and balanced chromosomal abnormalities (Kim et al., 2008). Stop mutations (S979X) in Nrx1 have been reported in a patient with autistic behaviors, severe ID, hyper-breathing, and some dysmorphic features (Zweier et al., 2009).

Pathophysiology

Loss of NL 3 in mice causes some features that are typically seen in ASD patients. *Nlgn3*-null mice display impaired ultrasound vocalization and altered social memory, possibly owing to olfactory deficiency. The animals, however, performed as well as wild types on direct social interactions and did not have learning deficits or stereotypies (Radyushkin et al., 2009). A recent study from NL3 knock-out mice has revealed disrupted hetero-synaptic competition and altered metabotropic glutamate receptor-dependent synaptic plasticity in the cerebellum (Baudouin et al., 2012). Interestingly, in this case it was possible to

rescue the observed deficits by re-expressing NL 3 in juvenile animals, highlighting the possibility of reverting neuronal circuit alterations after completion of development (Baudouin et al., 2012).
Mice carrying a knock-in allele with the R451C substitution displayed impaired social interactions associated with enhanced spatial learning (Tabuchi et al., 2007). These modifications were associated at the cellular level, with increased levels of the vesicular GABA transporter without apparent changes in the number of GABAergic synapses. In another animal model, a single amino-acid substitution (R704C) found in the neuroligin 4 gene and associated with autism (Yan et al., 2005) has been introduced into mouse neuroligin-3 by homologous recombination. Electrophysiological and morphological studies have revealed that the neuroligin-3 R704C mutation does not significantly alter synapse formation, but is responsible of a major and selective impairment of AMPA (but not GABA or NMDA) receptor-mediated synaptic transmission in pyramidal neurons of the hippocampus (Etherton et al., 2011). As for NL3, deletion of a NL4 in mice caused impaired social interactions and decreased ultrasonic vocalization, consistent with human studies illustrating that loss-of-function mutations cause ASDs and ID (Jamain et al., 2008). Mice lacking a-neurexins isoforms show a decreased presynaptic Ca^{2+} currents, especially N-type Ca^{2+} currents (Missler et al., 2003).These data illustrate that the both neurexins and their binding partners neuroligins, are essential for proper synapse assembly and function and, strength the concept that a synaptic dysfunction can be the cause of ASD.

Aim of the work

Compelling evidence indicates that cell adhesion molecules, once thought to function as the static backbones of synapses, can actually be dynamic regulators of synaptic plasticity that contribute to memory formation. Recently, an array of cell adhesion molecules, have been found to be engaged in a wide range of forms of synaptic plasticity (Dalva et al., 2007). These proteins all share two important features: first, they form homophilic or heterophilic protein-protein interactions spanning and maintaining the physical space of the synaptic cleft, and second, they interact with intracellular signaling partners on both sides of the synapses. Thus, these classes of adhesion molecules are well equipped to couple the functional and structural dynamics of synapses.
During my PhD course I combined electrophysiological recordings with molecular biology and immunocytochemistry to verify the hypothesis that gephyrin, the major scaffold molecule at inhibitory synapses, can transynaptically regulate synaptic strength. Selective intrabodies against gephyrin (scFv-gephyrin) developed in our laboratory (Zacchi et al., 2008) were

used to impair gephyrin function in cultured hippocampal neurons. When expressed in cultured rat hippocampal neurons as a fusion protein containing a nuclear localization signal, scFv-gephyrin were able to remove endogenous gephyrin from $GABA_A$ receptor clusters. Immunocytochemical experiments revealed a significant reduction in the number of synaptic gamma2-subunit containing $GABA_A$receptors and a significant decrease in the density of the GABAergic presynaptic marker, the vesicular GABA transporter (VGAT). These effects were associated with a slow down of the onset kinetics, a reduction in the amplitude and in the frequency of miniature inhibitory postsynaptic currents (Marchionni et al., 2009). The quantitative analysis of current responses to ultrafast application of GABA indicated that changes in onset kinetics resulted from modifications in the microscopic gating of $GABA_A$ receptors and in particular from a reduced entry into the desensitized state. In addition, hampering gephyrin function with scFv induced a significant reduction of $GABA_A$ receptor-mediated tonic conductance. The decrease in tonic conductance and in the frequency of miniature events suggest a reduced probability of GABA release probably secondary to the transynaptic action of gephyrin, Therefore, I first performed pair recordings from interconnected hippocampal cells in culture (the target cell expressing scFv) and found indeed a reduced probability of GABA release into scFv-gephyrin-transfected neurons as compared to controls. This effect was associated with a significant decrease in VGAT, the vesicular GABA transporter, and in NLG2, a protein that, interacting with neurexins, ensures the cross-talk between the post- and presynaptic sites. We were able to rescue these effects by over-expressing NLG 2 in gephyrin-deprived neurons.

Since a dysfunction of synaptic proteins of the NLs-Nrxn families may account for some forms of ASD, in another set of experiments I took advantage of knock-in mice carrying the R451C mutation of the *Nlgn3* gene (found in two Swedish brothers) to examine whether this mutation affects GABAergic and glutamatergic signaling in the hippocampus at early developmental stages. I found that an increased GABAergic innervation was responsible for the increase in frequency of network-driven GDPs, a hallmark of developmental networks, thought to play a crucial role in synapse formation and stabilization, The increased GABAergic but not glutamatergic innervations may affect in a developmental regulated way the excitatory/inhibitory balance, crucial for the refinement of neuronal circuits.. Although also in the present case, a modification of gephyrin clusters in R451C NL 3 knock-in mice was associated with changes in GABAergic innervations suggesting the involvement of a trans-synaptic signal, the role of NL3 mutation in this effect remains to be elucidated.

Materials and Methods

Ethical approval

All experiments were performed in accordance with the European Community Council Directive of November 24, 1986 (86/609EEC) and were approved by the local authority veterinary service and by SISSA ethical committee. All efforts were made to minimize animal suffering and to reduce the number of animal used.

Animals.

NL3^{R451C} mice (Tabuchi et al., 2007) were purchased from Jackson Laboratories (Maine, USA). Experiments were performed on off-spring male derived from heterozygous mating. Electrophysiological experiments were performed and analyzed blind before genotyping. This was carried out on tail biopsy DNA by PCR using a standard protocol. At least three mice for each genotype were used in a given experiment.

Neuronal and Cell Cultures

Primary cell cultures were prepared as described previously (Andjus et al., 1997). Briefly, 2–4-day-old (P2–P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 × *g*, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days. For paired recording experiments, neurons were plated at low density (~40,000 cells/ml). HEK-293 cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) and transiently transfected with various plasmid constructs using the standard calcium phosphate method. Cells were collected 24–48 h after transfection.

Construction of Plasmid Vectors, scFv-Gephyrin

Complementary DNAs encoding full-length FLAG-tagged gephyrin have been described previously (Zita et al., 2007). The N-terminal truncated gephyrin polypeptide (amino acids 2–188) fused to GFP is described by Maas *et al.* (Maas et al., 2006). It acts as a dominant negative protein due to its lack of dimerization motif and is able to deplete endogenous gephyrin clusters in neurites within 24 h of expression. The murine HA-tagged NLG1 and HA-tagged NLG2 were constructed as reported elsewhere (Scheiffele et al., 2006; Chih et al., 2006) HA-tagged NLG2Y770A point mutant was kindly provided by Dr. Varoqueaux

(Poulopoulos A., 2009). NLG2-GFP was constructed by using PCR-based mutagenesis. A PvuI restriction site was introduced 10 amino acids downstream of the sequence encoding for the transmembrane domain of NLG2-HA. This restriction site was then used to clone the EGFP coding sequence amplified using oligonucleotides containing PvuI consensus sites. The last 94 amino acids of the cytoplasmic domains of both NLGs were inserted into pGEX4T1 vector for bacterial expressions as glutathione *S*-transferase (GST)-NLGs 94-amino acid fusion proteins. All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations. The technique for isolating scFv-gephyrin has already been reported (Zacchi et al., 2008).

Neuronal Transfection and Immunocytochemistry

Hippocampal neurons in culture were transfected with EGFP alone or co-transfected with EGFP and scFv-gephyrin using the calcium phosphate transfection method. For each Petri dish, 3 µg of DNA was transfected in total. Reliable co-transfection was ensured by routinely transfecting 0.9 µg of EGFP and 2.1 µg of scFv-gephyrin and identified by the increased EGFP signal around the nucleus. For the rescue experiments, scFv-gephyrin and the full-length HA-tagged NLG2 (NLG2-HA), NLG2Y770A (NLG2Y770A-HA), and NLG1 (NLG1-HA) were co-transfected at a ratio of 1:2. Neurons were transfected at 7 days *in vitro* and used for immunostaining 48 h later. All steps were carried out at room temperature. After fixation with 4% paraformaldehyde in PBS for 10 min, neurons were quenched in 0.1 m glycine in PBS for 5 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min. For the rescue experiments, cells were fixed with precooled 4% paraformaldehyde in PBS for 5 min at 4 °C then 5 min at room temperature. They were then blocked in 0.2% BSA/1% FCS or 10% FCS in PBS for 30 min. After incubation with primary antibodies for 1 h, cells were incubated with Alexa Fluorophore-conjugated secondary antibodies (1:400) for 45 min. In the case of double-immunostaining, cells were incubated with biotinylated secondary antibodies (1:100, 45 min) followed by streptavidin-conjugated fluorophores (1:100, 30 min). The coverslips were washed in PBS, rinsed in water, and mounted with VectaShield (Vector Laboratories).

The antibodies used were as follows: mouse monoclonal anti-VGAT (1:200, Synaptic Systems), mouse monoclonal anti-VGLUT1 (1:200, Synaptic Systems), rabbit polyclonal anti-NGL2 (1:200, Synaptic Systems), biotinylated goat anti-mouse IgG (Vector Laboratories). All secondary antibodies were obtained from Invitrogen.

In Vitro Binding, Immunoprecipitation, and Western Blot Analysis

Transfections were performed with the calcium phosphate method. GST pulldown assays were performed as described previously (Zita et al., 2007). For NLGs and gephyrin co-immunoprecipitation, HEK 293 cells overexpressing NLG1-HA/NLG2-HA and gephyrin-FLAG were lysed in 50 mm Tris-HCl, pH 7.5, 100 mm NaCl, 0.1% Tween 20, 10% glycerol, 10 mm EDTA, 2 mm $MgCl_2$, and protease inhibitor mixture and immunoprecipitated by the anti-FLAG antibody. Analysis of NLG1/NLG2-gephyrin interactions was performed on postnuclear homogenates from neonatal rat brains using the following lysis buffer: 50 mm-Tris HCl, pH 7.5, 150 mm NaCl, 0.5% CHAPS, 1 mm EDTA, 10% glycerol, and protease inhibitor mixture. After a 2-h incubation with monoclonal anti-gephyrin antibody, an immunoprecipitation experiment was performed according to standard procedures. Primary antibodies were revealed by HRP-conjugated secondary antibodies (Sigma) followed by ECL (Amersham Biosciences). The following primary antibodies were used: mouse monoclonal anti-FLAG M2 (Sigma), mouse monoclonal anti-gephyrin 3B11 (Synaptic Systems), high affinity rat monoclonal anti-HA 3F10 (Roche), rabbit polyclonal anti-NLG2 (Synaptic Systems), and rabbit polyclonal anti-NL1 (Synaptic Systems).

Confocal Microscopy and Image Analysis

Fluorescence images were acquired on a TCS-SP confocal laser scanning microscope (Leica, Bensheim, Germany) with a 40× 1.4 NA oil immersion objective, additionally magnified 2-fold with the pinhole set at 1 Airy unit. Stacks of *z*-sections with an interval of 0.4 µm were sequentially scanned twice for each emission line to improve the signal/noise ratio. Cluster analysis was carried out using MetaMorph Imaging System (Universal Imaging, Westchester, PA). First a binary template was created using the EGFP staining to identify transfected neurons, then cluster intensities in regions overlapping with the binary template were analyzed. Images were segmented to select immunofluorescent puncta over background labeling, and clusters were defined as >3 pixels as determined by visual inspection. Integrated Morphometry Analysis function of MetaMorph was used to quantify the number and size of clusters (four or five cells from at least four different experiments). For the rescue experiments, NLG2 staining was used to create the binary template for the NL2-HA/scFv-gephyrin and NL2Y770A-HA/scFv-gephyrin co-transfected cells. As excessive NLG2-HA expression masks the rescuing effect and results in an overall increase in synaptic staining (similar to NL2-HA overexpression alone), cells with a moderate amount of NL2-HA/NL2Y770A-HA expression (as identified by the unsaturated NL2 fluorescence signal) was selected for the analysis of the rescue effect. Representative figures were prepared using ImageJ software.

Electrophysiological recordings from hippocampal neurons in culture

Spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded at 9 days *in vitro* from cultured hippocampal neurons (transfected at 7 days *in vitro* with different constructs) at 22–24 °C using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). In the case of transfected cells, mIPSCs or mEPSCs were recorded from single transfected cells surrounded by nontransfected ones. Patched cells were identified as putative principal cells on the basis of their passive membrane properties (V_{rest} and R_{input}) which were similar to those described in identified pyramidal neurons in culture at the same days *in vitro* (Yang et al., 1993). No differences were found in these parameters between control and transfected neurons. Pooled data gave a mean V_{rest} value of −51 ± 1 mV and a mean R_{input} value of 610 ± 43 MΩ (n = 47). Patch electrodes pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) had a resistance of 3–4 MΩ when filled with an intracellular solution containing 137 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mMBAPTA, 2 mM ATP, and 10 mM HEPES (the pH was adjusted to 7.3–7.4 with CsOH). IPSCs were recorded at a holding potential of −70 mV in the presence of 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM d-2-amino-5-phosphonopentanoic acid (d-AP5) to block AMPA and NMDA receptors, respectively. EPSCs were recorded in the presence of 10 μm bicuculline and 50 μM d-AP5 to block $GABA_A$ and NMDA receptors, respectively. Miniature PSCs were recorded in the presence of tetrodotoxin (TTX, 1 μM) to block sodium currents and propagated action potentials and the respective $GABA_A$ or AMPA/NMDA receptor antagonists.

For double patch recordings, pairs of action potentials (at 50-ms interval) were evoked in non transfected presynaptic neurons (in current clamp mode) by injecting depolarizing current pulses at a frequency of 0.1 Hz. IPSCs were detected from postsynaptic transfected (scFv-gephyrin) and non transfected (controls) neurons in voltage clamp mode at a holding potential of 0 mV (near the reversal potential for glutamate). In this case, the intracellular solutions contained 135 mM $KMeSO_4$, 10 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM Na_2ATP, and 0.4 mM Na_2GTP (the pH was adjusted to 7.3 with KOH). It is worth noting that the probability of finding interconnected cells was 10–20%. Only ∼6% of all neurons in a culture dish are GABAergic, and usually these cells are morphologically distinguishable (Benson et al., 1996). In all experiments, the cells were perfused with an external solution containing 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES, pH 7.4, with NaOH. Data were sampled at 10 kHz and low pass-filtered at 3 kHz. The stability of the patch was checked by repetitively monitoring the input and series resistances during the experiments. Cells exhibiting 15–20% changes were excluded from the analysis. The series resistance was 10–15 MΩ. All drugs (except TTX, which was purchased from Latoxan, Valence, France) were obtained

from Tocris (Cookson Ltd., Bristol, UK). All drugs were dissolved in external solution, except DNQX, which was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the bathing solution was 0.1%. At this concentration, dimethyl sulfoxide alone did not modify the shape or the kinetics of synaptic currents.

Hippocampal slice preparation.

Transverse hippocampal slices (300 μM) were obtained from neonatal (postnatal day 4-9) young (postnatal day 11–15) and adult (postnatal day 27-35) mice using a standard protocol (Griguoli et al., 2010). Briefly, after being anesthetized with CO_2, animals were decapitated. The brain was quickly removed from the skull and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 25 glucose, 3.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, and 1.3 $MgCl_2$, saturated with 95% O2 and 5% CO2 (pH 7.3–7.4). Transverse hippocampal slices were cut with a vibratome and stored at room temperature (22–24°C) in a holding bath containing the same solution as above. After incubation for at least 45 min, an individual slice was transferred to a submerged recording chamber and continuously superfused at 33–34°C with oxygenated ACSF at a rate of 3–4 ml/min.

Electrophysiological recordings in hippocampal slices.

Recordings were made with a patch-clamp amplifier (Axopatch 1D amplifier, Molecular Devices) from CA3 pyramidal cells visualized with an upright microscope equipped with differential interference contrast optics and infrared video camera, using the whole cell configuration of the patch-clamp technique. Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, Germany); when filled with an intracellular solution they had a resistance of 4-6 MΩ. The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting changes of 15 % were excluded from the analysis. The series resistance was < 25 MΩ.

Spontaneous glutamatergic and GABAergic postsynaptic currents were routinely recorded from a holding potential of -65, -70 mV in the presence of bicuculline (10 μM) and DNQX (20 μM), respectively. Miniature currents were recorded in the presence of TTX (1μM) to block sodium currents and propagated action potentials. For glutamatergic currents we used an intracellular solution containing (in mM): 125 Cs-methanesulphonate, 10 CsCl, 10 HEPES, 0.3 EGTA, 2 MgATP, 0.3 NaGTP, (pH adjusted to ~ 7.3 with CsOH). For GABAergic currents we used an intracellular solution containing (in mM): CsCl 137, Hepes 10, BAPTA 11, MgATP 2, $MgCl_2$ 2, $CaCl_2$ 1 and 5 QX-314 (pH adjusted to ~ 7.2-7.3 with CsOH).

Concentric bipolar electrodes were used to stimulate granule cells in the dentate gyrus in order to elicit mossy fiber-excitatory postsynaptic currents (MF-EPSCs)

in CA3 pyramidal neurons (frequency of stimulation: 0.1 Hz; stimulus duration 100-200 µs). Stimulus strength was adjusted to obtain at −65 mV stable 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)-mediated responses of ~100 pA. The NMDA component was recorded from the same neuron at +40 mV, using the same stimulus strength, after blocking the AMPA-mediated component with DNQX (20 µM).
In some experiments, extracellular field potentials were recorded using conventional glass microelectrodes (tip diameter 5–10 μM) filled with ACSF and placed into the stratum pyramidale of the CA3 area.

Data Analysis

The analysis of spontaneous events was performed with Clampfit 10.1 software (Axon Instruments, Foster City, CA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data.

For evoked IPSCs, transmission failures were identified visually. Mean IPSC amplitude was obtained by averaging successes and failures. The paired-pulse ratio (PPR), known to be inversely correlated to the initial release probability (Dobrunz and Stevens 1997), was calculated as the ratio between the mean amplitudes of IPSC2 over IPSC1. The coefficient of variation (CV^{-2}) was calculated as the square root of the ratio between the standard deviation of IPSC1 and the mean amplitude of IPSC1 (Korn and Faber, 1991).

The amplitude of the tonic current was estimated by the outward shift of the baseline current after the application of the $GABA_A$ receptor channel blocker picrotoxin (100 µM). Only current recordings that exhibited a stable baseline were included in the analysis. Baseline currents and their standard deviations were estimated by plotting 10 s periods of raw data in all point histograms. These were fitted with a Gaussian function. The peak of the fitted Gaussian was considered as the mean holding current (Glykys & Mody, 2007).
NMDA/AMPA ratio was measured by averaging 15 to 30 sweeps for each holding potential. The AMPA-mediated component was measured at the peak of the current obtained at – 65 mV, while the NMDA component was measured at the peak of the current obtained at +40 mV after blocking the AMPA component with DNQX. The weighted decay time constant for NMDA-mediated synaptic current was measured by dividing the area by its amplitude, independently of the fitting (Cathala et al., 2003).

Peak scaled non-stationary noise analysis was performed according to Traynelis et al., (1993) using the Mini Analysis program (version 6.0.1, Synaptosoft, Leonia, NJ). Miniature events were selected following the procedure described by Momiyama et al. (2003). Briefly, individual miniature events were analyzed by measuring the peak amplitude, the 10–90% rise time and the decay time constant. Measured parameters were then numbered according to the event number and tested by Spearman's rank order correlation test for time stability. After testing for correlation between rise time and amplitude and between rise time and decay time, miniature GABAergic events were used for noise analysis. Individual miniature events were aligned to the point of steepest rise time. The peak of the mean current response waveform was scaled to the response value at the corresponding point in time of each individual event before subtraction to generate the difference waveforms. The ensemble mean post synaptic current was binned into 50 bins of equal amplitude to assign similar weights to all phases of ensemble mean waveform. Variance was plotted against amplitude and individual points were fitted with the equation:

$$\sigma^2(I) = iI - I^2/N + \sigma_b^2 \quad (1)$$

where i is the unitary single-channel current, I is the mean current, N is the number of channels open at the current peak and σ_b^2 is the variance of the background noise. The single-channel chord conductance (Υ) was calculated as:

$$\Upsilon = i/(E_m - E_{rev}) \quad (2)$$

from the holding potential (E_m) of −70 mV, assuming a reversal potential (E_{rev}) of 0 mV.

Statistical analysis

All values are presented as mean ± SEM of n experiments. Statistical comparison was performed using unpaired t-test or one-way analysis of variance (ANOVA). Probability distributions were examined using the Kolmogorov–Smirnov test. A p value < 0.05 was considered as statistically significant.

RESULTS

-Paper N.1.-

Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures.

Varley ZK, Pizzarelli R, Antonelli R, Stancheva SH, Kneussel M, Cherubini E, Zacchi P.

Journal of Biological Chemistry 2011 ;286:20942-51.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2011/04/27/M111.234641.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 23, pp. 20942–20951, June 10, 2011
© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Gephyrin Regulates GABAergic and Glutamatergic Synaptic Transmission in Hippocampal Cell Cultures*[S]

Received for publication, February 24, 2011, and in revised form, April 12, 2011 Published, JBC Papers in Press, April 20, 2011, DOI 10.1074/jbc.M111.234641

Zeynep Kasap Varley[‡1], Rocco Pizzarelli[‡1], Roberta Antonelli[‡], Stefka H. Stancheva[‡], Matthias Kneussel[§], Enrico Cherubini[‡], and Paola Zacchi[‡¶2]

From the [‡]Neurobiology Department and Italian Institute of Technology Unit, International School for Advanced Studies (SISSA) and [¶]Cluster in Biomedicine, Via Bonomea 265, 34136 Trieste, Italy and [§]Center for Molecular Neurobiology, Zentrum für Molekulare Neurobiologie, University of Hamburg Medical School, 20251 Hamburg, Germany

Gephyrin is a scaffold protein essential for stabilizing glycine and GABA$_A$ receptors at inhibitory synapses. Here, recombinant intrabodies against gephyrin (scFv-gephyrin) were used to assess whether this protein exerts a transynaptic action on GABA and glutamate release. Pair recordings from interconnected hippocampal cells in culture revealed a reduced probability of GABA release in scFv-gephyrin-transfected neurons compared with controls. This effect was associated with a significant decrease in VGAT, the vesicular GABA transporter, and in neuroligin 2 (NLG2), a protein that, interacting with neurexins, ensures the cross-talk between the post- and presynaptic sites. Interestingly, hampering gephyrin function also produced a significant reduction in VGLUT, the vesicular glutamate transporter, an effect accompanied by a significant decrease in frequency of miniature excitatory postsynaptic currents. Overexpressing NLG2 in gephyrin-deprived neurons rescued GABAergic but not glutamatergic innervation, suggesting that the observed changes in the latter were not due to a homeostatic compensatory mechanism. Pulldown experiments demonstrated that gephyrin interacts not only with NLG2 but also with NLG1, the isoform enriched at excitatory synapses. These results suggest a key role of gephyrin in regulating transynaptic signaling at both inhibitory and excitatory synapses.

Speed and reliability of synaptic transmission are essential for information coding and require the presence of clustered neurotransmitter receptors at the plasma membrane in precise apposition to presynaptic release sites. The postsynaptic organization comprises a large number of proteins that ensure the correct targeting, clustering, and stabilization of neurotransmitter receptors. Among them, the tubulin-binding protein gephyrin plays a crucial role in the functional organization of inhibitory synapses (1). Through its self-oligomerizing properties, gephyrin can form a hexagonal lattice that traps glycine (2) and GABA$_A$ receptors in the right place at postsynaptic sites (3, 4) by linking them to the cytoskeleton. Disruption of endogenous gephyrin leads to reduced GABA$_A$ receptor clusters (3), an effect that has been shown to be accompanied by a loss of GABAergic innervation (5, 6). This observation suggests the existence of cross-talk between the post- and presynaptic sites. The retrograde control of presynaptic signaling may occur via neuroligins (NLGs),[3] postsynaptic cell adhesion molecules known to transynaptically interact with presynaptic neurexins (7). NLG1 is enriched at glutamatergic synapses (8, 9), whereas NLG2 is preferentially associated with GABAergic connections (10). Overexpression of NLGs has been shown to increase the number of GABAergic and glutamatergic synaptic contacts (11). Interestingly, increasing the expression level of PSD-95, the scaffold molecule that directly binds NLG1, caused an enhancement of the glutamatergic innervation at the expense of the GABAergic one. This effect was accompanied by the recruitment of NLG2 to glutamatergic synapses (11–13). Moreover, the recent demonstration of a direct interaction between NLG2 and gephyrin (14) suggests a role for this protein in regulating transynaptic signaling at inhibitory connections. Altogether, these findings have led to the hypothesis that scaffolding molecules can establish and maintain the proper excitatory (E)/inhibitory (I) balance necessary for the correct functioning of neuronal networks, by modulating neuroligin localization and function at particular synapses (15–17). Understanding the molecular mechanisms involved in the maintenance of a proper E/I balance is a challenge as an alteration of this parameter underlies several devastating forms of neurological diseases including autism spectrum disorders (18). Previous studies on cultured hippocampal neurons have demonstrated that removal of gephyrin with single chain antibody fragments (scFv-gephyrin) (19) produces changes in the gating properties of GABA$_A$ receptors associated with a decrease in GABAergic innervation (6).

In the present study, scFv-gephyrin fragments were used to characterize further the transynaptic contribution of gephyrin to maintaining and stabilizing GABAergic synapses. Double patch experiments from monosynaptically connected cells revealed a reduction in the probability of GABA release to scFv-

* This work was supported by a grant from Ministero Istruzione, Università e Ricerca (MIUR) (to E. C.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Neurobiology Sector, SISSA, Via Bonomea 265, 34136 Trieste, Italy. Tel.: 39-040-3787773; Fax: 39-040-3787702; E-mail: zacchi@sissa.it.

[3] The abbreviations used are: NLG, neuroligin; D-AP5, D-2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EGFP, enhanced green fluorescent protein; E/I, excitatory/inhibitory; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; PPR, paired-pulse ratio; scFv, single chain antibody fragment; TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid; TTX, tetrodotoxin; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter.

Downloaded from www.jbc.org at SISSA, on October 9, 2012

gephyrin-transfected cells. Moreover, transfection with scFv-gephyrin affected not only GABA but also glutamate release as demonstrated by the reduction in frequency of spontaneous and miniature glutamatergic synaptic events. Immunocytochemical data revealed a significant reduction in the number of NLG2 clusters together with a decrease of VGAT and VGLUT, the vesicular GABA and glutamate transporters, respectively. Finally, biochemical experiments demonstrated that gephyrin can form a complex not only with NLG2 but also with NLG1 in the brain, suggesting a role of this scaffold protein in regulating both excitatory and inhibitory synaptic transmission.

EXPERIMENTAL PROCEDURES

Neuronal and Cell Cultures—All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609 EEC) and were approved by the local authority veterinary service. Primary cell cultures were prepared as described previously (20). Briefly, 2–4-day-old (P2–P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 × *g*, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days. For paired recording experiments, neurons were plated at low density (~40,000 cells/ml).

HEK-293 cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) and transiently transfected with various plasmid constructs using the standard calcium phosphate method. Cells were collected 24–48 h after transfection.

Construction of Plasmid Vectors, scFv-Gephyrin—Complementary DNAs encoding full-length FLAG-tagged gephyrin have been described previously (21). The N-terminal truncated gephyrin polypeptide (amino acids 2–188) fused to GFP is described by Maas *et al.* (22). It acts as a dominant negative protein due to its lack of dimerization motif and is able to deplete endogenous gephyrin clusters in neurites within 24 h of expression. The murine HA-tagged NLG1 and HA-tagged NLG2 were constructed as reported elsewhere (23, 24). HA-tagged NLG2Y770A point mutant was kindly provided by Dr. Varoqueaux (14). NLG2-GFP was constructed by using PCR-based mutagenesis. A PvuI restriction site was introduced 10 amino acids downstream of the sequence encoding for the transmembrane domain of NLG2-HA. This restriction site was then used to clone the EGFP coding sequence amplified using oligonucleotides containing PvuI consensus sites.

The last 94 amino acids of the cytoplasmic domains of both NLGs were inserted into pGEX4T1 vector for bacterial expressions as glutathione *S*-transferase (GST)-NLGs 94-amino acid fusion proteins. All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations. The technique for isolating scFv-gephyrin has already been reported (19).

Neuronal Transfection and Immunocytochemistry—Hippocampal neurons in culture were transfected with EGFP alone or co-transfected with EGFP and scFv-gephyrin using the calcium phosphate transfection method. For each Petri dish, 3 μg of DNA was transfected in total. Reliable co-transfection was ensured by routinely transfecting 0.9 μg of EGFP and 2.1 μg of scFv-gephyrin and identified by the increased EGFP signal around the nucleus. For the rescue experiments, scFv-gephyrin and the full-length HA-tagged NLG2 (NLG2-HA), NLG2Y770A (NLG2Y770A-HA), and NLG1 (NLG1-HA) were co-transfected at a ratio of 1:2.

Neurons were transfected at 7 days *in vitro* and used for immunostaining 48 h later. All steps were carried out at room temperature. After fixation with 4% paraformaldehyde in PBS for 10 min, neurons were quenched in 0.1 M glycine in PBS for 5 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min. For the rescue experiments, cells were fixed with precooled 4% paraformaldehyde in PBS for 5 min at 4 °C then 5 min at room temperature. They were then blocked in 0.2% BSA/1% FCS or 10% FCS in PBS for 30 min. After incubation with primary antibodies for 1 h, cells were incubated with Alexa Fluorophore-conjugated secondary antibodies (1:400) for 45 min. In the case of double-immunostaining, cells were incubated with biotinylated secondary antibodies (1:100, 45 min) followed by streptavidin-conjugated fluorophores (1:100, 30 min). The coverslips were washed in PBS, rinsed in water, and mounted with VectaShield (Vector Laboratories).

The antibodies used were as follows: mouse monoclonal anti-VGAT (1:200, Synaptic Systems), mouse monoclonal anti-VGLUT1 (1:200, Synaptic Systems), rabbit polyclonal anti-NGL2 (1:200, Synaptic Systems), biotinylated goat anti-mouse IgG (Vector Laboratories). All secondary antibodies were obtained from Invitrogen.

In Vitro Binding, Immunoprecipitation, and Western Blot Analysis—Transfections were performed with the calcium phosphate method. GST pulldown assays were performed as described previously (21). For NLGs and gephyrin co-immunoprecipitation, HEK 293 cells overexpressing NLG1-HA/NLG2-HA and gephyrin-FLAG were lysed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 10% glycerol, 10 mM EDTA, 2 mM $MgCl_2$, and protease inhibitor mixture and immunoprecipitated by the anti-FLAG antibody. Analysis of NLG1/NLG2-gephyrin interactions was performed on postnuclear homogenates from neonatal rat brains using the following lysis buffer: 50 mM-Tris HCl, pH 7.5, 150 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 10% glycerol, and protease inhibitor mixture. After a 2-h incubation with monoclonal anti-gephyrin antibody, an immunoprecipitation experiment was performed according to standard procedures. Primary antibodies were revealed by HRP-conjugated secondary antibodies (Sigma) followed by ECL (Amersham Biosciences). The following primary antibodies were used: mouse monoclonal anti-FLAG M2 (Sigma), mouse monoclonal anti-gephyrin 3B11 (Synaptic Systems), high affinity rat monoclonal anti-HA 3F10 (Roche), rabbit polyclonal anti-NLG2 (Synaptic Systems), and rabbit polyclonal anti-NLG1 (Synaptic Systems).

Confocal Microscopy and Image Analysis—Fluorescence images were acquired on a TCS-SP confocal laser scanning microscope (Leica, Bensheim, Germany) with a 40× 1.4 NA oil immersion objective, additionally magnified 2-fold with the pinhole set at 1 Airy unit. Stacks of *z*-sections with an interval of 0.4 μm were sequentially scanned twice for each emission line

Downloaded from www.jbc.org at SISSA, on October 9, 2012

to improve the signal/noise ratio. Cluster analysis was carried out using MetaMorph Imaging System (Universal Imaging, Westchester, PA). First a binary template was created using the EGFP staining to identify transfected neurons, then cluster intensities in regions overlapping with the binary template were analyzed. Images were segmented to select immunofluorescent puncta over background labeling, and clusters were defined as >3 pixels as determined by visual inspection. Integrated Morphometry Analysis function of MetaMorph was used to quantify the number and size of clusters (four or five cells from at least four different experiments). For the rescue experiments, NLG2 staining was used to create the binary template for the NLG2-HA/scFv-gephyrin and NLG2Y770A-HA/scFv-gephyrin co-transfected cells. As excessive NLG2-HA expression masks the rescuing effect and results in an overall increase in synaptic staining (similar to NLG2-HA overexpression alone), cells with a moderate amount of NLG2-HA/NLG2Y770A-HA expression (as identified by the unsaturated NLG2 fluorescence signal) was selected for the analysis of the rescue effect. Representative figures were prepared using ImageJ software.

Electrophysiological Recordings—Spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded at 9 days *in vitro* from cultured hippocampal neurons (transfected at 7 days *in vitro* with different constructs) at 22–24 °C using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). In the case of transfected cells, mIPSCs or mEPSCs were recorded from single transfected cells surrounded by nontransfected ones. Patched cells were identified as putative principal cells on the basis of their passive membrane properties (V_{rest} and R_{input}) which were similar to those described in identified pyramidal neurons in culture at the same days *in vitro* (25). No differences were found in these parameters between control and transfected neurons. Pooled data gave a mean V_{rest} value of -51 ± 1 mV and a mean R_{input} value of 610 ± 43 megohms ($n = 47$). Patch electrodes pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) had a resistance of 3–4 megohms when filled with an intracellular solution containing 137 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 2 mM ATP, and 10 mM HEPES (the pH was adjusted to 7.3–7.4 with CsOH). IPSCs were recorded at a holding potential of −70 mV in the presence of 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM d-2-amino-5-phosphonopentanoic acid (D-AP5) to block AMPA and NMDA receptors, respectively. EPSCs were recorded in the presence of 10 μM bicuculline and 50 μM D-AP5 to block $GABA_A$ and NMDA receptors, respectively. Miniature PSCs were recorded in the presence of tetrodotoxin (TTX, 1 μM) to block sodium currents and propagated action potentials and the respective $GABA_A$ or AMPA/NMDA receptor antagonists.

For double patch recordings, pairs of action potentials (at 50-ms interval) were evoked in nontransfected presynaptic neurons (in current clamp mode) by injecting depolarizing current pulses at a frequency of 0.1 Hz. IPSCs were detected from postsynaptic transfected (scFv-gephyrin) and nontransfected (controls) neurons in voltage clamp mode at a holding potential of 0 mV (near the reversal potential for glutamate). In this case, the intracellular solutions contained 135 mM $KMeSO_4$, 10 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM Na_2ATP, and 0.4 mM Na_2GTP (the pH was adjusted to 7.3 with KOH). It is worth noting that the probability of finding interconnected cells was 10–20%. Only ~6% of all neurons in a culture dish are GABAergic, and usually these cells are morphologically distinguishable (26). In all experiments, the cells were perfused with an external solution containing 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES, pH 7.4, with NaOH. Data were sampled at 10 kHz and low pass-filtered at 3 kHz. The stability of the patch was checked by repetitively monitoring the input and series resistances during the experiments. Cells exhibiting 15–20% changes were excluded from the analysis. The series resistance was 10–15 megohms. All drugs (except TTX, which was purchased from Latoxan, Valence, France) were obtained from Tocris (Cookson Ltd., Bristol, UK). All drugs were dissolved in external solution, except DNQX, which was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the bathing solution was 0.1%. At this concentration, dimethyl sulfoxide alone did not modify the shape or the kinetics of synaptic currents.

Data Analysis—The analysis of spontaneous events was performed with Clampfit 10.1 software (Axon Instruments, Foster City, CA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data.

For evoked IPSCs, transmission failures were identified visually. Mean IPSC amplitude was obtained by averaging successes and failures. The paired-pulse ratio (PPR), known to be inversely correlated to the initial release probability (27), was calculated as the ratio between the mean amplitudes of IPSC2 over IPSC1. The coefficient of variation (CV^{-2}) was calculated as the square root of the ratio between the standard deviation of IPSC1 and the mean amplitude of IPSC1 (28).

Values are given as mean ± S.E. Unless otherwise stated, significance of differences was assessed by Student's *t* test. The differences were considered significant when $p < 0.05$.

RESULTS

Impairing Gephyrin Function with scFv-Gephyrin Reduces the Probability of GABA Release—As recently reported (6), transfecting cultured hippocampal neurons with scFv-gephyrin reduced the number of gephyrin and synaptic $\gamma 2$ subunit-containing $GABA_A$ receptor clusters. These effects were associated with a severe impairment of both phasic and tonic $GABA_A$ receptor-mediated inhibition. The mechanisms underlying these effects relied on changes in GABAergic innervation as suggested by the concomitant reduction in the number and size of presynaptic VGAT clusters.

According to the quantal theory, the synaptic efficacy E, the mean amplitude of unitary IPSCs, can be defined as $E = mQ$, where m is the quantal content or mean number of quanta released per presynaptic action potential and Q is the quantal size or amplitude of the unitary IPSC (29). Whereas Q depends on both pre- and postsynaptic mechanisms, m depends on presynaptic factors, namely the number of release sites N and the

Downloaded from www.jbc.org at SISSA, on October 9, 2012

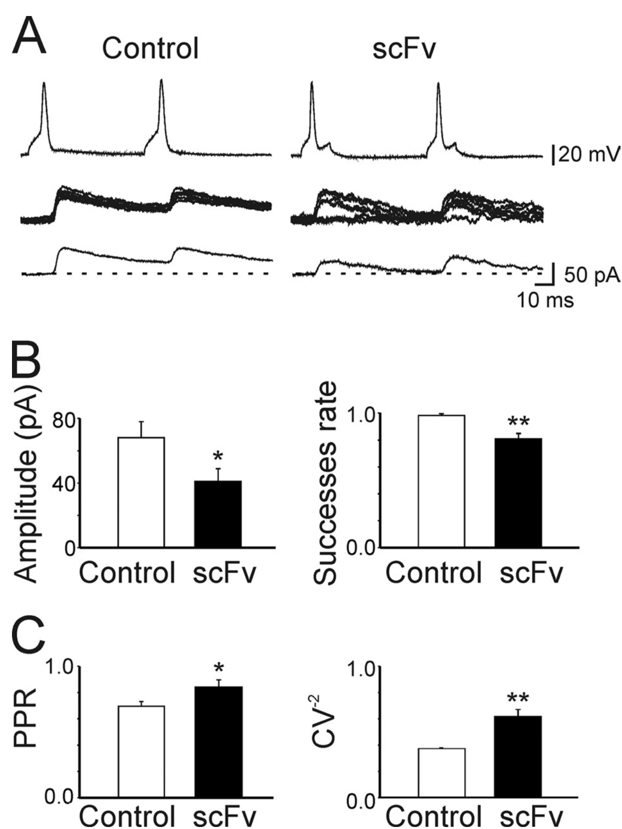


FIGURE 1. **Hampering gephyrin function with scFv-gephyrin reduces the probability of GABA release.** *A*, pair recordings obtained from two interconnected neurons. The postsynaptic cell was transfected with scFv-gephyrin (*right*). As control a neighboring nontransfected cell was used (*left*). *Top traces* are pairs of action potentials evoked in presynaptic cells at a 50-ms interval by depolarizing current steps of variable amplitude every 10 s. *Middle traces* are monosynaptic IPSCs (successes and failures) evoked at 0 mV (E_{GABA} −70 mV) by presynaptic action potentials. *Bottom traces* are averaged responses. *B*, mean amplitude and successes rate obtained in monosynaptically connected cells in control (*white columns*; $n = 7$) and in scFv-transfected neurons (*black columns*). *C*, PPR and CV^{-2} of monosynaptically connected neurons ($n = 6$) recorded from control and scFv-transfected cells. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.E.

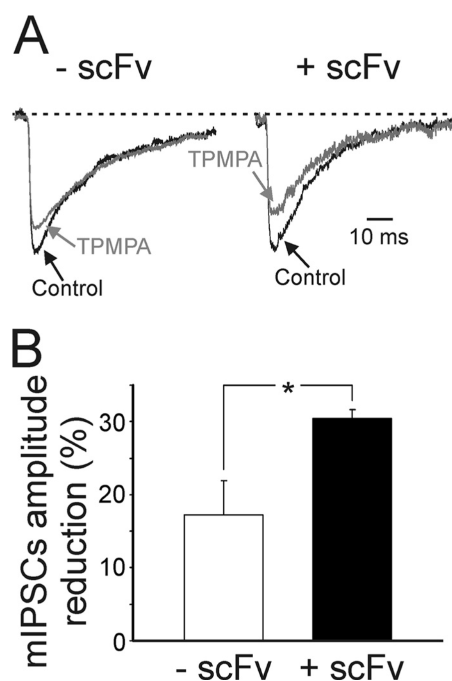


FIGURE 2. **The removal of gephyrin with scFv reduces synaptic GABA transient in the cleft.** *A*, representative traces of mIPSCs recorded at −70 mV (*dashed lines*) from a control (*left*) and from a scFv-gephyrin-transfected cell (*right*) in the absence (*black*) or in the presence of TPMPA 100 μM (gray). In both cases, mIPSC amplitudes are normalized to those obtained in pre-drug conditions. Each *trace* is the average of 20–30 individual traces. *B*, each column representing the mean TPMPA-induced reduction in amplitude of mIPSCs in control (*white*, $n = 7$) and scFv-gephyrin-transfected cells (*black*, $n = 6$; *, $p < 0.05$). Note the significantly larger TPMPA inhibition in transfected cells. *, $p < 0.05$. *Error bars*, S.E.

probability of release (*P*). To see whether a decrease in quantal content could account for the observed effects, simultaneous recordings were obtained from pairs of interconnected neurons (the postsynaptic one expressing or not expressing scFv-gephyrin; see "Experimental Procedures"). As shown in Fig. 1, IPSCs evoked in nontransfected cells by pairs of presynaptic action potentials (50 ms apart, delivered at a frequency of 0.1 Hz, Control) were highly reliable and usually did not exhibit synaptic failures. In contrast, with respect to control, IPSCs from scFv-gephyrin-transfected cells ($n = 6$) exhibited a significant reduction in amplitude (from 68.2 ± 9.7 pA to 41.1 ± 7.8 pA; $p < 0.05$, Mann-Whitney Rank test) and in successes rate (from 0.98 ± 0.01 to 0.80 ± 0.03; $p < 0.01$, Mann-Whitney Rank test; Fig. 1, *A* and *B*). These effects were associated with a significant increase in the PPR (from 0.69 ± 0.03 to 0.84 ± 0.05; $p < 0.05$; Fig. 1*C*), which is considered an index of presynaptic release probability (27, 28). Furthermore, the coefficient of variation (CV^{-2}) was significantly increased (from 0.37 ± 0.007 to 0.61 ± 0.05; $p < 0.01$; Fig. 1*C*), indicating changes in quantal content (28).

To assess further whether gephyrin depletion affects presynaptic GABA release, as an additional approach we used 100 μM TPMPA, a weak competitive $GABA_A$ receptor antagonist that has a very fast dissociation constant and competes with synaptically released GABA for the ligand binding site on $GABA_A$ receptors (30, 31). The reduction of mIPSC amplitude by TPMPA would therefore be influenced by relative changes in synaptic GABA transient in the cleft (31). As shown in Fig. 2, in scFv-gephyrin-transfected cells, the block of mIPSCs by TPMPA was significantly ($p < 0.05$) larger than controls (30.4 ± 1.3% *versus* 17.2 ± 4.7%; $n = 6$ for scFv-gephyrin and 7 for controls; $p < 0.5$; Fig. 2, *A* and *B*), indicating that for scFv-gephyrin-transfected cells, GABA concentration in the cleft was lower than control. Overall, these data strongly suggest that hampering gephyrin function with scFv-gephyrin reduces the release of GABA from presynaptic terminals.

Gephyrin 2–188, a Dominant Negative Form of Gephyrin, Mimics the Effect of scFv-Gephyrin on GABAergic Function— To validate the results obtained with scFv-gephyrin, a truncated gephyrin polypeptide comprising the N-terminal (amino acids 2–188) of gephyrin fused with EGFP, known to act as a dominant negative protein, was used (22). Due to the lack of dimerization motif, this polypeptide interferes with the endogenous gephyrin lattice formation and depletes gephyrin clusters in neurites within 24 h of expression on cultured neurons.

Immunocytochemical experiments on hippocampal neurons transfected with gephyrin 2–188 revealed a significant reduction in the number of VGAT clusters (without an effect on their

Downloaded from www.jbc.org at SISSA, on October 9, 2012

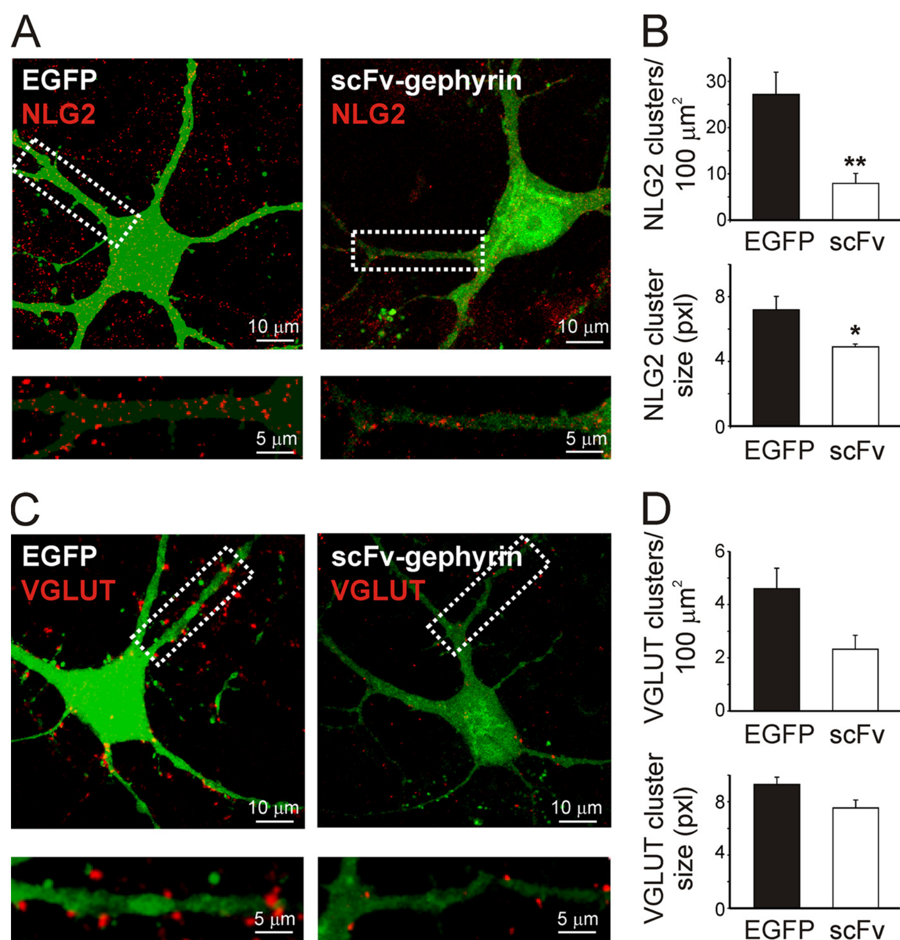


FIGURE 3. **scFv-gephyrin reduces the number and size of NLG2 and VGLUT clusters.** *A*, neurons transfected (*green*) with EGFP (*left*) or EGFP/scFv-gephyrin (*right*) and immunostained for NLG2 (*red*). *Bottom panels* are magnifications of the *white boxes* marked on *top*. *B*, quantification of NLG2 clusters density (*top*) and cluster size (*bottom*). *C* and *D*, as in *A* and *B*, but neurons were immunostained for the presynaptic glutamatergic marker VGLUT (*red*). Note the significant reduction in the density and size of NLG2 and VGLUT clusters. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.E.

size), indicating an effect on GABAergic innervation similar to that observed for scFv-gephyrin (supplemental Fig. 1, *A* and *B*). As with scFv-gephyrin, this effect was accompanied by a significant reduction in amplitude and frequency of spontaneous and miniature IPSCs (in cells transfected with gephyrin 2–188 the reduction in amplitude of sIPSCs and mIPSCs was 54 ± 9% and 79 ± 9% of controls, respectively; the reduction in frequency of sIPSCs and mIPSCs was 32 ± 7% and 37 ± 1% of controls, respectively; supplemental Fig. 1, *C–E*). These data further support the hypothesis that gephyrin not only regulates postsynaptic organization of synaptic $GABA_A$ receptors but also GABAergic innervation.

Gephyrin Removal Reduces the Density and Size of Neuroligin 2 Clusters—How can gephyrin interfere with GABA release? One possibility is that this protein interacts with cell adhesion molecules such as neuroligins which, by binding neurexins, ensure the cross-talk between the pre- and postsynaptic sites (7). Of particular interest is NLG2, because this protein is known to play a pivotal role in the organization of GABAergic synapses (14). To verify whether disrupting gephyrin affects NLG2 distribution, hippocampal neurons transfected with scFv-gephyrin were immunostained for NLG2.

As shown in Fig. 3, *A* and *B*, scFv-gephyrin-transfected neurons exhibited a significant reduction in the density of NLG2-positive clusters compared with EGFP-transfected controls (7.9 ± 2.1 clusters/100 μm^2 for scFv-gephyrin *versus* 27.2 ± 4.8 clusters/100 μm^2 for EGFP; $p < 0.01$; $n = 9$). In addition, the average size of these clusters was smaller for scFv-gephyrin- than for EGFP-transfected neurons (4.9 ± 0.2 μm^2 for scFv-gephyrin *versus* 7.2 ± 0.8 μm^2 for EGFP; $p < 0.05$; $n = 9$). NLG2 did not relocalize to glutamatergic synapses because the synaptic fraction co-localized with VGLUT was barely detectable (4.1 ± 0.01% in control and 5.3 ± 0.02% in scFv-gephyrin-transfected cells, respectively; these values were not significantly different; $p > 0.05$; data not shown).

Impairing Gephyrin Function with scFv-Gephyrin Reduces Glutamatergic Innervation—The interaction of NLGs with scaffolding proteins is crucial for ensuring the correct excitatory/inhibitory balance, critical for the proper functioning of neuronal networks. Therefore, the following experiments were performed to assess whether disrupting gephyrin function with scFv-gephyrin can affect not only GABAergic but also glutamatergic transmission.

To this aim, cultured hippocampal neurons transfected with scFv-gephyrin were immunostained for VGLUT, a widely used marker for presynaptic glutamatergic terminals (32). Compared with controls (EGFP-transfected cells) in scFv-gephyrin-transfected cells VGLUT-immunopositive clusters were signif-

Downloaded from www.jbc.org at SISSA, on October 9, 2012

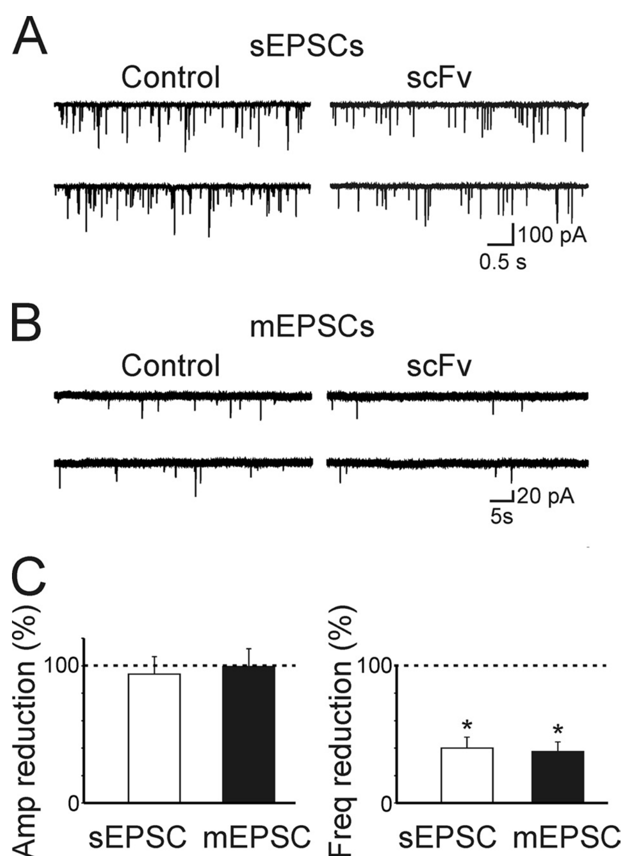


FIGURE 4. **scFv-gephyrin reduces the frequency but not the amplitude of sEPSCs and mEPSCs.** *A*, samples of spontaneous EPSCs recorded from controls and scFv-gephyrin-transfected neurons at a holding potential of −70 mV in the presence of 10 μM bicuculline and 50 μM D-AP5. *B*, samples of miniature EPSCs recorded from controls and scFv-gephyrin-transfected neurons at a holding potential of −70 mV in the presence of 1 μM TTX. *C*, each *column* reprsents the reduction in amplitude (*left*) and in frequency (*right*) of sIPSC (*white*) and mIPSCs (*black*) obtained from scFv-gephyrin-transfected neurons ($n = 12$) and expressed as percentage of controls ($n = 12$; *dashed lines*). *, $p < 0.05$. *Error bars*, S.E.

icantly reduced in density and size (Fig. 3, *C* and *D*). In particular, the density of VGLUT clusters was reduced from 4.6 ± 0.8 clusters/100 μm^2 in EGFP to 2.3 ± 0.5 clusters/100 μm^2 in scFv-gephryin ($p < 0.05$; $n = 12$). The size of these clusters was reduced from 9.3 ± 0.5 μm^2 to 7.5 ± 0.6 μm^2 ($p <$ 0.05). Furthermore, whole cell voltage clamp recordings performed in the presence of bicuculline (10 μM) and D-AP5 (50 μM), to block $GABA_A$ and NMDA receptors, respectively, revealed a significant reduction in frequency (but not in amplitude) of spontaneous EPSCs (the frequency reached 40 ± 8%; $p < 0.05$; $n = 12$; the amplitude 95 ± 13%; $p > 0.05$; $n = 12$) recorded from scFv-gephyrin-transfected neurons compared with controls (Fig. 4, *A* and *C*). Similarly, in scFv-gephyrin-transfected cells, the frequency of miniature EPSCs recorded in the presence of TTX was significantly reduced with respect to controls (to 37 ± 7%; $p < 0.05$; from 0.78 ± 0.14 Hz to 0.32 ± 0.05 Hz; $n = 12$) whereas the amplitude was unchanged (to 100 ± 13%; $p > 0.05$; from 34 ± 6 pA to 34 ± 5 pA; $n = 7$; Fig. 4, *B* and *C*). Altogether, these results strongly support the involvement of gephyrin in regulating not only GABAergic but also glutamatergic synaptic transmission.

The Loss of GABAergic but Not Glutamatergic Innervation in Gephyrin-deprived Neurons Can Be Rescued by Overexpressing NLG2—To assess further the possibility that the reduced GABAergic innervation in scFv-gephyrin-transfected cells is mediated by NLG2 which may convey information in a retrograde way from post- to presynaptic sites, NLG2 was co-expressed with scFv-gephyrin. In immunocytochemical experiments, co-expression of NLG2 with scFv-gephyrin induced a significant increase in the density of VGAT-positive clusters compared with cells transfected with scFv-gephyrin alone (180 ± 8%; from 10.6 ± 0.7 clusters/100 μm^2 to 19.1 ± 0.9 clusters/100 μm^2; $p < 0.01$; $n = 11$ and 8 for scFv and scFv/NLG2, respectively), restoring VGAT cluster density to control levels (Fig. 5, *A* and *B*). In line with previous studies (9), overexpression of NLG2 alone led to a 2-fold increase in the density of VGAT clusters compared with EGFP-transfected controls (data not shown).

Parallel electrophysiological experiments from cultured neurons revealed no changes in amplitude and frequency of spontaneous mIPSCs between cells co-transfected with scFv-gephyrin/NLG2 and controls (neighboring nontransfected cells). On average, the frequency of mIPSCs was 0.211 ± 0.040 Hz and 0.213 ± 0.044 Hz ($p = 0.97$) whereas the amplitude was −55 ± 4 pA and −53 ± 6 pA ($p = 0.7$) in control ($n = 7$) and in co-transfected neurons ($n = 8$), respectively (Fig. 5*B*).

To see whether the observed rescue was gephyrin-dependent, similar experiments were performed using the NLG2 mutant (NLG2Y770A-HA) which lacks gephyrin binding (14). In this case, the number of VGAT-positive clusters/100 μm^2 was 19.8 ± 1.7 ($n = 9$), a value not significantly different from that obtained with NLG2 ($p > 0.5$; supplemental Fig. 2*A*).

In electrophysiological recordings, no changes in amplitude or frequency of mIPSCs were detected between scFv-gephyrin/NLG2Y770A-transfected cells and controls. The frequency of mIPSCs was 0.22 ± 0.07 Hz and 0.16 ± 0.06 Hz in control ($n = 7$) and in scFv-gephyrin/NLG2Y770A-transfected cells ($n = 6$), respectively ($p > 0.05$; supplemental Fig. 2, *B* and *C*). The amplitude of mIPSCs was 42 ± 5 pA and 32 ± 5 pA in the absence or in the presence of scFv-gephyrin/NLG2Y770A, respectively (supplemental Fig. 2, *B* and *C*).

Altogether, these experiments indicate that overexpression of NLG2 is able to rescue the loss of GABAergic innervation induced by scFv-gephyrin. However, our data with NLG2Y770A suggest that this effect only partially relies on the direct recruitment of gephyrin by NLG2 at inhibitory synapses (see "Discussion").

It is possible that the observed reduction in glutamatergic innervation following gephyrin depletion with scFv-gephyrin represents a homeostatic compensatory mechanism to prevent hyperexcitability and to maintain the right E/I balance within the neuronal network (33). If this is the case, rescuing GABAergic innervation should lead to a concomitant change in glutamatergic transmission. However, this was not the case because overexpressing NLG2 in gephyrin-depleted neurons failed to restore VGLUT immunoreactive puncta (0.5 ± 0.1 and 0.6 ± 0.1 clusters/100 μm^2 for scFv and scFv/NLG2, respectively; $p >$ 0.5; Fig. 5*C*) as well as the frequency of mEPSCs to control levels. The frequency of mEPSCs was 0.64 ± 0.16 Hz and 0.26 ±

Downloaded from www.jbc.org at SISSA, on October 9, 2012

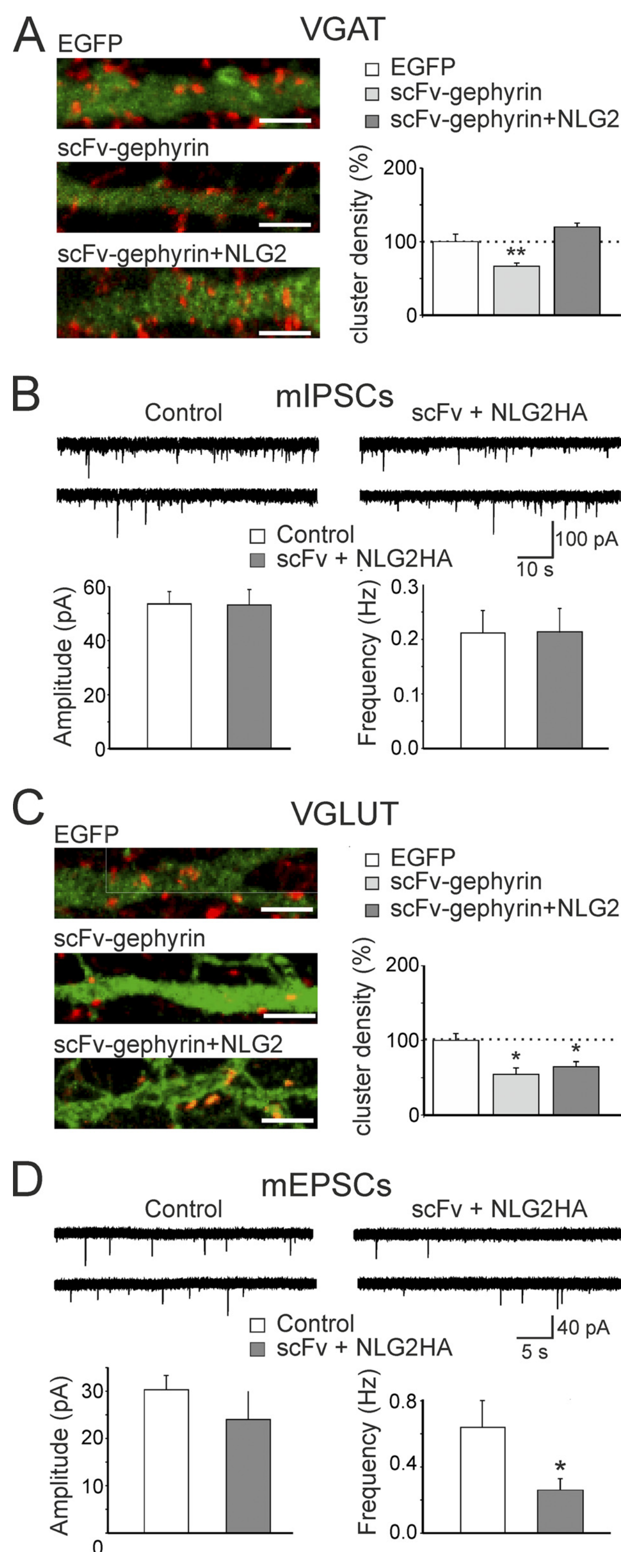


FIGURE 5. **Co-expression of NLG2 with scFv-gephyrin restores the loss of GABAergic but not glutamatergic innervation.** *A*, *left*, representative images of neurons transfected with EGFP (*top*), scFv-gephyrin (*middle*) or co-transfected with scFv-gephyrin and NLG2-HA (*bottom*). Dendrites were visualized by EGFP signal or NLG2 staining (*green*). Neurons were immunostained for VGAT (*red*). *Scale bars*, 5 μm. *Right*, quantification of VGAT cluster densities relative to the mean value obtained from EGFP-transfected neurons (*dashed line*). **, $p < 0.01$. *C* and *D*, as in *A* and *B* but for neurons immunostained for VGLUT (*red*). *Scale bars*, 5 μm. *B*, samples of spontaneous mIPSCs recorded from cells co-transfected with scFv-gephyrin plus NLG2-HA and from neighboring nontransfected cells (*Control*) at a holding potential of −70 mV in the presence of 1 μM TTX, 20 μM DNQX, and 50 μM D-AP5. The *columns* below the *traces* represent the mean amplitude (*left*) and frequency (*right*) of mIPSC from control (*white*; $n = 7$) and from scFv-gephyrin-transfected cells (*gray*; $n = 8$). *C*, as in *A* but for cells immunostained for VGLUT. *D*, as in *B* but for mEPSCs. These were recorded from cells co-transfected with scFv-gephyrin plus NLG2-HA ($n = 9$) and from neighboring nontransfected cells (*Control*, $n = 8$) in the presence of 1 μM TTX and 10 μM bicuculline. *Error bars*, S.E.

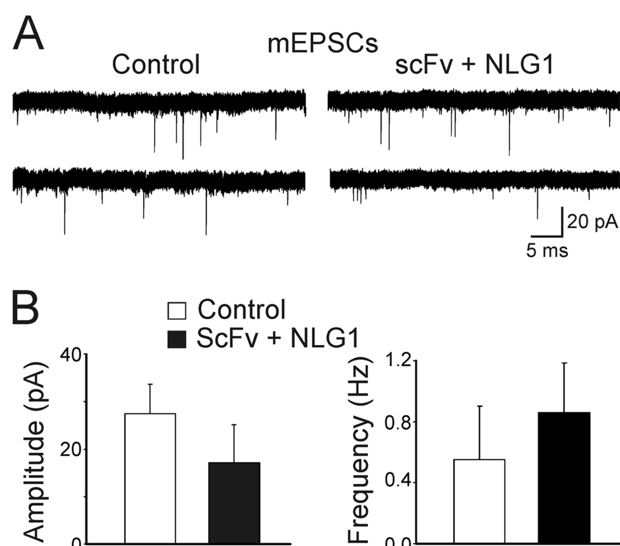


FIGURE 6. **Co-expression of NLG1 with scFv-gephyrin restores glutamatergic innervation.** *A*, samples of spontaneous mIPSCs recorded from cells co-transfected with scFv-gephyrin plus NLG1 and from neighboring nontransfected cells (*Control*) at a holding potential of −70 mV in the presence of 1 μM TTX and 10 μM bicuculline are shown. *B*, each column represents the mean amplitude (*left*) and frequency (*right*) of mIPSC from control (*white*, $n = 7$) and from scFv-gephyrin/NLG1-transfected cells (*black*, $n = 5$).

0.07 Hz in the absence or presence of NLG2 overexpression; $p < 0.05$; the amplitude of mEPSCs was 29 ± 3 pA and 25 ± 7 pA in the absence ($n = 8$) or in the presence ($n = 9$) of NLG2 overexpression ($p > 0.05$; Fig. 5*D*).

However, the co-expression of NLG1 with scFv-gephyrin was able to rescue the frequency of mEPSCs to control levels. The frequency of mEPSCs was 0.55 ± 0.35 Hz and 0.86 ± 0.33 Hz in control ($n = 7$) and in scFv-gephyrin/NLG1-transfected cells ($n = 5$), respectively ($p > 0.05$; Fig. 6). No changes in mEPSC amplitude were detected between control and scFv-gephyrin/NLG1-transfected cells (27 ± 6 pA in the absence and 17 ± 8 pA in the presence of NLG1 overexpression; $p > 0.05$; Fig. 6). Overall, these data indicate that the observed NLG1-induced rescue of glutamatergic function in gephyrin-deprived neurons is at least partially dependent on gephyrin activity.

Gephyrin Interacts Directly with NLG1—It has been recently reported that at inhibitory synaptic contacts gephyrin binds NLG2 directly (14). The amino acid sequence identified as gephyrin-binding motif on NLG2 is highly conserved in all NLGs, and indeed gephyrin binds to all four NLGs in yeast two-hybrid assays (14). To test whether gephyrin can form a complex with NLG1 in mammalian cells, lysates of HEK-293 cells transfected with gephyrin-FLAG were subjected to a pull-down assay with beads loaded with GST-NLG1 cytoplasmic domain ($NLG1_{CD}$), GST-$NLG2_{CD}$, or with GST alone as a negative control. In agreement with previous observations (14), $NLG2_{CD}$ was able to precipitate a consistent amount of gephy-

Downloaded from www.jbc.org at SISSA, on October 9, 2012

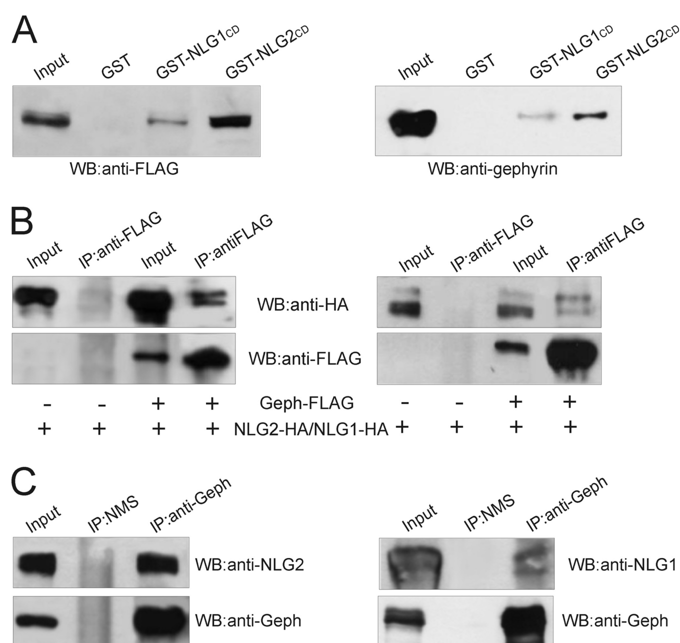


FIGURE 7. **Gephyrin interacts with NLG2 and NLG1.** *A*, GST-NLG1/2_{CD} pull-down assay using lysates of HEK-293 cells transfected with gephyrin-FLAG (*left*) and rat brain lysates (*right*). *B*, lysates of HEK-293 cells transfected with either NLG2-HA (*left*) or NLG1-HA (*right*) in the presence of gephyrin-FLAG or with the vector alone (as a negative control) immunoprecipitated with monoclonal anti-FLAG antibodies. Immunoprecipitates were analyzed by Western blotting using anti-HA and anti-FLAG monoclonal antibodies. *C*, co-immunoprecipitation experiments on rat brain lysates using a monoclonal anti-gephyrin antibody and normal mouse serum (*NMS*) as negative control. Immunoprecipitates were analyzed by Western blotting using a monoclonal anti-gephyrin antibody and a polyclonal antibody against NLG2 and NLG1.

rin-FLAG (Fig. 7*A*, *left*). Interestingly, a small but significant fraction of gephyrin-FLAG was also found in complex with GST-$NLG1_{CD}$ (Fig. 7). Similar pulldown experiments were then performed to assay the ability of endogenous gephyrin present on neonatal rat brain homogenates to interact with NLG1 and NLG2. Also in this case gephyrin was not only associated with GST-$NLG2_{CD}$ fusion protein but also with GST-$NLG1_{CD}$ (Fig. 7*A*, *right*). Here, the immunoblot analysis was performed using a monoclonal antibody raised against the C-terminal domain of gephyrin.

We then performed immunoprecipitation experiments to investigate the presence of NLG1-HA/gephyrin-FLAG complexes *in vitro*. HEK-293 cells were co-transfected with plasmids encoding for NLG1/2-HA and gephyrin-FLAG, or NLG1/2-HA alone, and cell lysates were immunoprecipitated with the anti-FLAG monoclonal antibody. The bound protein complexes were analyzed by Western blotting using anti-HA and anti-FLAG for NLG1 and gephyrin detection, respectively. As shown in Fig. 7*B* (*right*), NLG1-HA was immunoprecipitated only from cells co-expressing gephyrin-FLAG. The same experimental conditions were also applied to detect the expected presence of NLG2-HA/gephyrin-FLAG complexes in mammalian cells. Indeed, we found that a lower amount of gephyrin-FLAG was able to precipitate a higher amount of NLG2-HA compared with NLG1-HA, thus supporting previous *in vitro* observations. Finally, endogenous NLG1 and NLG2 were found in native complexes with gephyrin upon co-immunoprecipitation from mouse brain homogenates (Fig. 7*C*). These data suggest that gephyrin, by interacting directly with NLG2 and to a lesser extent with NLG1, may affect not only GABAergic but also glutamatergic synaptic transmission.

DISCUSSION

The tubulin-binding protein gephyrin is a core protein of inhibitory postsynaptic densities that interacts with the cytoskeleton to stabilize inhibitory receptors in precise apposition to presynaptic active zones (1). In a previous study, we have demonstrated that disrupting endogenous gephyrin with selective scFv-gephyrin altered the gating properties of $GABA_A$ receptors, an effect that was found to be associated with modifications of GABAergic innervation (6). In the present study we hypothesized that hampering gephyrin function affects not only the number of release sites (as suggested by the reduction in VGAT clusters) but also the probability of GABA release. In support of this view, in double patch experiments from interconnected neurons, we found that, with respect to controls, scFv-gephyrin-expressing cells exhibited a significant decrease in the amplitude of individual synaptic currents accompanied by a clear increase in the number of transmitter failures and a reduction in the PPR. Changes in transmitter failures and in PPR are consistent with a decrease in release probability (27, 28). This would lead to a reduction in GABA concentration in the synaptic cleft as suggested by the TPMPA experiments.

The role of gephyrin in ensuring a correct communication between pre- and postsynaptic elements of synapses was further validated by the experiments in which a truncated form of gephyrin (gephyrin 2–188; 22) was used. This gephyrin mutant lacks the dimerization motif, but it can still interact with endogenous gephyrin molecules, producing dominant negative effects on postsynaptic gephyrin clusters. Similar to scFv-gephyrin, overexpression of gephyrin 2–188 caused a reduction in GABAergic innervation and a decrease in frequency of spontaneous and miniature IPSCs, further confirming a key role of gephyrin in maintaining the stability of GABAergic connections within the neuronal network. The ability of gephyrin to influence presynaptic innervation was already suggested by Yu *et al.*, even though no mechanistic interpretation was provided (5).

The presynaptic action of gephyrin on GABA release implies the coordinated activity of other signaling molecules that interact directly or indirectly with gephyrin to ensure the corrected cross-talk between the post- and presynaptic elements of the synapse. Possible candidates are NLGs, specialized cell adhesion molecules that functionally couple the postsynaptic densities with the transmitter release machinery by forming transynaptic complexes with their presynaptic binding partners, neurexins (7). In particular, NLG2 is preferentially concentrated at inhibitory synapses (10) and binds gephyrin directly through a conserved cytoplasmic domain (14). Consistent with this finding, hampering gephyrin function with scFv-gephyrin promoted a significant decrease in the total number and size of NLG2 clusters upon scFv-induced gephyrin removal. It is interesting to note that in a recent study (34), knocking down gephyrin with siRNA led to a shift of endogenous NLG2 from inhibitory to excitatory synapses, in the absence of any change in the density of NLG2 clusters. In the present experiments instead

Downloaded from www.jbc.org at SISSA, on October 9, 2012

we have observed a clear reduction in the density of NLG2 clusters without a detectable relocalization of this protein to glutamatergic synapses. Because scFv-mediated removal of gephyrin is associated with a significant reduction of synaptic γ2-containing $GABA_A$ receptors (6) and evidence has been provided for the reciprocal stabilization of NLG2 by $GABA_A$ receptors (35), the reduction of NLG2 staining could be a consequence of the loss of gephyrin-dependent $GABA_A$ receptor clustering. We cannot exclude the possibility that scFv-gephyrin may affect the function of additional gephyrin-bound factors important for the efficient localization of NLG2 to and from GABAergic terminals. Conventional kinesin (KIF5) and the dynein motor complex have been shown to be involved in microtubule-dependent transport of gephyrin, thus contributing to postsynaptic remodeling (22, 36). Because microtubule motors transport and remodel a variety of transmembrane and submembrane postsynaptic proteins (37, 38), similar mechanisms may account for NLG2 transport.

The role of NLG-neurexin complex as a coordinator between postsynaptic and presynaptic sites has been investigated at excitatory CA3-CA1 synapses in the hippocampus. This study has revealed a retrograde modulation of neurotransmitter release by PSD-95-NLG complex (39). The authors found that overexpression of the glutamatergic scaffold protein PSD-95 enhanced release probability via a mechanism involving the NLG-neurexin complex.

Along the same line, at GABAergic synapses, evidence has been provided that knocking down NLG2 produces a reduction in quantal content associated with a decrease in quantal size of unitary responses (40). In agreement with our electrophysiological data, these findings suggest a crucial role of gephyrin-NLG2 interaction on GABA release.

In our experiments, the reduction in the probability of GABA release after scFv-gephyrin transfection seems to involve a mechanism that only partially relies on the direct NLG2-gephyrin interaction. Hence, the NLG2 point mutant, NLG2Y770A, unable to bind gephyrin was as effective as the wild-type protein in rescuing GABAergic transmission in scFv-gephyrin-deprived neurons. Two possible hypotheses, which are not mutually exclusive, can be put forward to explain our results.

First, although the NLG2Y770A mutant is impaired in gephyrin binding activity it still maintains the ability to induce the activation of collybistin (14), a gephyrin partner known to promote its synaptic targeting *in vivo* (14). Interestingly, in collybistin knock-out mice, a reduction in frequency of mIPSCs similar to that detected in the present experiments was observed (41), thus suggesting a possible involvement of collybistin in transynaptic signaling.

Second, NLGs form homomultimers through the extracellular acetylcholinesterase-homologous domain (42). Therefore, it is possible that overexpressing NLG2Y770A in hippocampal neurons containing endogenous NLG2 allows the formation of multimers. As a consequence this mutant recruited at inhibitory synapses would act in concert with endogenous NLG2 to rescue GABAergic transmission.

Unexpectedly, hampering gephyrin function with scFv-gephyrin produced a significant reduction not only of GABAergic but also of glutamatergic innervation as assessed by the significant decrease in density of VGLUT-positive puncta associated with a significant reduction in frequency, but not in amplitude, of spontaneous and miniature glutamatergic events. This effect was not due to a homeostatic plasticity mechanism because overexpressing NLG2 in gephyrin-depleted neurons failed to reestablish glutamatergic innervation. However, a rescue was obtained by co-expressing NLG1 with scFv-gephyrin. Because the overexpression of NLG1 alone has been shown to enhance glutamatergic innervation by severalfold (9) the fact that this does not occur in gephyrin-deprived neurons suggests that the scaffold protein contributes to modulate NLG1-dependent transynaptic signaling. In support of this observation, co-immunoprecipitation experiments revealed the existence of native complexes not only between gephyrin and NLG2 but also with NLG1, which is localized primarily at excitatory synapses (8), thus confirming and extending previous data obtained with the yeast two-hybrid system (14). Moreover, in favor of the possible involvement of gephyrin in regulating transynaptic signaling at excitatory synapses is the observation that, at least in immature hippocampal neurons *in vitro* (as those used in the present study), this protein has been found localized opposite to glutamatergic release sites (43, 44). The presence of gephyrin at both GABAergic and glutamatergic synapses may be relevant for neuronal development. Altogether, these results show that gephyrin interacts, at least in immature neurons, with both NLG2 and NLG1 to regulate both excitatory and inhibitory inputs converging on the same neuron thus controlling the E/I balance at the network level.

Acknowledgments—We are grateful to P. Scheiffele (Biozentrum, Basel, Switzerland) for providing NLG1-HA and NLG2-HA constructs and F. Varoqueaux (Max Planck, Göttingen) for the NLG2Y770A-HA. We thank B. Pastore for excellent technical assistance on hippocampal neuronal cultures.

REFERENCES

1. Fritschy, J. M., Harvey, R. J., and Schwarz, G. (2008) *Trends Neurosci.* **31,** 257–264
2. Sola, M., Bavro, V. N., Timmins, J., Franz, T., Ricard-Blum, S., Schoehn, G., Ruigrok, R. W., Paarmann, I., Saiyed, T., O'Sullivan, G. A., Schmitt, B., Betz, H., and Weissenhorn, W. (2004) *EMBO J.* **23,** 2510–2519
3. Kneussel, M., Brandstätter, J. H., Laube, B., Stahl, S., Müller, U., and Betz, H. (1999) *J. Neurosci.* **19,** 9289–9297
4. Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J. M., Pangalos, M. N., and Moss, S. J. (2008) *J. Neurosci.* **28,** 1356–1365
5. Yu, W., Jiang, M., Miralles, C. P., Li, R. W., Chen, G., and de Blas, A. L. (2007) *Mol. Cell. Neurosci.* **36,** 484–500
6. Marchionni, I., Kasap, Z., Mozrzymas, J. W., Sieghart, W., Cherubini, E., and Zacchi, P. (2009) *Neuroscience* **164,** 552–562
7. Südhof, T. C. (2008) *Nature* **455,** 903–911
8. Song, J. Y., Ichtchenko, K., Südhof, T. C., and Brose, N. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 1100–1105
9. Chih, B., Engelman, H., and Scheiffele, P. (2005) *Science* **307,** 1324–1328
10. Varoqueaux, F., Jamain, S., and Brose, N. (2004) *Eur J. Cell Biol.* **83,** 449–456
11. Levinson, J. N., Chéry, N., Huang, K., Wong, T. P., Gerrow, K., Kang, R., Prange, O., Wang, Y. T., and El-Husseini, A. (2005) *J. Biol. Chem.* **280,** 17312–17319
12. Gerrow, K., Romorini, S., Nabi, S. M., Colicos, M. A., Sala, C., and El-Husseini, A. (2006) *Neuron* **49,** 547–562
13. Prange, O., Wong, T. P., Gerrow, K., Wang, Y. T., and El-Husseini, A.

Downloaded from www.jbc.org at SISSA, on October 9, 2012

(2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 13915–13920
14. Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K., Jedlicka, P., Schwarzacher, S. W., Betz, H., Harvey, R. J., Brose, N., Zhang, W., and Varoqueaux, F. (2009) *Neuron* **63,** 628–642
15. Craig, A. M., and Kang, Y. (2007) *Curr. Opin. Neurobiol.* **17,** 43–52
16. Dalva, M. B., McClelland, A. C., and Kayser, M. S. (2007) *Nat. Rev. Neurosci.* **8,** 206–220
17. Gerrow, K., and El-Husseini, A. (2006) *Front. Biosci.* **11,** 2400–2419
18. Rubenstein, J. L., and Merzenich, M. M. (2003) *Genes Brain Behav.* **2,** 255–267
19. Zacchi, P., Dreosti, E., Visintin, M., Moretto-Zita, M., Marchionni, I., Cannistraci, I., Kasap, Z., Betz, H., Cattaneo, A., and Cherubini, E. (2008) *J. Mol. Neurosci.* **34,** 141–148
20. Andjus, P. R., Stevic-Marinkovic, Z., and Cherubini, E. (1997) *J. Physiol.* **504,** 103–112
21. Zita, M. M., Marchionni, I., Bottos, E., Righi, M., Del Sal, G., Cherubini, E., and Zacchi, P. (2007) *EMBO J.* **26,** 1761–1771
22. Maas, C., Tagnaouti, N., Loebrich, S., Behrend, B., Lappe-Siefke, C., and Kneussel, M. (2006) *J. Cell Biol.* **172,** 441–451
23. Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000) *Cell* **101,** 657–669
24. Chih, B., Gollan, L., and Scheiffele, P. (2006) *Neuron* **51,** 171–178
25. Yang, J., Thio, L. L., Clifford, D. B., and Zorumski, C. F. (1993) *Brain Res. Dev. Brain Res.* **71,** 19–26
26. Benson, D. L., Watkins, F. H., Steward, O., and Banker, G. (1994) *J. Neurocytol.* **23,** 279–295
27. Dobrunz, L. E., and Stevens, C. F. (1997) *Neuron* **18,** 995–1008
28. Korn, H., and Faber, D. S. (1991) *Trends Neurosci.* **14,** 439–445
29. Katz, B. (1969) *The Release of Neural Transmitter Substances*, Liverpool University Press, Liverpool, UK
30. Jones, M. V., Jonas, P., Sahara, Y., and Westbrook, G. L. (2001) *Biophys. J.* **81,** 2660–2670
31. Barberis, A., Petrini, E. M., and Cherubini, E. (2004) *Eur. J. Neurosci.* **20,** 1803–1810
32. Yu, W., and De Blas, A. L. (2008) *J. Neurochem.* **104,** 830–845
33. Turrigiano, G. G., and Nelson, S. B. (2004) *Nat. Rev. Neurosci.* **5,** 97–107
34. Levinson, J. N., Li, R., Kang, R., Moukhles, H., El-Husseini, A., and Bamji, S. X. (2010) *Neuroscience* **165,** 782–793
35. Dong, N., Qi, J., and Chen, G. (2007) *Mol. Cell. Neurosci.* **35,** 14–23
36. Maas, C., Belgardt, D., Lee, H. K., Heisler, F. F., Lappe-Siefke, C., Magiera, M. M., van Dijk, J., Hausrat, T. J., Janke, C., and Kneussel, M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 8731–8736
37. Hirokawa, N., and Takemura, R. (2005) *Nat. Rev. Neurosci.* **6,** 201–214
38. Kneussel, M. (2005) *EMBO Rep.* **6,** 22–27
39. Futai, K., Kim, M. J., Hashikawa, T., Scheiffele, P., Sheng, M., and Hayashi, Y. (2007) *Nat. Neurosci.* **10,** 186–195
40. Gibson, J. R., Huber, K. M., and Südhof, T. C. (2009) *J Neurosci* **29,** 13883–13897
41. Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskaia, M., Harvey, R. J., Harvey, K., O'Sullivan, G. A., Laube, B., Hülsmann, S., Geiger, J. R., and Betz, H. (2007) *EMBO J.* **26,** 3888–3899
42. Comoletti, D., Grishaev, A., Whitten, A. E., Tsigelny, I., Taylor, P., and Trewhella, J. (2007) *Structure* **15,** 693–705
43. Studler, B., Sidler, C., and Fritschy, J. M. (2005) *J. Comp. Neurol.* **484,** 344–355
44. Anderson, T. R., Shah, P. A., and Benson, D. L. (2004) *Neuropharmacology* **47,** 694–705

Downloaded from www.jbc.org at SISSA, on October 9, 2012

This paper is available online at www.jbc.org

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 35, p. 31062, September 2, 2011
© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

VOLUME 286 (2011) PAGES 20942–20951
DOI 10.1074/jbc.A111.234641

Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures.

Zeynep Kasap Varley, Rocco Pizzarelli, Roberta Antonelli, Stefka H. Stancheva, Matthias Kneussel, Enrico Cherubini, and Paola Zacchi

PAGE 20949:

Lines 16 and 17 under "Discussion" should read "by a clear increase in the number of transmitter failures and in the PPR . . . "

VOLUME 286 (2011) PAGES 24553–24560
DOI 10.1074/jbc.A110.202341

mTORC2 protein-mediated protein kinase B (Akt) serine 473 phosphorylation is not required for Akt1 activity in human platelets.

Samantha F. Moore, Roger W. Hunter, and Ingeborg Hers

mTORC2 is a protein complex and not a protein. Akt and not protein kinase B is used throughout the article. Because of this, the title of the article should read "mTORC2 Protein Complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation Is Not Required for Akt1 Activity in Human Platelets."

Downloaded from www.jbc.org at SISSA, on October 9, 2012

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

-Paper N.2-

Developmental regulation of GABAergic signalling in the hippocampus of neuroligin 3 R451C knock-in mice: an animal model of Autism.

Pizzarelli R and CherubiniE

submitted

Developmental regulation of GABAergic signalling in the hippocampus of neuroligin 3 R451C knock-in mice: an animal model of Autism

Rocco Pizzarelli and Enrico Cherubini

Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), via Bonomea, 265, 34136 Trieste, Italy

Running title: The $NL3^{R451C}$ mutation and GABAergic signalling in the hippocampus

Key words: neuroligin 3 mutation, developing hippocampus; $GABA_A$-mediated synaptic transmission

Total number of words (excluding references and Figure legends): 6079

Corresponding Author: Enrico Cherubini, Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), 34014 Trieste, Italy, Via Bonomea 265, 34136 Trieste, Italy. Tel: +39-040-3787704; Fax: +39-040-3787702; e-mail: cher@sissa.it

Key points summary

- Autism spectrum disorders (ASDs) are often associated with single mutations of genes involved in synaptic organization, including those encoding for neuroligins which bridge the cleft and ensure the cross-talk between the pre and postsynaptic elements of the synapse.
- Here, transgenic mice carrying the human R451C mutation of *Nlgn3*, have been used to investigate whether changes in synaptic signaling occur in the CA3 hippocampal region at early stages of postnatal development.
- An enhancement of correlated network activity such as Giant Depolarizing Potentials, a hallmark of developmental networks, was observed at birth.
- This depended on the increased GABAergic drive to CA3 principal cells, since it was associated with an enhanced frequency of miniature GABAergic (but not glutamatergic) synaptic currents.
- An early alteration in the excitatory/inhibitory balance, thought to be crucial for the refinement of neuronal circuits, may account for the behavioral deficits observed in children with ASDs.

Abstract

Autism Spectrum Disorders (ASDs) comprise an heterogeneous group of neuro-developmental abnormalities, mainly of genetic origin, characterized by impaired social interactions, communications deficits and stereotyped behaviors. In a small percentage of cases, ASDs have been found to be associated with single mutations in genes involved in synaptic function. One of these involves the postsynaptic cell adhesion molecule neuroligin (NL) 3. NLs interact with presynaptic neurexins (Nrxs) to ensure a correct cross talk between post and presynaptic specializations. Here, transgenic mice carrying the human R451C mutation of *Nlgn3*, were used to study GABAergic signaling in the hippocampus early in postnatal life. Whole cell recordings from CA3 pyramidal neurons in slices from NL3 ^{R451C} knock-in mice revealed an enhanced frequency of Giant Depolarizing Potentials, as compared to controls. This effect was probably

dependent on an increased GABAergic drive to principal cells as demonstrated by the enhanced frequency of miniature $GABA_A$-mediated (GPSCs), but not AMPA-mediated postsynaptic currents (EPSCs). Change in frequency were associated with an acceleration of mGPSCs decays possibly of postsynaptic origin. However, peak scaled non-stationary fluctuation analysis did not reveal any change in unitary synaptic conductance or in the number of $GABA_A$ receptor channels. The increased frequency of mEPSCs detected in adult NL3 ^{R451C} mice may represent a late form of compensatory homeostatic correction to counter the excessive $GABA_A$-mediated inhibition. Therefore, it is reasonable to assume that alterations in the excitatory/inhibitory balance, crucial for the refinement of neuronal circuits early in postnatal development, accounts for the behavioral deficits observed in ASDs patients.

Abbreviations

ASD, Autism Spectrum Disorders; DNQX, 6,7-Dinitroquinoxaline-2,3-dione; EPSCs, excitatory postsynaptic currents; GDPs, Giant depolarizing Potentials; GPSCs, $GABA_A$-mediated postsynaptic currents; KI, knock-in; KO, knock-out; K-S, Kolmogorov-Smirnov; MF, mossy fibers; NL, neuroligin; NO-711, 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride hydrochloride; Nrx, neuroexin; P, postnatal; SR 95531, 6-Imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid; TPMPA, (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid; TTX, tetrodotoxin; WT, wild type.

Introduction

ASDs comprise a heterogeneous group of pathological conditions characterized by impaired social interactions, deficits in verbal and non verbal communication, limited interest in the surrounding environment associated with stereotyped and repetitive behavior (American Psychiatric Association, 2000). ASDs are among the most heritable neuro-developmental disorders with a high incidence in infancy and early childhood (Weintraub, 2011). It is believed that the genetic predisposition together with environmental factors contribute to alter normal brain development leading to an impaired connectivity between brain regions, ultimately weakening the specialized functions of cortical areas (Geschwind & Levitt, 2007).

Interestingly, a small percentage of cases with idiopathic ASD have been found to be associated with single mutations in genes involved in synapses organization, pointing to synaptic dysfunction as one possible cause of autism (Südhof, 2008). Among these, mutations of genes encoding for cell adhesion molecules of the neuroligin (NL; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005), neurexin (Nrx; Kim et al,. 2008) families or for SHANK3, a scaffold protein involved in the structural organization of dendritic spines and a binding partner of NLs (Durand et al., 2007), have received particular attention. Nrxs and NLs bridge the cleft thus providing the functional link between pre- and post-synaptic elements of the synapse. Over-expression of NLs in non-neuronal cells co-cultured with neurons induces structural differentiation of both excitatory and inhibitory presynaptic terminals in contacting axons (Scheiffele et al., 2000; Fu et al., 2003; Graf et al., 2004; Sara et al., 2005). Conversely, Nrxs trigger postsynaptic differentiation by aggregating NLs and neurotransmitter receptors on the dendritic surface (Graf et al., 2004). The bidirectional signaling through the NL-Nrx is crucial for synapse development and stabilization (Scheiffele et al., 2000; Varoqueaux et al., 2006; Chubykin et al., 2007; Poulopoulus et al., 2009; Ko et al., 2009).

Interestingly, one missense mutation causing R451C substitution within a highly conserved region of the extracellular esterase-homology domain of the *Nlgn3* gene was detected in two male siblings, one with autism, severe intellectual disabilities and seizures and the other with Asperger syndrome (Jamain et al., 2003). To gain insights into the possible mechanisms of ASD, this mutation has been introduced into the endogenous *Nlgn3* in mice by gene targeting (Tabuchi

et al., 2007). Previous work from juvenile and adult NL3^{R451C} knock-in mice have revealed deficits in social interaction, reminiscent of those found in ASD patients, associated with modifications in GABAergic (Tabuchi et al., 2007) and glutamatergic (Etherton et al., 2011) synaptic transmission.

These studies however, did not address the question whether the R451C mutation of NL3 affects GABAergic signaling at early developmental stages when GABA exerts a critical role in synapse formation and stabilization (Pizzarelli & Cherubini, 2011). This information is important because developmental disorders such as ASDs can be diagnosed early in infancy when an immediate therapeutic intervention may maximize potential benefits.

We examined synaptic transmission in the CA3 hippocampal area during the first two weeks of postnatal life when rapid morphological changes occur at both pre and postsynaptic levels Amaral & Dent, 1981) and in adulthood. We found that the R451C mutation selectively affects GABAergic signaling and correlated network activity from birth.

Methods

Ethical approval

All experiments were performed in accordance with the European Community Council Directive of November 24, 1986 (86/609EEC) and were approved by the local authority veterinary service and by SISSA ethical committee. All efforts were made to minimize animal suffering and to reduce the number of animal used.

Animals

$NL3^{R451C}$ mice (Tabuchi et al., 2007) were purchased from Jackson Laboratories (Maine, USA). Experiments were performed on off-spring male derived from heterozygous mating. Electrophysiological experiments were performed and analyzed blind before genotyping. This was carried out on tail biopsy DNA by PCR using a standard protocol. At least three mice for each genotype were used in a given experiment.

Hippocampal slice preparation.

Transverse hippocampal slices (300 μM) were obtained from neonatal (postnatal day 4-9) young (postnatal day 11–15) and adult (postnatal day 27-35) mice using a standard protocol (Griguoli et al., 2010). Briefly, after being anesthetized with CO_2, animals were decapitated. The brain was quickly removed from the skull and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 25 glucose, 3.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, and 1.3 $MgCl_2$, saturated with 95% O2 and 5% CO2 (pH 7.3–7.4). Transverse hippocampal slices were cut with a vibratome and stored at room temperature (22–24°C) in a holding bath containing the same solution as above. After incubation for at least 45 min, an individual slice was transferred to a submerged recording chamber and continuously superfused at 33–34°C with oxygenated ACSF at a rate of 3–4 ml/min.

Electrophysiological recordings.

Recordings were made with a patch-clamp amplifier (Axopatch 1D amplifier, Molecular Devices) from CA3 pyramidal cells visualized with an upright microscope equipped with

differential interference contrast optics and infrared video camera, using the whole cell configuration of the patch-clamp technique. Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, Germany); when filled with an intracellular solution they had a resistance of 4-6 MΩ. The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting 15% changes were excluded from the analysis. The series resistance was < 25 MΩ.

Spontaneous glutamatergic and GABAergic postsynaptic currents were routinely recorded from a holding potential of -65, -70 mV in the presence of bicuculline (10 µM) and DNQX (20 µM), respectively. Miniature currents were recorded in the presence of TTX (1µM) to block sodium currents and propagated action potentials. For glutamatergic currents we used an intracellular solution containing (in mM): 125 Cs-methanesulphonate, 10 CsCl, 10 HEPES, 0.3 EGTA, 2 MgATP, 0.3 NaGTP, (pH adjusted to ~ 7.3 with CsOH). For GABAergic currents we used an intracellular solution containing (in mM): CsCl 137, Hepes 10, BAPTA 11, MgATP 2, $MgCl_2$ 2, $CaCl_2$ 1 and 5 QX-314 (pH adjusted to ~ 7.3 with CsOH).

Concentric bipolar electrodes were used to stimulate granule cells in the dentate gyrus in order to elicit 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)-mediated excitatory postsynaptic currents (EPSCs) in CA3 pyramidal neurons (frequency of stimulation: 0.1 Hz; stimulus duration 100-200 µs). Stimulus strength was adjusted to obtain at −65 mV stable EPSCs of ~100 pA amplitude. The NMDA component was recorded from the same neuron at +40 mV, using the same stimulus strength, after blocking the AMPA-mediated component with DNQX (20 µM). While AMPA-mediated EPSCs were recorded close to the reversal potential of GABA, NMDA currents were elicited in the presence of DNQX (20 µM) and bicuculline (10 µM) to block AMPA and $GABA_A$ receptors, respectively.

In some experiments, extracellular field potentials were recorded using conventional glass microelectrodes (tip diameter 5–10 μM) filled with ACSF and placed into the stratum pyramidale of the CA3 area.

Drugs

The following drugs were used: 6,7-Dinitroquinoxaline-2,3-dione (DNQX), 6-Imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid (SR 95531) hydrobromide, picrotoxin and bicuculline, purchased from Ascent Scientific; (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) and *N*-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX 314), purchased from Tocris Bioscence; tetrodotoxin (TTX) from Latoxan. Bumetanide and 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride hydrochloride (NO-711), from Sigma-Aldrich. Zolpidem was a gift of Dr. A. Barberis (Italian Institute of Technology, Genova). Stock solutions were made in distilled water and then aliquoted and frozen at –20°C. DNQX and picrotoxin were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the membrane potential, input resistance, or the firing properties of CA3 pyramidal neurons. Drugs were applied in the bath by gravity by changing the superfusion solution to one differing only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 2-3 min.

Data analysis

Data were acquired at 20 kHz , filtered with a cut-off frequency of 2 kHz and stored on computer in order to perform off-line analysis. Miniature AMPA and $GABA_A$-mediated postsynaptic currents were analyzed using Clampfit 10.0 (Axon Instruments). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace by one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data. To minimize the contribution of the unquantifiable effects of cable filtering, detailed kinetic analysis of miniature synaptic currents was limited to events with a rise time ≤ 1 ms. The rise time was estimated as the 10–90% time needed to reach the peak of the synaptic current. The weighted decay was obtained by dividing miniatures charge transfer by their amplitude.

The amplitude of the tonic current was estimated by the outward shift of the baseline current after the application of the $GABA_A$ receptor channel blocker picrotoxin (100 μM). Only current

recordings that exhibited a stable baseline were included in the analysis. Baseline currents and their standard deviations were estimated by plotting 10 s periods of raw data in all point histograms. These were fitted with a Gaussian function. The peak of the fitted Gaussian was considered as the mean holding current (Glykys & Mody, 2007).

NMDA/AMPA ratio was measured by averaging 15 to 30 sweeps for each holding potential. The AMPA-mediated component was measured at the peak of the current obtained at – 65 mV, while the NMDA component was measured at the peak of the current obtained at +40 mV after blocking the AMPA component with DNQX. The weighted decay time constant for NMDA-mediated synaptic current was measured by dividing the area by its amplitude, independently of the fitting (Cathala et al., 2003).

Peak scaled non-stationary noise analysis was performed according to Traynelis et al., (1993) using the Mini Analysis program (version 6.0.1, Synaptosoft, Leonia, NJ). Miniature events were selected following the procedure described by Momiyama et al. (2003). Briefly, individual events were analyzed by measuring the peak amplitude, the 10–90% rise time and the decay time constant. Measured parameters were then numbered according to the event number and tested by Spearman's rank order correlation test for time stability. After testing for correlation between rise time and amplitude and between rise time and decay time, miniature GABAergic events were used for noise analysis. Individual events were aligned to the point of steepest rise time. The peak of the mean current response waveform was scaled to the response value at the corresponding point in time of each individual event before subtraction to generate the difference waveforms. The ensemble mean post synaptic current was binned into 50 bins of equal amplitude to assign similar weights to all phases of ensemble mean waveform. Variance was plotted against amplitude and individual points were fitted with the equation:

$$\sigma^2(I) = iI - I^2/N + \sigma_b^2 \quad (1)$$

where i is the unitary single-channel current, I is the mean current, N is the number of channels open at the current peak and σ_b^2 is the variance of the background noise. The single-channel chord conductance (Υ) was calculated as:

$$\Upsilon = i/(E_m - E_{rev}) \quad (2)$$

from the holding potential (E_m) of −70 mV, assuming a reversal potential (E_{rev}) of 0 mV.

Statistical analysis

All values are presented as mean ± SEM of *n* experiments. Statistical comparison was performed using unpaired *t*-test or one-way analysis of variance (ANOVA). Probability distributions were examined using the Kolmogorov–Smirnov test. A p value < 0.05 was considered as statistically significant.

Results

Spontaneous network-driven events are enhanced in NL3^{R451C} mice

As ASDs are neurodevelopmental disorders, we first verified whether the NL3^{R451C} mutation alters correlated network activity, the so-called Giant Depolarizing Potentials (GDPs), a hallmark of developmental networks (Ben-Ari et al., 1989; Ben-Ari et al., 2007). GDPs which represent a primordial form of synchrony between neurons, thought to be essential for proper circuit maturation (Ben-Ari et al., 2007; Blankenship & Feller, 2010), are generated by the synergistic action of glutamate and GABA, both of which are depolarizing and excitatory at early development stages (Cherubini et al., 1991).

We focused on the CA3 region of the hippocampus where GABAergic interneurons are centrally involved in GDPs generation (Allene et al., 2012). GDPs were recorded simultaneously from single cells and from population of neurons as extracellular field potentials (Figure 1A and B).

GDPs occurred more frequently in NL3^{R451C} knock-in mice respect to WT (0.69 ± 0.009 Hz, n=17, and 0.046 ± 0.006 Hz, n=24 in knock-in and control mice, respectively; *p*=0.043; unpaired t-test). As shown in the cumulative inter-event-interval plot of Figure 1C, the curve obtained from NL3^{R451C} knock-in mice was shifted to the left respect to that obtained from WT animals (*p*<0.05; K-S test). In addition, as illustrated in Figure 1C, in NL3^{R451C} knock-in mice the cumulative probability curve related to charge transfers through the currents underlying GDPs was shifted to the right respect to controls and was significantly different from that obtained from WT animals (p<0.01 ; K-S test).

Enhanced basal GABAergic but not glutamatergic transmission in young NL3^{R451C} knock-in mice

Since GDPs are generated by the synergistic action of GABA and glutamate we next examined whether changes in spontaneous miniature synaptic currents could account for the observed effects. Miniature $GABA_A$- and AMPA-mediated postsynaptic currents (mGPSCs and mEPSCs, respectively) were recorded at three different postnatal ages (P4-P9, P11-P15, P27-P35) in the presence of TTX (1 µM) and bicuculline or DNQX, respectively. As shown in the cumulative distribution plots of Figure 2, significant differences in the inter-event intervals distributions but

not amplitude of mGPSCs were observed in all groups examined (p<0.05 for all three groups; K-S test). However due to the large variability between individual neurons, the mean frequency values from knock-in mice were significantly larger than those obtained from WT animals only at P11-P15. Mean frequency values were: P4-P9 (WT, 0.8 ± 0.1 Hz, n=19; $NL3^{R451C}$, 0.97 ± 0.18 Hz n=18 ; p>0.05; unpaired t-test). P11-P15 (WT, 4.47 ± 0.4 Hz n=18; $NL3^{R451C}$, 6.48 ± 0.75 Hz; n=20; p<0.05; unpaired t-test); P27-P35 (WT, 11.9 ± 2 Hz, n=9; $NL3^{R451C}$, 14.4 ± 1.44 Hz, n=12 p>0.05; unpaired t-test). Respective amplitude values were: P4-P9 (WT, 39.8 ± 2.8 pA n=2 ; $NL3^{R451C}$, 41.9 ± 2.4 pA; n=18); P11-P15 (WT, 42.9 ± 2.1 pA; n=18; $NL3^{R451C}$, 45.9 ± 2.4 pA; n=20) P27-P35 (WT, 48.5 ± 4.5 pA; n=9; $NL3^{R451C}$, 49.3 ± 4.8 pA n=12); p>0.05 for all three groups.

In contrast, cumulative distribution plots of inter-event-intervals of mEPSCs recorded from $NL3^{R451C}$ and WT mice were significantly different only at P28-P35 (p<0.05; K-S test; Figure 3). No significant differences in cumulative amplitude distributions were observed between $NL3^{R451C}$ and WT mice (p>0.05; K-S test). Mean frequency values were: P4-P9 (WT, 0.76 ± 0.12 Hz; n=9; $NL3^{R451C}$, 0.6 ± 0.12; Hz n=11); P11-P15 (WT, 0.97 ± 0.19 Hz; n=11; $NL3^{R451C}$, 0.91 ± 0.13 Hz; n=12); P27-P35 (WT, 2.7 ± 0.8 Hz; n=9; $NL3^{R451C}$, 3.8 ± 0.6 Hz; n=17). Mean amplitude values were P4-P9 (WT, 23.5 ± 3 pA; n=9; $NL3^{R451C}$, 21.9 ± 1.7 pA; n=11); P11-P15 (WT, 25.7 ± 1.7 pA; n=11; $NL3^{R451C}$, 24.8 ± 1 pA; n=12); P27-P35 (WT, 23.5 ± 2.9 pA, n=9; $NL3^{R451C}$, 25.5 ± 1.3 pA;n=17); p>0.05 for all three groups).

Altogether, these results strongly support the involvement of the $NL3^{R451C}$ mutation in the enhancement of GABAergic transmission at early stages of postnatal development.

Furthermore, to assess whether the $NL3^{R451C}$ mutation differentially affects AMPA- and NMDA-mediated synaptic transmission, AMPA- and NMDA-mediated postsynaptic currents evoked by granule cell stimulation were examined at P4-P9 from CA3 principal cells at -65 mV and +40 mV, respectively. While AMPA-mediated EPSCs were recorded close to the reversal potential of GABA, NMDA currents were elicited in the presence of DNQX (20 μM) and bicuculline (10 μM) to block AMPA and $GABA_A$ receptors, respectively. As illustrated in Figure S1, no significant differences were observed in the NMDA/AMPA ratio (WT 0.24 ± 0.06, n=6 ; KI 0.28 ± 0.04 n=4 ; p>0.05) between the two different genotypes.

In addition, similar decay time values of NMDA-mediated EPSCs were detected in both genotypes (WT 88.17 ± 6 ms, n=6; NL3^{R451C} 85.1 ± 20 ms; n=4; p>0.05; Supplementary Figure 1), indicating that at MF-CA3 synapses the NL3^{R451C} mutation does not alter glutamatergic synaptic transmission and the postsynaptic expression of NMDA receptor subunits. As the stronger effect of the NL3^{R451C} mutation on GABAergic synaptic transmission was observed during the second postnatal week we concentrated our attention on this developmental stage (P11-P15)

The NL3^{R451C} knock-in mutation affects GABA release

According to the quantal theory, the synaptic efficacy *E*, the mean amplitude of unitary GPSCs, can be defined as $E=mQ$, where m is the quantal content or the mean number of quanta released per presynaptic action potential and Q is the quantal size or amplitude of the unitary postsynaptic current (Katz, 1969). While Q depends on both pre (GABA content in synaptic vesicles) and postsynaptic ($GABA_A$ receptors) mechanisms, m depends on presynaptic factors, namely the number of release sites N and the probability of release (P) at each individual site. Therefore, presynaptic changes in GABA release may be related to modifications either in Q or m or both.

To assess whether the increase in frequency of mGPSCs observed in NL3^{R451C} mice is due to changes in GABA release, we analyzed GABA transients in the cleft. We took advantage of the weak competitive $GABA_A$ receptor antagonist TPMPA which has a very fast dissociation constant and competes with synaptically released GABA for the same ligand binding site on $GABA_A$ receptors (Jones et al., 2001; Barberis et al., 2004). A different blocking effect of TPMPA on mGPSCs in WT and in NL3^{R451C} mice would reflect relative changes in synaptic GABA transients since the fast dissociation time constant of TPMPA is comparable with the duration of GABA synaptic transient in the cleft (Mozrzymas et al., 1999; Barberis et al., 2000; Jones et al., 2001). Bath application of TPMPA (200 μM) caused a more pronounced reduction of mGPSCs amplitude in WT than in NL3^{R451C} mice (30.5 ± 2.2 %; n=8 and 18.4 ± 3.7 %; n=7 in WT and NL3^{R451C} mice, respectively, p<0.05; one-way ANOVA), suggesting an increased GABA transient in the synaptic cleft of NL3^{R451C} mice respect to controls (Figure 4).

To test whether this effects was associated with an increased number of available $GABA_A$ receptors on the postsynaptic membrane (opened by a single GABA quantum, on the assumption that $GABA_A$ receptors are not saturated by the content of a single vesicle, Barberis et al., 2004), we used the peak-scaled non-stationary fluctuation analysis (see methods; Traynelis et al., 1993). We used only stable recordings of mGPSCs with no time-dependent changes in either peak amplitude, 10-90% rise time and decay time (electrotonic filtering was excluded on the basis of no correlation between the 10-90% rise time and the decay time; Momyiama et al., 2003).

For each cell a parabolic variance *versus* mean curve was obtained (see individual samples in Figure 5A and B). By fitting data points with equation 1 gave an estimated unitary currents of 2 ± 0.27 pA and 2.6 ± 0.25 pA in WT and $NL3^{R451C}$ mice, respectively, corresponding to a weighted mean channel conductance of 29.2 ± 3.8 pS (n=8) and 37 ± 3.5 pS (n=9). These values were not significantly different ($p > 0.05$; Figure 5C). In addition, no significant differences in the number of $GABA_A$ receptor channels were found between WT and knock-in mice (25.7 ± 4.8, n=8 and 22.4 ± 2.1, n=9, in WT and $NL3^{R451C}$ mice, respectively; $p>0.05$; Figure 5C) indicating that changes in frequency of mGPSCs could not be attributed to modifications in the number of receptor channels opened at the peak of a spontaneous miniature GABAergic events.

The $NL3^{R451C}$ knock-in mutation alters the decay kinetic of $GABA_A$ receptors

Next, we examined the kinetic properties of miniature events. As shown in Figure 6A, respect to WT animals, synaptic currents obtained from $NL3^{R451C}$ KI mice at P9-P11 displayed a significantly faster decay time (the weighted decay time was 9.14 ± 0.4 ms; n=9; and 7.43 ± 0.5 ms; n=9; in WT and $NL3^{R451C}$ mice, respectively; $p<0.05$) in the absence of any change in the rise time (0.53 ± 0.02 ms; n=9 ; and 0.47 ±0.02 ms; n= 9; in WT and $NL3^{R451C}$ mice, respectively; $p>0.05$). This led to a leftward shift of the cumulative probability curve obtained from $NL3^{R451C}$ respect to WT mice (the two cumulative curves were significantly different; $p<0.05$, K-S test; Figure 6B).

The faster decay time of mGPSCs observed in $NL3^{R451C}$ mice may result from a differential expression of $GABA_A$ receptor subunits in the postsynaptic membrane. One possibility is that the $NL3^{R451C}$ KI mutation accelerates the developmental switch from α2 to α1 subunits of $GABA_A$

receptors, known to produce currents with faster decay kinetics (Laurie et al. 1992; Cherubini & Conti, 2001). To elucidate whether in NL3^{R451C} mice more α1 containing GABA$_A$ receptors are recruited at synapses respect to α2, we examined the prolongation of current decay induced by zolpidem, known to selectively enhance the activity of α1 subunit-containing receptors (Pritchett & Seeburg, 1990). Since the effects of zolpidem on miniature GABAergic currents reflects the degree of receptor occupancy (Perrais & Ropert, 1999), we analyzed only mGPSCs duration. Application of zolpidem (100 nM) prolonged the decay time of mGPSCs in both WT and NL3^{R451C} mice. As shown in Figure 6C and D, in comparison with WT animals, the prolongation of synaptic decay was more pronounced in NL3^{R451C} KI mice (WT, 14 ± 2%, n=6; NL3^{R451C}, 23 ± 4 %, n=7). However the difference between the two genotypes did not reach a significant level (p>0.05, one-way ANOVA).

Recent studies have shown that intracellular chloride concentration may interfere with the gating properties and the expression of GABA$_A$ receptors thus regulating the shape of GABA$_A$-mediated postsynaptic currents (Houston et al., 2009; Succol et al., 2012). In particular, in the cerebellum, the developmental shift from α3 to α1 containing GABA$_A$ receptor subunits responsible for the faster decay of synaptic events has been attributed to changes in polarity of GABA action from the depolarizing to the hyperpolarizing direction (Succol et al., 2012). Therefore, the following experiments were undertaken to assess whether a different GABAergic drive (due to changes in $[Cl^-]_{in}$) could differentially affect network activity in the two genotypes at P11-P15. To this aim spontaneous EPSCs were recorded at -65 mV (close to the reversal of GABA, see methods) from WT and NL3^{R451C} KI mice, before and after application of gabazine (10 μM; a selective GABA$_A$ receptor antagonist) or bumetanide (10 μM; a selective blocker of the chloride importer NKCC1). In percentage these drugs produced a similar reduction in frequency (but not in amplitude) of spontaneous glutamatergic events (gabazine: 59.8 ± 12.2 %; n=6; 61.6 ± 12.3 %; n=9; bumetanide: 52.2 ± 10.8 %; n=9; 51.2 % ± 11.7; n=7; in WT and in NL3^{R451C} knock-in mice, respectively; p>0.05; Supplementary Figure 2).

The similar reduction in frequency of spontaneous EPSCs induced by gabazine- and bumatanide-detected in WT and NL3^{R451C} KI mice suggests comparable changes in GABAergic drive and $[Cl^-]_{in}$ in the two genotypes and possibly reflects the action of gabazine and bumetanide on

presynaptic $GABA_A$ receptors (Jang et al., 2006; Nakamura et al., 2007; Trigo et al., 2008; Beltran & Gutierrez, 2012) and NKCC1 chloride importer on axon initial segments (Khiroug et al., 2008), respectively.

The $NL3^{R451C}$ KI mutation does not alter the tonic $GABA_A$-mediated conductance

Once released, GABA rapidly diffuses across the synaptic cleft to occupy synaptic $GABA_A$ receptors. Part of the neurotransmitter escapes the cleft and invades the extracellular space to occupy extrasynaptic high affinity receptors and to generate a persistent $GABA_A$-mediated conductance (Farrant & Nusser, 2005) which is involved in a number of physiological and pathological processes (Brickley & Mody, 2012). Recent studies have demonstrated a down-regulation of $GABA_A$-mediated tonic conductance in an animal model of X Fragile syndrome, a common inherited cause of mental retardation with language deficit and autistic behavior (Curia et al., 2008; Olmos-Serrano et al., 2010). Therefore, in the following experiments we searched for differences in $GABA_A$-mediated tonic conductance between WT and $NL3^{R451C}$ mice. The tonic conductance was obtained by measuring the shift in the holding current following the application of the $GABA_A$ receptor channel blocker picrotoxin (100 µM). This drug caused a similar shift in holding current in WT and in $NL3^{R451C}$ mice (54 ± 11 pA, n=11, and 49 ± 15 pA, n=6, in WT and in $NL3^{R451C}$ mice, respectively; p=0.8; Figure 7).

Furthermore, to test whether GABA transporters differentially affect ambient GABA in WT and $NL3^{R451C}$ mice, we applied NO-711, which selectively blocks the neuronal and glial GABA transporter GAT-1 (Borden, 1996; Semyanov et al., 2004). NO-711 (10 µM) produced a similar inward shift of the baseline current in both WT and $NL3^{R451C}$ mice (41.5 ± 12.8 pA and 42 ± 7.5 pA in WT, n=6, and knock-in animals, respectively; n=7; p=0.7 in both; Figure 8 A-C). Addition of picrotoxin caused in the two genotypes a similar outward shift in baseline currents (71 ± 20 pA; n= 6 and 71 ± 12 pA; n=7; in WT and in $NL3^{R451C}$ mice, respectively, p=0.98). These data indicate that the $NL3^{R451C}$ mutation does not affect GABA transporters and the tonic $GABA_A$-mediated conductance.

Discussion

The present results provide evidence that the $NL3^{R451C}$ mutation selectively affects correlated network activity and GABAergic signaling already at birth. A previous study from layer 2/3 pyramidal neurons in acute slices of somatosensory cortex obtained from juvenile (P13-P16) $NL3^{R451C}$ KI mice has revealed an increased inhibitory synaptic transmission. These animals exhibited enhanced spatial learning abilities associated with deficits in social interaction (Tabuchi et al., 2007; but see Chadman et al., 2008 for the behavioral phenotype), similarly to those found in some forms of ASDs. The authors suggested that the NL3 mutation enhances GABAergic transmission without changing the release probability since they failed, at least in the barrel cortex, to detect major modifications in short-term synaptic plasticity.

Our data on GDPs indicate that the $NL3^{R451C}$ mutation affects GABA release. During the first week of postnatal life, GDPs are generated by the interplay between GABA and glutamate, both of them depolarizing and excitatory. Therefore changes in frequency and shape of spontaneous giant events can be attributed to modifications in the GABAergic, in the glutamatergic drive to principal cells or in both. A close examination of spontaneous miniature events, occurring during the first week of postnatal life, revealed an increased frequency, but not in amplitude of mGPSCs, suggesting a presynaptic type of action. This was further supported by TPMPA experiments that, as expected for an enhanced GABA transient in the cleft, showed a reduced blocking effect of the fast-off $GABA_A$ antagonist on miniature events in $NL3^{R451C}$ KI mice. Although an increased number of available postsynaptic $GABA_A$ receptors, if these are not saturated by the content of a single GABA containing vesicle (Barberis et al., 2004; Hartman et al., 2006) may account for these results, this was not the case since a similar number of receptor channels were revealed with peak-scaled non-stationary fluctuation analysis in both WT and $NL3^{R451C}$ knock-in mice, indicating that the observed effects were not postsynaptic in origin. Presynaptic changes in GABA release can be attributed to modifications in the probability of GABA release, in the number of release sites or in the content of GABA in single synaptic vesicles. Changes in probability of GABA release seem unlikely considering that we examined miniature events generated by the release of a single quantum. Our data do not allow distinguishing between the other two possibilities (changes in the number of release sites or in

vesicle GABA content). However, in agreement with previous data from Südhof group showing an enhancement of presynaptic GABAergic marker VGAT (but not VGlut1) in the hippocampus of $NL3^{R451C}$ KI mice (Tabuchi et al., 2007), it is likely that an GABAergic innervation may contribute to the enhancement of GABA release.

In the present experiments we did not characterize which subtype of GABAergic interneuron is involved in the observed effects. However, several lines of evidence suggest the involvement of parvalbumin-positive cells: i. highly interconnected, parvalbumin positive hub neurons, essential for triggering network oscillations in the form of GDPs in the immature hippocampus (Bonifazi et al., 2009) have been found to be occasionally parvalbumin-positive (Picardo et al. 2011). These cells which persist in adulthood (Picardo et al., 2011), retain the capacity of coordinating the timing of neuronal activity, thus contributing to generate theta and gamma rhythms (Bartos et al., 2007; Klausberger & Somogyi, 2008; Wulff et al., 2009), known to be involved in high cognitive functions (Singer, 1993). ii. In ASD mouse models ($NL3^{R451C}$ knock-in mice and in animals prenatally exposed to the histone deacetylase inhibitor, valproate) an asymmetric reduced expression of parvalbumin-positive interneurons was found across hemispheres in parietal and occipital cortices (Gogolla et al., 2009). Since parvalbumin-positive neurons normally drive experience-dependent circuit development (Fagiolini et al., 2004; Hensch, 2005), the selective disruption of these cells may alter neuronal networks during a critical period of postnatal development (Pizzarelli & Cherubini, 2011). Whatever is the subtype of GABAergic interneuron involved, the present data unveil an alteration of the excitatory/inhibitory (E/I) balance, known to exert a key role in the refinement of cortical circuits early in postnatal life (Le Blanc & Fagiolini, 2011).

The increased frequency (but not amplitude) of spontaneous miniature glutamatergic events detected in adult (but not in neonatal or juvenile $NL3^{R451C}$ mice) may represent a late form of compensatory homeostatic correction, developed to counter the excessive $GABA_A$-mediated inhibition. In this respect, the possibility that changes in the expression of AMPA- and NMDA receptors containing the NR2B subunits, observed in the CA1 region of adult $NL3^{R451C}$ mice may underlie the same phenomenon, cannot be excluded (Etherton et al., 2011).

Interestingly, as compared to WT animals, $NL3^{R451C}$ knock-in mice exhibited mGPSCs with faster decay kinetics. The speed-up of mGPSCs kinetics may depend on different pre, postsynaptic mechanisms or both (Barberis et al., 2011). At the postsynaptic level, this can be attributed to different expression of $GABA_A$ receptors containing subunits. In particular, receptors containing the $\alpha1$ or $\alpha2$ subunits (along with β and $\gamma2$ subunits) exhibit synaptic currents with fast and slow kinetics, respectively (Cherubini & Conti, 2001) and the tendency of zolpidem to prolong more the synaptic decay in mGPSCs from $NL3^{R451C}$ respect to WT, strongly suggest an increased recruitment of $\alpha1$ respect to $\alpha2$ subunits at synapses. $GABA_A$ receptor subunits expression may be altered by activity-dependent changes of intracellular chloride (Succol et al., 2012). However, no differences in intracellular chloride levels were detected between WT and $NL3^{R451C}$ mice making this possibility unlikely. At presynaptic level, the spatio-temporal profile of GABA concentration in the synaptic cleft may affect agonist binding as well as the gating properties of $GABA_A$ receptors resulting in modifications of the shape of synaptic signals. However, in contrast with the present observations, in these cases changes in the decay kinetics should be associated with changes in amplitude and in the rise time of synaptic currents (Barberis et al., 2011). Finally, the similar values of $GABA_A$-mediated tonic conductance measured in WT and $NL3^{R451C}$ mice, allow excluding the involvement of extrasynaptic receptors in the observed kinetics modifications.

How the E/I balance regulates brain functions ? Recent studies have highlighted the role of transynaptic signaling *via* NLs and Nrxs in assembling and stabilizing pre and postsynaptic components (Südhof, 2008). In particular, the NLs which in vertebrate are encoded by four genes (*Nlgn1-4* with various splice variants) form homo-dimers through their extracellular domains (Missler et al., 2012). While NL1 is preferentially associated with glutamatergic synapses (Song et al., 1999), NL2 and NL4 with GABAergic synapses (Varoqueaux et al., 2004; Graf et al., 2004; Dong et al., 2007; Hoon et al., 2011). Interestingly, we have recently found that gephyrin, a core protein of inhibitory postsynaptic densities that interacts with the cytoskeleton to stabilize inhibitory receptors in precise opposition to presynaptic active zones, transynaptically acts *via* NL2 on GABA release, thus directly contributing to maintain an appropriate E/I balance (Marchionni et al., 2009; Varley et al., 2011). Hampering gephyrin function not only alters

$GABA_A$ receptors clusterization and their gating properties but also the probability of GABA release, an effect mediated by NL2 since it could be rescued by over expressing this protein in gephyrin-deprived neurons (Varley et al., 2011). Although NL3 is highly expressed in the brain where is localized at both excitatory and inhibitory synapses (Budreck & Scheiffele, 2007), its functional role remains to be elucidated. However, the developmental pattern of NL3 expression, whose peak is coincident with that of synaptogenesis strongly suggests the involvement of this protein in synapse formation and stabilization.

As already outlined by Tabuchi et al., (2007), the $NL3^{R451C}$ mutation results in a gain of function since no effects on miniature GABAergic currents were detected in NL3 KO mice. It is worth noting that although mutated NL genes or associated proteins have been found only in a small number of young patients, they provide crucial information on the synaptic abnormalities which possibly affect ASDs.

References

Allene C, Picardo MA, Becq H, Miyoshi G, Fishell G & Cossart R (2012). Dynamic changes in interneuron morphophysiological properties mark the maturation of hippocampal network activity. *J Neurosci* **32**, 6688-6698.

Amaral DG & Dent JA (1981). Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J Comp Neurol* **195**, 51-86.

American Psychiatric Association (2000). Diagnostic and Statistical manual of Mental Disorders DC, USA, 2000.

Barberis A, Cherubini E & Mozrzymas JW (2000). Zinc inhibits miniature GABAergic currents by allosteric modulation of GABAA receptor gating. *J Neurosci* **20**, 8618-8627.

Barberis A, Petrini EM & Cherubini E (2004). Presynaptic source of quantal size variability at GABAergic synapses in rat hippocampal neurons in culture. *Eur J Neurosci* **20**, 1803-1810.

Barberis A, Petrini EM & Mozrzymas JW (2011). Impact of synaptic neurotransmitter concentration time course on the kinetics and pharmacological modulation of inhibitory synaptic currents. *Front Cell Neurosci* **5**, Epub 2011 Jun 22.

Bartos M, Vida I & Jonas P (2007). Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. *Nat Rev Neurosci* **8**, 45-56.

Beltran JQ & Gutierrez R (2012). Co-release of glutamate and GABA from single, identified mossy fiber giant boutons. *J Physiol* [Epub ahead of print] PMID: 22711957

Ben-Ari Y, Cherubini E, Corradetti R & Gaiarsa JL (1989). Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol* **416**, 303-325.

Ben-Ari Y, Gaiarsa JL, Tyzio R & Khazipov R (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev* **87**, 1215-1284.

Blankenship AG & Feller MB (2010). Mechanisms underlying spontaneous patterned activity in developing neural circuits. *Nat Rev Neurosci* **11**,18-29.

Bonifazi P, Goldin M, Picardo MA, Jorquera I, Cattani A, Bianconi G, Represa A, Ben-Ari Y & Cossart R (2009). GABAergic hub neurons orchestrate synchrony in developing hippocampal networks. *Science* **326**, 1419-1424.

Borden LA (1996). GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem Int* **29**, 335–356.

Brickley SG & Mody I (2012). Extrasynaptic GABAA-R: Their function in the CNS and Implications for disease. *Neuron* **73**, 23-34.

Budreck EC & Scheiffele P (2007). Neuroligin 3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. *Eur J Neurosci* **26**, 1738-1748.

Cathala L, Brickley S, Cull-Candy S & Farrant M (2003). Maturation of EPSCs and intrinsic membrane properties enhances precision at a cerebellar synapse. *J Neurosci* **23**, 6074-6085.

Chadman KK, Gong S, Scattoni ML, Boltuck SE, Gandhy SU, Heintz N & Crawley JN (2008). Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice. *Autism Res* **1**, 147-158.

Cherubini E, Gaiarsa JL & Ben-Ari Y (1991). GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci* **14**, 515-519.

Cherubini E & Conti F (2001). Generating diversity at GABAergic synapses. *Trends Neurosci* **24**, 155-162.

Chubykin AA, Atasoy D, Etherton MR, Brose N, Kavalali E, Gibson JR & Sudhof TC (2007). Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. *Neuron* **54**, 919-931.

Curia G, Papouin T, Séguéla P & Avoli M (2009). Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome. *Cerebral Cortex* **19**,1515-1520.

Dong N, Qi J & Chen G (2007). Molecular reconstitution of functional GABAergic synapses with expression of neuroligin-2 and GABAA receptors. *Mol Cell Neurosci* **35**, 14-23.

Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, Gillberg IC, Anckarsäter H, Sponheim E, Goubran-Botros H, Delorme R, Chabane N, Mouren-Simeoni MC, de Mas P, Bieth E, Rogé B, Héron D, Burglen L, Gillberg C, Leboyer M & Bourgeron T (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet* **39**, 25-27.

Etherton M, Földy C, Sharma M, Tabuchi K, Liu X, Shamloo M, Malenka RC & Südhof TC (2011). Autism-linked neuroligin-3 R451C mutation differentially alters hippocampal and cortical synaptic function. *Proc Natl Acad Sci USA* **108**,13764-13769.

Fagiolini M, Fritschy JM, Löw K, Möhler H, Rudolph U & Hensch TK (2004). Specific GABAA circuits for visual cortical plasticity. *Science* **303**, 1681-1683.

Farrant M & Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nature Rev. Neurosci* **6**, 215-229.

Fu Z, Washbourne P, Ortinski P & Vicini S (2003). Functional excitatory synapses in HEK293 cells expressing neuroligin and glutamate receptors. *J Neurophysiol* **90**, 3950-3957.

Geschwind DH & Levitt P (2007). Autism spectrum disorders: developmental disconnection syndromes. *Curr Opin Neurobiol* **17**, 103-111.

Glykys J & Mody I (20070. The main source of ambient GABA responsible for tonic inhibition in the mouse hippocampus. *J Physiol* **582**, 1163-78.

Gogolla N, Leblanc JJ, Quast KB, Südhof TC, Fagiolini M &Hensch TK (2009). Common circuit defect of excitatory-inhibitory balance in mouse models of autism. *J Neurodev Disord* **1**, 172-181.

Graf ER, Zhang X, Jin SX, Linhoff MW & Craig AM (2004). Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* **119**, 1013-1026.

Griguoli M, Maul A, Nguyen C, Giorgetti A, Carloni P & Cherubini E (2010). Nicotine blocks the hyperpolarization-activated current Ih and severely impairs the oscillatory behavior of oriens-lacunosum moleculare interneurons. *J Neurosci* **30**, 10773-10783.

Hartman KN, Pal SK, Burrone J & Murthy VN (2006). Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons. *Nat Neurosci* **9**, 642-649.

Hensch TK (2005). Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* **6**, 877-888.

Hoon M, Soykan T, Falkenburger B, Hammer M, Patrizi A, Schmidt KF, Sassoe-Pognetto M, Lowel S, Moser T, Taschenberger H, Brose N & Varoqueaux F (2011). Neuroligin-4 is localized to glycinergic postsynapses and regulates inhibition in the retina. *Proc Natl Acad Sci USA* **108**, 3053-3058.

Houston CM, Bright DP, Sivilotti LG, Beato M & Smart TG (2009). Intracellular chloride ions regulate the time course of GABA-mediated inhibitory synaptic transmission. J Neurosci **29**, 10416-10423.

Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C & Bourgeron T (2003). Mutations of the x-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* **34**, 27-29.

Jang IS, Nakamura M, Ito Y & Akaike N (2006). Presynaptic GABAA receptors facilitate spontaneous glutamate release from presynaptic terminals on mechanically dissociated rat CA3 pyramidal neurons. *Neuroscience* **138**, 25-35.

Jones MV, Jonas P, Sahara Y & Westbrook GL (2001). Microscopic kinetics and energetics distinguish GABAA receptor agonists from antagonists. *Biophys J* **81**, 2660–2670.

Katz B (1969). The release of neural transmitter substances. Liverpool University Press

Kim HG, Kishikawa S, Higgins AW, Seong IS, Donovan DJ, Shen Y, Lally E, Weiss LA, Najm J, Kutsche K, Descartes M, Holt L, Braddock S, Troxell R, Kaplan L, Volkmar F, Klin A, Tsatsanis K, Harris DJ, Noens I, Pauls DL, Daly MJ, MacDonald ME, Morton CC, Quade BJ & Gusella JF (2008). Disruption of neurexin 1 associated with autism spectrum disorder. *Am J Hum Genet* **82**, 199-207.

Khirug S, Yamada J, Afzalov R, Voipio J, Khiroug L & Kaila K (2008). GABAergic depolarization of the axon initial segment in cortical principal neurons is caused by the Na-K-2Cl cotransporter NKCC1. *J Neurosci* **28**, 4635-4639.

Klausberger T & Somogyi P (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* **321**, 53-57.

Ko J, Zhang C, Arac D, Boucard AA, Brunger AT & Sudhof TC (2009). Neuroligin-1 performs neurexin-dependent and neurexin-indipendent functions in synapses validation. *EMBO J* **28**, 3244-3255.

Laumonnier F, Bonnet-Brilhault F, Gomot M, Blanc R, David A, Moizard MP, Raynaud M, Ronce N, Lemonnier E, Calvas P, Laudier B, Chelly J, Fryns JP, Ropers HH, Hamel BC, Andres C, Barthélémy C, Moraine C & Briault S (2004). X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet* **74**, 552-557.

Laurie DJ, Wisden W & Seeburg PH (1992). The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* **12**, 4151-4172.

LeBlanc JJ, Fagiolini M (2011) Autism: a "critical period" disorder? *Neural Plast* 921680. Epub 2011 Aug 3.

Marchionni I, Kasap Z, Mozrzymas JW, Sieghart W, Cherubini E & Zacchi P (2009). New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture. *Neuroscience* **164**, 552-562.

Missler M, Südhof TC & Biederer T (2012). Synaptic cell adhesion. *Cold Spring Harb Perspect Biol*. **4**, a005694. doi: 10.1101/cshperspect.a005694

Momiyama A, Silver RA, Hausser M, Notomi T, Wu Y, Shigemoto R & Cull-Candy SG (2003). The density of AMPA receptors activated by a transmitter quantum at the climbing fibre-Purkinje cell synapse in immature rats. *J Physiol* **549**,75-92.

Mozrzymas JW, Barberis A, Michalak K & Cherubini E (1999). Chlorpromazine inhibits miniature GABAergic currents by reducing the binding and by increasing the unbinding rate of GABAA receptors. *J Neurosci* **19**, 2474-2488.

Nakamura M, Sekino Y & Manabe T (2007) GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus. *J Neurosci* **27**,1365-1373.

Olmos-Serrano JL, Paluszkiewicz SM, Martin BS, Kaufmann WE, Corbin JG & Huntsman MM (2010). Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. *J Neurosci* **30**, 9929–9938.

Perrais D & Ropert N (1999). Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic GABAA receptors in central synapses. *J Neurosci* **19**, 578-588.

Picardo MA, Guigue P, Bonifazi P, Batista-Brito R, Allene C, Ribas A, Fishell G, Baude A & Cossart R (2011). Pioneer GABA cells comprise a subpopulation of hub neurons in the developing hippocampus. *Neuron* **71**, 695-709.

Pizzarelli R & Cherubini E (2011). Alterations of GABAergic signaling in Autism Spectrum Disorders. *Neural Plast* 297153. Epub 2011 Jun 23.

Poulopoulus A, Aramuni G, Meyer G, Soykan T, Hoon M, Papadopoulos T, Zhang M, Paarmann I, Fuchs C, Harvey K, Brose N, Zhang W & Varoqueaux F (2009). Neuroligin2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* **63**, 628-642.

Pritchett DB & Seeburg PH (1990). Gamma-aminobutyric acidA receptor alpha 5-subunit creates novel type II benzodiazepine receptor pharmacology. *J Neurochem* **54**,1802-1804.

Sara Y, Biederer T, Atasoy D, Chubykin A, Mozhayeva MG, Südhof TC & Kavalali ET (2005). Selective capability of SynCAM and neuroligin for functional synapse assembly. *J Neurosci* **25**, 260-270.

Scheiffele P, Fan J, Choih J, Fetter R & Serafini T (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* **101**, 657-669.

Semyanov A, Walker MC, Kullmann DM & Silver RA (2004). Tonically active GABA A receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27**, 262-269.

Singer W (1993). Synchronization of cortical activity and its putative role in information processing and learning. *Annu Rev Physiol* **55**, 349-374.

Song JY, Ichtchenko K, Sudhof TC & Brose N (1999). Neuroligin1 is a postsynaptic cell adhesion molecule of excitatory synapses. *Proc Natl acad Sci USA* **108**, 16502-16509.

Succol F, Fiumelli H, Benfenati F, Cancedda L & Barberis A (2012). Intracellular chloride concentration influences the GABAA receptor subunit composition. *Nat Commun* **3**, 738. doi: 10.1038/ncomms1744.

Südhof TC (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* **455**, 903-911.

Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM & Südhof TC (2007). A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice *Science* **318**, 71-76.

Traynelis SF, Silver RA & Cull-Candy SG (1993). Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. *Neuron* **11**, 279-289.

Trigo FF, Marty A & Stell BM (2008). Axonal GABAA receptors. *Eur J Neurosci* **28**, 841-848.

Varley ZK, Pizzarelli R, Antonelli R, Stancheva SH, Kneussel M, Cherubini E & Zacchi P (2011). Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. *J Biol Chem* **286**, 20942-20951.

Varoqueaux F, Jamain S & Brose N (2004). Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol* **83**, 449-456.

Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, Zhang W, Sudhof T & Brose N (2006). Neuroligins determines synapses maturation and function. *Neuron* **51**, 741-754.

Weintraub K (2011). The prevalence puzzle: Autism counts. *Nature* **479**, 22-24.

Wulff P, Ponomarenko AA, Bartos M, Korotkova TM, Fuchs EC, Bähner F, Both M, Tort AB, Kopell NJ, Wisden W & Monyer H (2009). Hippocampal theta rhythm and its coupling with gamma oscillations require fast inhibition onto parvalbumin-positive interneurons. *Natl Acad Sci USA***106**, 3561-3566.

Yan J, Oliveira G, Coutinho A, Yang C, Feng J, Katz C, Sram J, Bockholt A, Jones IR, Craddock N, Cook EH Jr, Vicente A & Sommer SS (2005). Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients. *Mol Psychiatry* **10**, 329-332.

Author contributions

EC and RP: conceived and designed the experiments

RP: performed the experiments, analyzed data

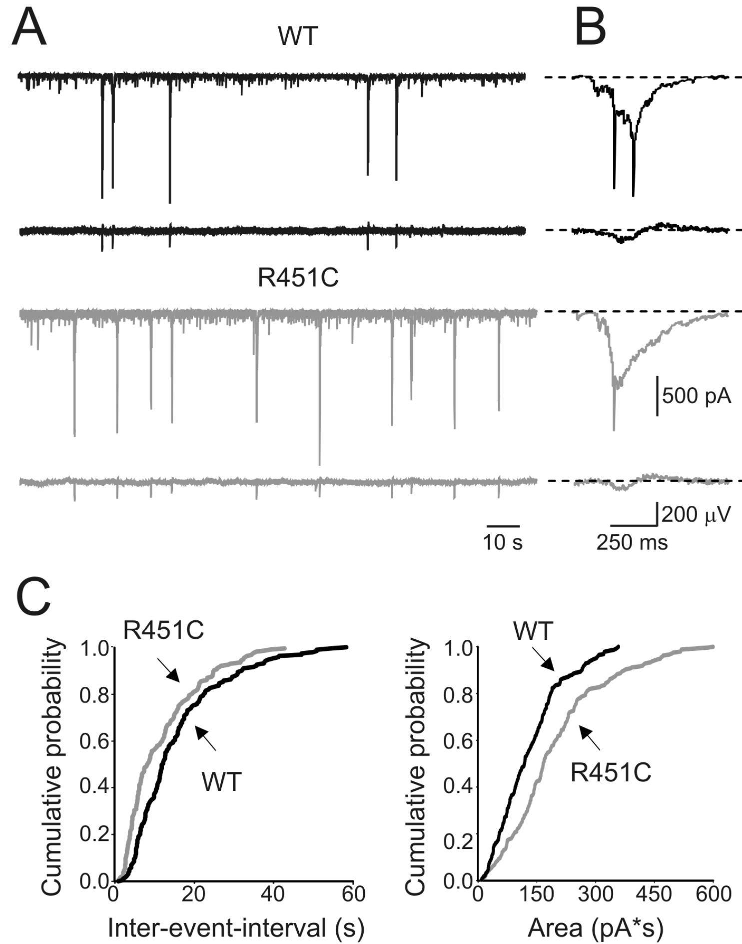
EC: wrote the paper

Both authors approved the final version of the manuscript

Acknowledgements

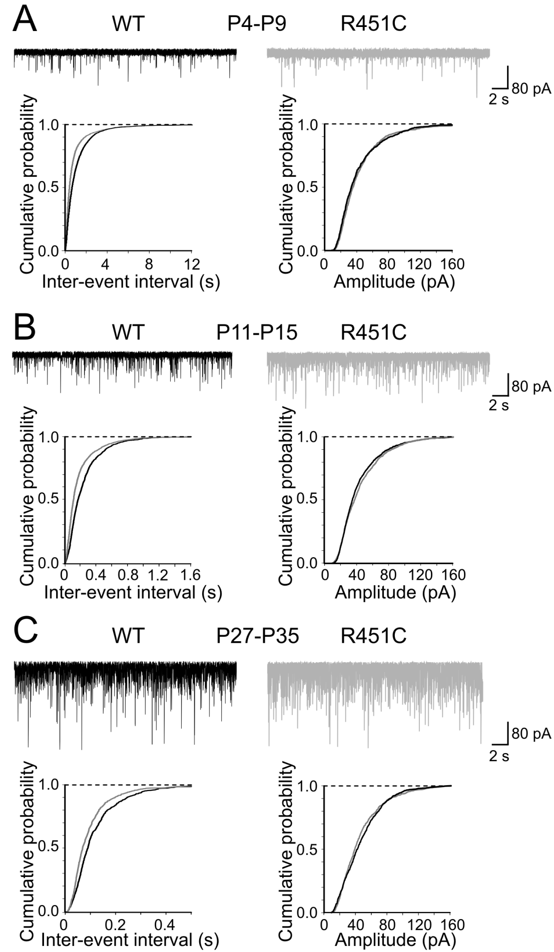
The authors are grateful to Drs. Andrea Barberis and Paola Zacchi for useful suggestions and for carefully reading the manuscript; to Jessica Franzot for genotyping. This study was supported by a Telethon grant to EC (GGP11043).

Figure 1



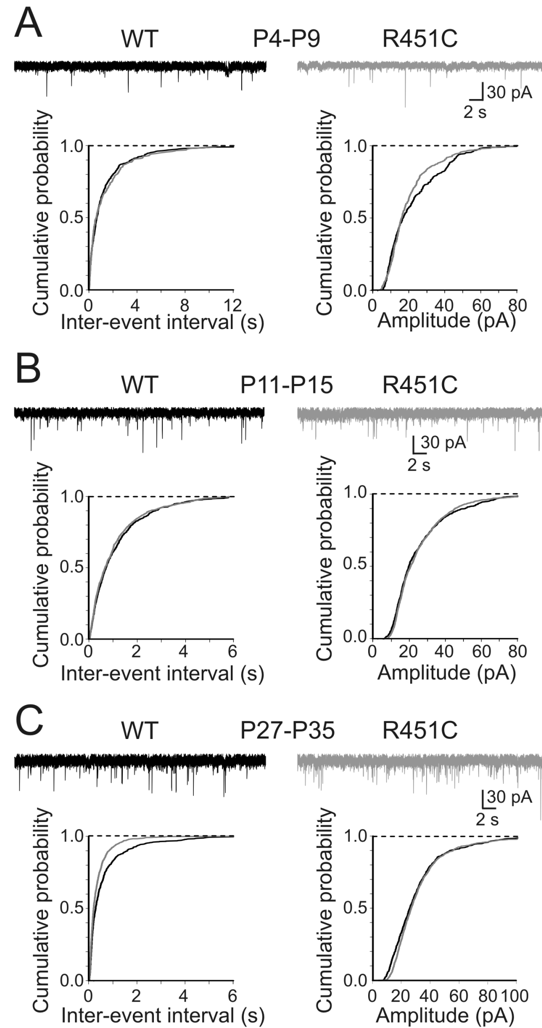
The NL3^{R451C} knock-in mutation enhances correlated network activity in the immature hippocampus. A. Whole cell voltage-clamp (upper traces) and extracellular field recordings (lower traces) of GDPs in hippocampal slices obtained from WT (black) and NL3^{R451C} mice (grey). B. GDPs from the traces in A shown on an expanded time scale. C. Cumulative probability plots of inter-event intervals and areas of inward currents underlying GDPs recorded from WT (n=24) and NL3^{R451C} mice (n=17). Curves in the plots referring to WT and R451C KI mice are significantly different ($p<0.01$; $p<0.01$; K-S test).

Figure 2



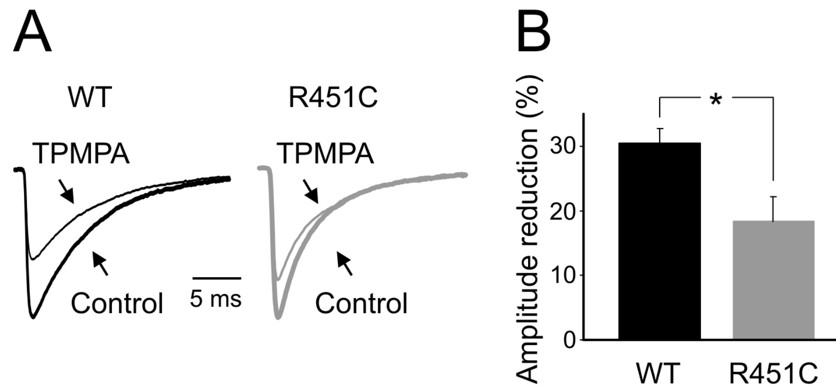
The NL3^{R451C} knock-in mutation enhances the frequency of mGPSCs. Samples traces of mGPSCs recorded from CA3 principal cells at P4-P9 (A), P11-P15 (B) and P27-P35 (C) from WT (black) and in NL3^{R451C} knock-in mice (grey). Below, cumulative probability plots of inter-event intervals (left) and amplitude (right) of obtained from WT and NL3^{R451C} knock-in mice. Changes in inter-event-interval ($p<0.05$; K-S test) but not in amplitude ($p>0.05$; K-S test) were significantly different at all developmental stages examined .

Figure 3



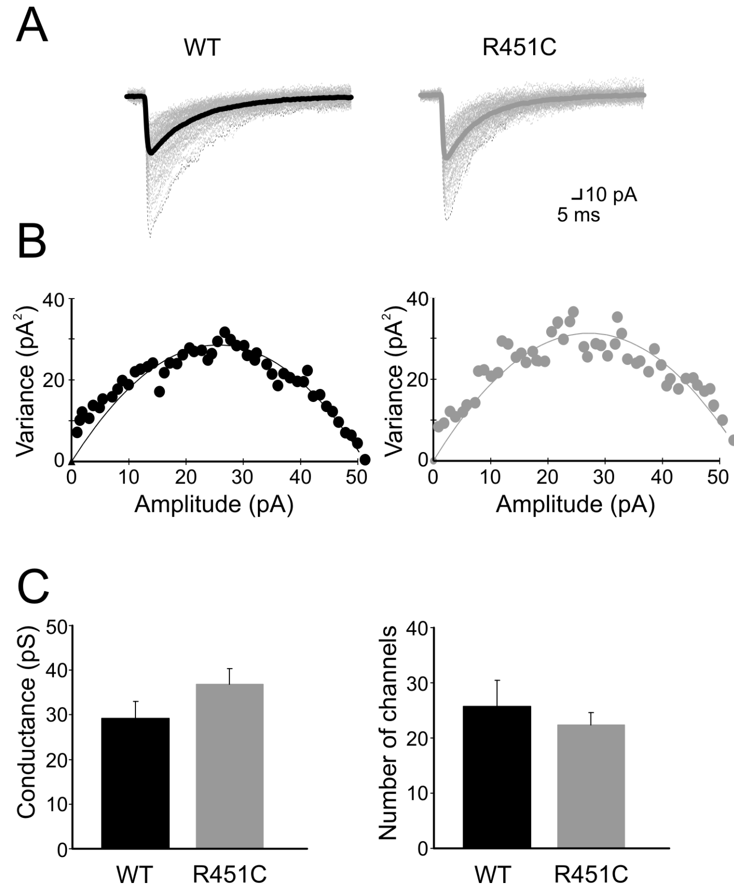
The NL3^{R451C} knock-in mutation does not affect spontaneous miniature glutamatergic events. A. Samples traces of mEPSCs recorded from CA3 principal cells at P4-P9 from WT (black) and in NL3^{R451C} knock-in mice (grey). Below, cumulative probability plots of inter-event intervals (left) and amplitude (right) of obtained from P4-P9 (A), P11-P15 (B) and P27-P35 (C) WT and NL3^{R451C} knock-in mice. B. and C. as A but for later developmental stages. Note changes in frequency (but not amplitude of miniature events at P27-P35 (the two curves are significantly different; $p<0.05$; K-S test).

Figure 4



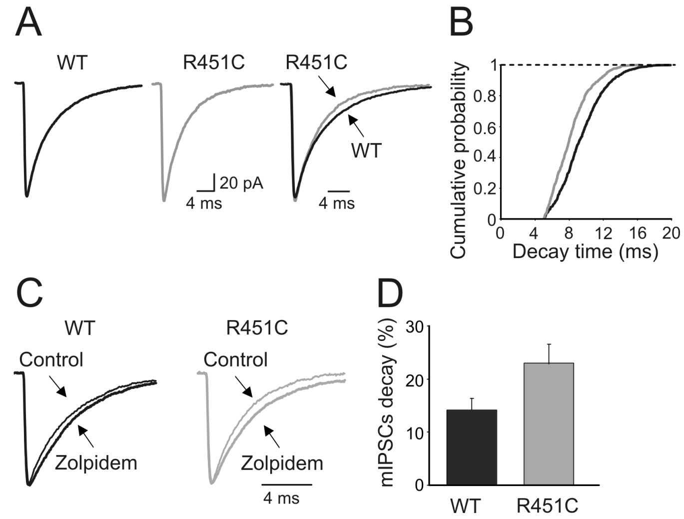
Increased synaptic GABA transient in NL3^{R451C} knock-in mutant mice. A. Sample traces of mGPSCs recorded from NL3^{R451C} mutants (grey) and littermate (black), in the absence (tick line, Control) and in the presence of TPMPA (200 μM; thin line). The amplitudes of mGPSCs from NL3^{R451C} knock-in mice were normalized to those obtained from WT animals. B. Each column represents the mean TPMPA-induced reduction in amplitude of mGPSCs in WT (black: n= 8) and NL3^{R451C} mice (grey; n= 7). * p<0.05.

Figure 5



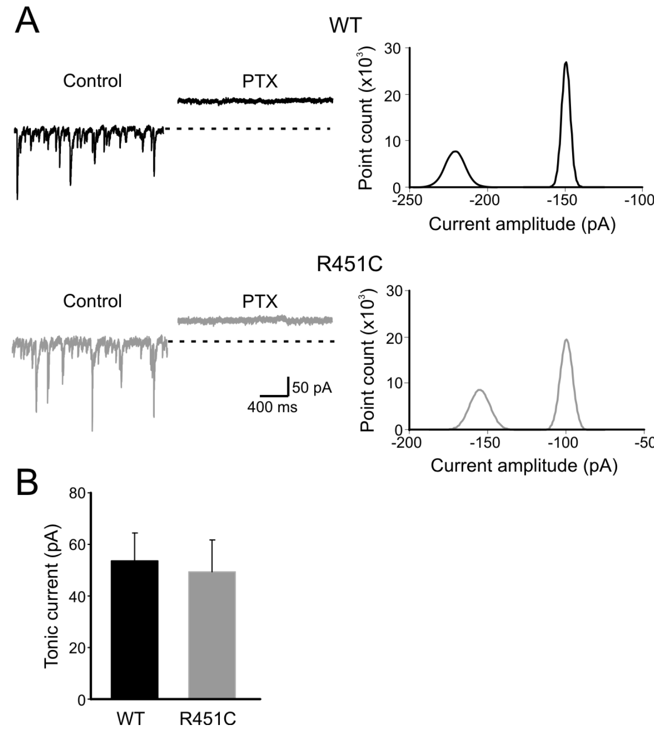
The NL3^{R451C} knock-in mutation does not affect single channel conductance and/or the number of GABA$_A$ receptor channels. A. Individual mGPSCs (dotted lines) are shown with the average currents (thick lines) in WT and R451C mice. B. Current/variance relationships for mGPSCs shown in A. C. Summary plots of weighted mean channel conductance and number of GABA$_A$ receptor channels in WT (black; n=8) and in NL3^{R451C} knock-in mice (grey; n=9).

Figure 6



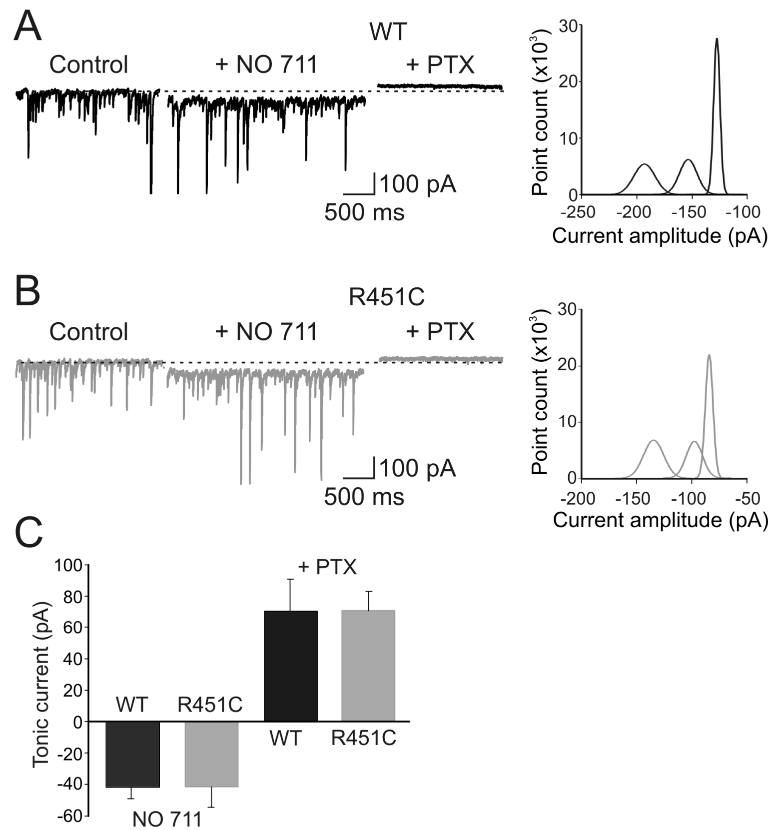
Faster decay of mGPSCs in NL3^{R451C} knock-in mice. A. Sample traces of mGPSCs (each trace is the average of > 50 individual traces) obtained from NL3^{R451C} knock-in mice (grey) and control littermates (WT, black). On the right the two traces are superposed. B. Cumulative probability plot of decay times from WT (black; n= 9) and NL3^{R451C} knock-in mice (grey; n= 8). The two curves are significantly different (p<0.01 ; K-S test). C. Sample traces of mGPSCs from NL3^{R451C} (grey) and WT mice (black), before (Control) and during application of zolpidem (100 nM). D. Summary plots of normalized changes in mGPSCs decays induced by zolpidem in WT (black, n= 6) and in NL3^{R451C} (grey, n= 7) mice.

Figure 7



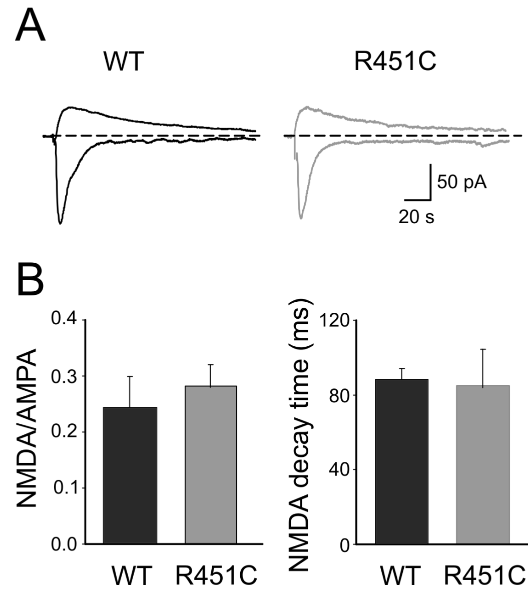
The NL3^{R451C} knock-in mutation does not alter tonic GABA$_A$-mediated conductance. A. Left: representative trace of spontaneous GPSCs recorded from a P11 pyramidal neurons in hippocampal slices obtained from a WT (black) and NL3^{R451C} KI mice (grey) before (Control) and during bath application of picrotoxin (PTX; 100 μM). Right: all-point histogram of 10 ms traces from the cell recorded on the left in control conditions and in the presence of picrotoxin. B. Summary data obtained from WT (black; n= 11) and NL3^{R451C} KI mutant mice (grey; n=6).

Figure 8



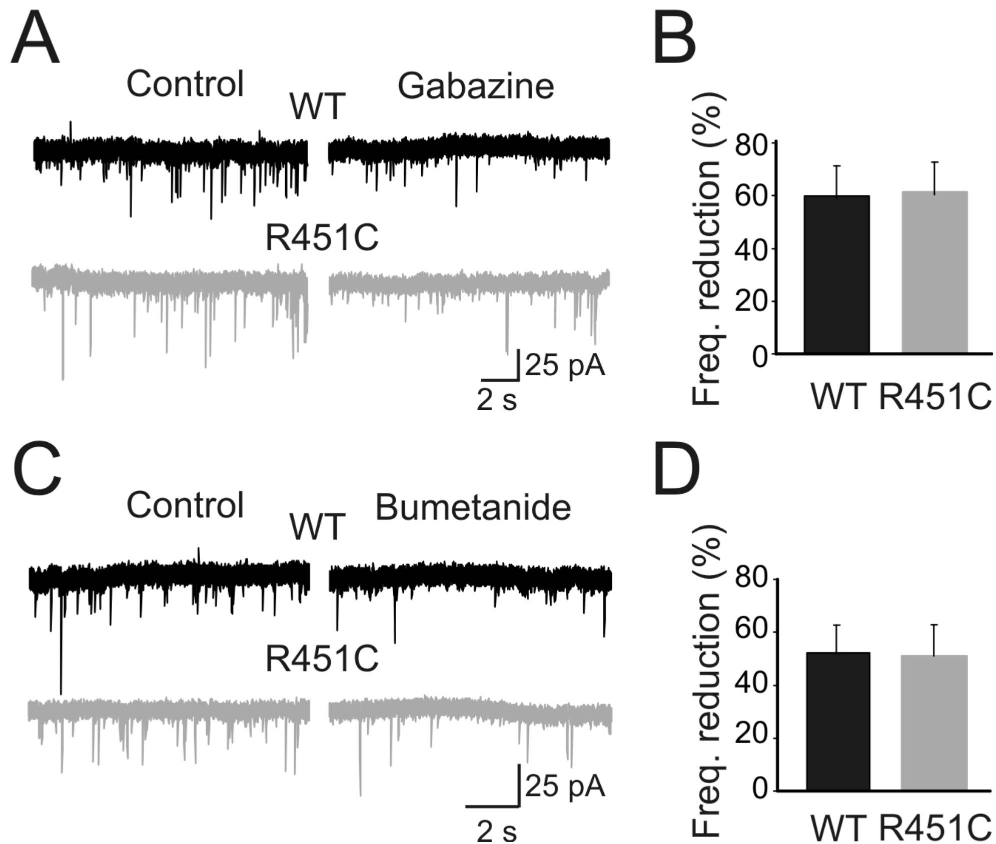
The NL3^{R451C} knock-in mutation does not alter tonic GABA$_A$-mediated inhibition. A. Left: representative traces of spontaneous GPSCs recorded in a P 12 pyramidal cell in hippocampal slice obtained from a WT animal before (Control), during application of the GAT-1 blocker NO-711 (10 µM) and NO-711 plus picrotoxin (100 µM). Right: all-point histogram of 10 ms traces from the cell recorded on the left in control conditions, in the presence of NO-711 and NO-711 plus picrotoxin. B. As in A but from NL3^{R451C} KI mutant mouse. C. Summary data obtained from WT (black; n= 6) and NL3^{R451C} knock-in mutant mice (grey; n= 7).

Supplementary Figure 1



The $NL3^{R451C}$ knock-in mutation does not affect excitatory glutamatergic responses at immature MF-CA3 synapses. A. Sample traces of measurements of the ratio of NMDA *versus* AMPA receptor-mediated synaptic currents in $NL3^{R451C}$ mutants (grey) and in littermate controls (black).B. summary graphs of NMDA/AMPA ratio (WT 0.24 ±0.06, n=6 ; KI 0.28 ±0.04 n=4 ; p> 0.05) and NMDA decay times (WT 88.17± 6 ms, n=6; KI 85.1± 20 ms, n=4, p>0.05).

Supplementary Figure 2



Gabazine and bumetanide similarly reduce spontaneous glutamatergic activity in WT and in NL3^{R451C} knock-in mice. A. Sample traces of AMPA-mediated spontaneous EPSCs recorded at P11-P15 from WT (black) and NL3^{R451C} knock-in mice (grey) in control and in the presence of gabazine (10 μM) from a holding potential of -65 mV. B. Each column represents the normalized reduction in frequency of spontaneous EPSCs in WT (black; n=6) and in NL3^{R451C} knock-in mice (grey; n=9). C-D. As in A-B but in the presence of bumetanide (WT n=9; KI n=7. Note similar gabazine- and bumetanide-induced reduction in frequency of spontaneous glutamatergic events in both genotypes.

-Paper N.3-

Alterations of GABAergic signaling in autism spectrum disorders.

Pizzarelli R, Cherubini E.

Neural Plasticity 2011;2011:297153

Hindawi Publishing Corporation
Neural Plasticity
Volume 2011, Article ID 297153, 12 pages
doi:10.1155/2011/297153

Review Article

Alterations of GABAergic Signaling in Autism Spectrum Disorders

Rocco Pizzarelli and Enrico Cherubini

Neurobiology Sector and IIT Unit, International School for Advanced Studies (SISSA), Via Bonomea 265, 34136 Trieste, Italy

Correspondence should be addressed to Enrico Cherubini, cher@sissa.it

Received 16 January 2011; Accepted 1 April 2011

Academic Editor: Tommaso Pizzorusso

Copyright © 2011 R. Pizzarelli and E. Cherubini. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Autism spectrum disorders (ASDs) comprise a heterogeneous group of pathological conditions, mainly of genetic origin, characterized by stereotyped behavior, marked impairment in verbal and nonverbal communication, social skills, and cognition. Interestingly, in a small number of cases, ASDs are associated with single mutations in genes encoding for neuroligin-neurexin families. These are adhesion molecules which, by regulating transsynaptic signaling, contribute to maintain a proper excitatory/inhibitory (E/I) balance at the network level. Furthermore, GABA, the main inhibitory neurotransmitter in adult life, at late embryonic/early postnatal stages has been shown to depolarize and excite targeted cell through an outwardly directed flux of chloride. The depolarizing action of GABA and associated calcium influx regulate a variety of developmental processes from cell migration and differentiation to synapse formation. Here, we summarize recent data concerning the functional role of GABA in building up and refining neuronal circuits early in development and the molecular mechanisms regulating the E/I balance. A dysfunction of the GABAergic signaling early in development leads to a severe E/I unbalance in neuronal circuits, a condition that may account for some of the behavioral deficits observed in ASD patients.

1. Introduction

Autism spectrum disorders (ASDs) comprise a complex and heterogeneous group of pathological conditions including autism, Rett and Asperger syndromes, and pervasive developmental disorder-otherwise nonspecified, characterized by impaired social interactions, deficits in verbal and nonverbal communication, and a limited interest in the surrounding environment associated with stereotyped and repetitive behaviors [1]. The incidence of these disorders, which varies between 10 and 20 per 10000 children, has risen dramatically over the past two decades mainly because of the use of broader diagnostic criteria and the increased attention of the medical community [2]. Clinical signs are usually present at the age of 3 years, but prospective studies of infants at risk have demonstrated that deficits in social responsiveness, communication, and play could be present already at the age of 6–12 months.

ASDs are the most heritable neurodevelopmental disorders of early childhood. Genetic factors are thought to account for ~80% of autism cases, and since autism is a spectrum of disorders, it is conceivable that in most cases different genes act in combination in different individuals [3]. Genes, interacting with epigenetic factors, may influence neuronal migration, axon pathfinding, dendritic development, synaptogenesis, and pruning, thus contributing to alter neuronal connectivity and information processing [4].

Interestingly, a small percentage of ASDs patients carry single mutations in genes encoding for synaptic cell adhesion molecules of the neurexin (NRXN)-neuroligin (NLG) families [5]. These include mutations in genes encoding for NRXN1 [6, 7], for NLG3, NLG4 [8–10], and for Shank3 [11]. Although rare, these mutations provide crucial information on the synaptic abnormalities which possibly affect ASDs patients and point to synapses dysfunction as a possible site of autism origin. Synapses are specialized intercellular junctions which transfer information from a neuron to a target cell, usually another neuron.

Several lines of evidence suggest that an impairment of GABAergic transmission contributes to the development

of ASDs. GABA, the main inhibitory neurotransmitter in adulthood is released by interneurons which contain the GABA synthesizing enzymes glutamic acid decarboxylase (GAD)65 and GAD67. GABAergic interneurons, which constitute a heterogeneous group of cells, differently classified in virtue of their anatomical, physiological, and molecular features [12], represent only 10%–15% of the total neuronal population. Nevertheless, they provide the functional balance, complexity, and computational architecture of neuronal circuits [13]. They play a key role in regulating neuronal excitability *via* feedback and feed-forward inhibition. Axons of different inhibitory cells target different postsynaptic subcellular compartments, allowing them to selectively control the output of pyramidal cells [14], thus providing the temporal structure that orchestrates the activity of neuronal ensembles leading to coherent network oscillations [15].

While in the mature brain GABA acts as an inhibitory transmitter, during the embryonic and the perinatal period, this neurotransmitter depolarizes targeted cells and triggers calcium influx. GABA-mediated calcium signaling regulates a variety of different developmental processes from cell proliferation migration, differentiation, synapse maturation, and cell death [16]. Although the geometry and the cellular and subcellular selectivity of GABAergic axons are mainly genetically determined, axonal branching and arborization are regulated by activity and experience and often require brain-derived neurotropic factor (BDNF, [17]). Thus, sensory stimulation contributes to shape neuronal circuits, whereas sensory deprivation significantly retards their maturation [18–20].

Considering the multifacet of GABA activities particularly during development, it is not surprising that disturbance of GABAergic signaling can result in aberrant information processing, as found in neurodevelopmental disorders such as ASDs. In particular, it has been hypothesized that at least some forms of autism result from an imbalance between excitation and inhibition in local circuits involved in sensory, mnemonic, social, and emotional processes. The resulting hyperexcitability could disrupt the normal formation of cortical maps leading to a relatively unstable state [21]. The cortex is organized in vertical mini columns of functionally related glutamatergic and GABAergic neurons that process thalamic inputs. Local GABAergic circuits contribute to control the functional integrity of minicolumns *via* lateral inhibition. Interestingly, analysis of postmortem tissues from ASDs patients has revealed alterations in the number of mini columns, in the horizontal spacing separating cell columns, and in their internal structure [22]. The abnormal cytoarchitecture is often associated with an increased expression of calbindin-, calretinin- and parvalbumin-positive GABAergic interneurons [23]. In addition, changes in GAD65 and GAD67 [24], in the mRNA encoding for these enzymes [25–27], in $GABA_A$ [28, 29] and $GABA_B$ receptors [30] have been found in brain samples from ASDs patients. The altered GABAergic function may reduce the threshold for developing seizures as demonstrated by the high comorbidity of ASDs with epilepsy (one third of ASDs patients have seizures [31]). This further strengthens the hypothesis that an unbalance between excitation and inhibition contributes to these devastating neurological disorders.

This paper will focus on the functional role of GABA in regulating developmental processes, their experience-dependent refinement and, at the network level, the balance between excitation and inhibition. In addition, the implications that an altered GABAergic signaling may have in neurodevelopmental disorders such as ASDs will be discussed taking into account different animal models.

2. GABA, a Pioneer Neurotransmitter in Neuronal Circuits Formation

The construction of the brain relies on a series of well-defined genetically and environmentally driven factors whose disruption leads to pathological disorders including ASDs. During central nervous system development, a sequence of temporally related events during which neurons proliferate, migrate, differentiate, and establish proper synaptic connections occurs [16]. Further refinement of immature networks needs adaptive processes involving experience- or activity-dependent mechanisms, which lead to the formation of new synapses and elimination of others. Using imaging techniques and electrophysiological approaches, several patterns of coherent activity have been characterized early in development [32]. Uncorrelated spontaneous activity consisting of calcium action potentials has been suggested to play a crucial role in regulation of cortical neurogenesis at late embryonic stages [16, 33]. At birth, synchronous neuronal activity can be detected in the hippocampus and in the neocortex. This relies firstly on the activation of intrinsic conductances and gap junctions and later on synapse-driven events. Thus, small cell assemblies coupled to gap junctions generate nonsynaptic spontaneous plateau assemblies (SPAs, [32], Figure 1).

These involve small groups of neurons and are associated with sustained intrinsic membrane potential oscillations. SPAs are modulated by oxytocin, a maternal hormone essential for labour induction, which transiently converts GABA action from excitatory to inhibitory [34]. As the network matures and the density of functional synapses increases, synaptic-driven network oscillations replace SPAs. A downregulation in the expression of connexins *via* CREB signalling, following activation of NMDA receptors, may lead to SPAs silencing [35]. Two different patterns of network-driven synaptic oscillations have been described: the giant depolarizing potentials or GDPs [36] and early network oscillations or ENOs [37]. These are reminiscent of "long oscillations" and "spindle bursts", respectively, recorded from the rat somatosensory cortex *in vivo* [38] or of discontinue activity patterns observed in the EEG of preterm babies [39]. While ENOs (which usually precede GDPs) were initially thought to constitute the cortical counterpart of hippocampal GDPs, they have been shown to coexist with GDPs in the neocortex [32]. In the neocortex, ENOs critically depend on the activation of NMDA receptors [37]. In addition, evidence has been provided that extrasynaptic NMDA receptors activated by ambient glutamate generate a tonic

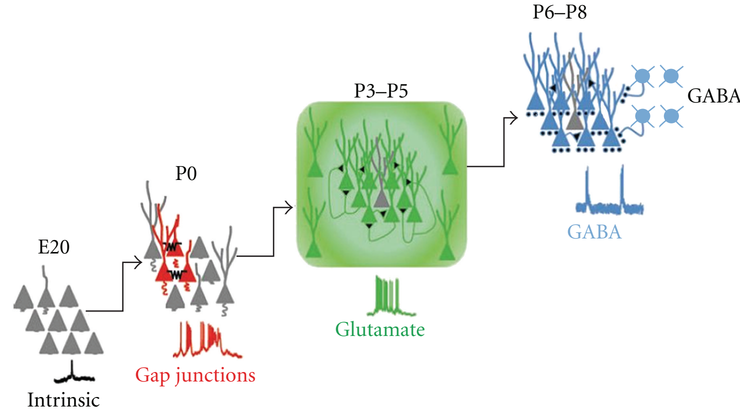


Figure 1: Patterns of electrical activity observed at late embryonic/early postnatal stages in the cortex. E20: uncorrelated calcium spikes; P0: Spontaneous Plateau Assemblies (SPAs) synchronized by gap junctions. P3–P5: early network oscillations (ENOs) mediated by glutamate. P6–P8: giant depolarizing potentials (GDPs) mediated by GABA and glutamate. (Modified from [32]).

current, which contributes to depolarize the membrane, to enhance cell excitability and to convert silent synapses into functional ones [40]. The activation of NMDA receptors by "ambient" glutamate would be facilitated by changes in subunits composition [41], in voltage dependence of the magnesium block [42] and in the high affinity for glutamate of extrasynaptic NMDA receptors.

In the hippocampus, GDPs are generated by the synergistic action of glutamate and GABA, which in the immediate postnatal period, orchestrates neuronal ensembles *via* its depolarizing and excitatory action [43]. Before synapses formation, GABA depolarizes targeted neurons *via* a paracrine type of action. GABA released in a calcium- and SNARE-independent way by nonconventional release sites such as growth cones and astrocytes diffuses away to activate extrasynaptic receptors [44]. The absence of an efficient uptake system will enable GABA to accumulate in the extracellular space and to reach a concentration sufficient to exert its distal action. The depolarizing action of GABA would activate voltage-dependent calcium channels and would facilitate the relief of the voltage-dependent magnesium block from NMDA receptors, thus allowing calcium entry and activation of second messengers.

Using network dynamics imaging, online reconstruction of functional connectivity and targeted whole-cell recordings, it has been recently demonstrated that, in immature hippocampal slices, functional hubs composed of subpopulations of GABAergic interneurons with large axonal arborizations are able to synchronize large neuronal ensembles [45]. The depolarizing action of GABA in immature neurons results from an outwardly directed flux of chloride. Chloride homeostasis is controlled by the Na-K-2Cl cotransporter NKCC1 and by the K-Cl cotransporter KCC2 that enhance and lower $[Cl^-]_i$, respectively [46]. Due to the low expression of the KCC2 extruder at birth, chloride accumulates inside the neuron *via* NKCC1. The progressive increase in the expression of KCC2 is responsible for the developmental shift of GABA from the depolarizing to the hyperpolarizing direction. KCC2 extrudes K^+ and Cl^- using the electrochemical gradient for K^+. Cl^- extrusion is weak in immature neurons and increases with neuronal maturation.

The functional role of the depolarizing action of GABA on early circuits development has been assessed by manipulating the expression levels of KCC2 and NKCC1, respectively. Thus, the premature expression of KCC2, has been shown to convert the action of GABA from excitatory to inhibitory and to impair the morphological maturation of cortical cells, without altering their radial migration [47]. This effect can be mimicked by overexpressing the inwardly rectifying K^+ channel which lowers the membrane potential and reduces cell excitability, strongly suggesting that membrane depolarization caused by the early GABA excitation is essential for the functional maturation of cortical circuits *in vivo*. On the other hand, knocking down the expression of NKCC1 to abolish $GABA_A$-mediated excitation, leads to a significant reduction in AMPA receptor-mediated synaptic transmission associated with a disruption of dendritic arborization and spines density further indicating that the depolarizing and excitatory action of GABA plays a permissive role in the formation of excitatory synapses [48]. Interestingly, these effects could be rescued by over expressing a mutant form of voltage-independent NMDA receptors, indicating that GABA depolarization cooperates with NMDA receptor to regulate the formation of excitatory synapses. It is worth noting that GDPs and associated calcium transients act as coincidence detectors for enhancing, in an associative type of manner, synaptic efficacy at emerging GABAergic [49], and glutamatergic synapses [50]. Using a "pairing" procedure, consisting of correlating GDPs-associated calcium rise with stimulation of mossy fibers or Schaffer collaterals, in the CA3 and CA1 region, respectively, we found that this procedure produced a strong and persistent potentiation of synaptic responses (Figure 2).

In the absence of pairing, no significant changes in synaptic efficacy could be detected. Similar results were obtained by progressively increasing the interval between GDPs and mossy fiber/Schaffer collateral stimulation. Pairing-induced potentiation was prevented when the cells were loaded with

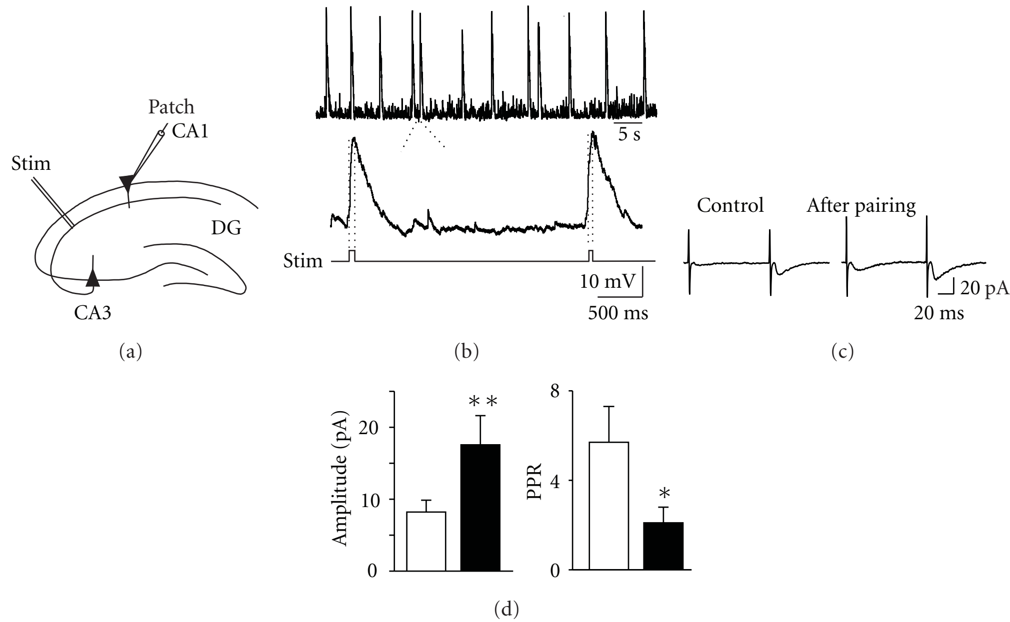


Figure 2: Pairing GABA-mediated GDPs with Schaffer collateral stimulation persistently enhances synaptic strength at glutamatergic CA3-CA1 connections. (a) Experimental paradigm. (b) The rising phase of GDPs (between the dashed lines) was used to trigger synaptic stimulation (stim) (c) EPSCs evoked in CA1 principal cells by minimal stimulation of Schaffer collateral, before and after pairing (average of 19 responses). (d) Each bar represents the mean peak amplitude of synaptic responses including failures ($n = 8$) and the paired pulse ratio (PPR; $n = 8$), obtained before (open) and after (closed) pairing. (Modified from [50]).

the calcium chelator BAPTA or when nifedipine (but not the NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid) was added to the extracellular medium, suggesting that activity-dependent changes in synaptic efficacy depend on calcium rise through voltage-dependent calcium channels and not *via* NMDA receptors.

Immature neurons are characterized by an elevated number of "silent" synapses [40]. These are synapses that do not conduct at rest either, because the neurotransmitter is not released when the presynaptic terminal is invaded by an action potential (presynaptically silent), or because they are unable to detect the release of the neurotransmitter due to the lack of the respective receptors on the subsynaptic membrane (postsynaptically silent). Silent synapses can be converted into active ones by activity-dependent processes and this represents the most common mechanism for LTP induction, not only during development but also in the mature brain [51]. Interestingly, the pairing procedure was able to convert silent synapses into active ones. In particular, in double pulse experiments, pairing caused the appearance of responses to the first stimulus and increased the number of successes to the second one, indicating that an increased probability of transmitter release accounts for long-term increase in synaptic strength. Therefore, calcium entry through voltage-dependent calcium channels, activated by the depolarizing action of GABA during GDPs, is instrumental in enhancing the number of functional GABAergic and glutamatergic synapses and/or the probability of GABA and glutamate release in a Hebbian way. This may contribute to refine neuronal connectivity before the establishment of the adult neuronal circuit.

3. Molecular Determinants of GABAergic Synapses Formation

In the adult brain, information processing relies on the integration of excitatory and inhibitory circuits which use glutamate and GABA/glycine as neurotransmitters, respectively. The so-called excitatory/inhibitory (E/I) balance represents a critical condition for the correct functioning of neuronal networks and it is essential for nearly all brain functions, including representation of sensory information and cognitive processes. The E/I balance is maintained *via* highly regulated homeostatic mechanisms [52]. Neurons are able to compensate for experimental perturbations by modulating ion channels, receptors, signaling pathways, and neurotransmitters. At the molecular level, these processes require chromatin remodeling, changes in gene expression and repression, changes in protein synthesis, turnover and cytoskeleton rearrangement [53]. A disruption of the homeostatic control, due to the lack of compensatory changes, leads to an imbalanced E/I ratio and to the developmental of neuropsychiatric disorders including mental retardation, epilepsy and ASDs [21].

During brain maturation, the development of a proper E/I balance is achieved with the shift of GABA action from the depolarizing to the hyperpolarizing direction, a process that in rodents starts appearing toward the end of the first, beginning of the second postnatal week [54]. Disturbances in the E/I balance may also occur when the formation or maintenance of one class of synapses prevails over the others. The selective loss of excitatory or inhibitory synapses can take place during the initial period of synapse formation

and consolidation or late in development during activity-dependent refinement of neuronal circuits and may involve mutations in genes encoding for ion channels or $GABA_A$ receptor subunits. These would lead to circuits with abnormal activity and prone to seizures [55]. For example, the disruption in mice of the *gabrb3* gene, which encodes for $\beta 3$ subunits of $GABA_A$ receptors, highly expressed during development, is sufficient to cause phenotypic traits which parallel those present in the Angelman syndrome [56]. Thus, mice lacking the $\beta 3$ subunits exhibit a major reduction of $GABA_A$ receptors, thalamic disinhibition and seizures associated with learning and memory deficits, poor motor skills on a repetitive task, hyperactivity, and a disturbed rest-activity cycle, all features characteristic of children affected by this neurological disorder. The cellular and molecular mechanisms underlying these phenomena are still poorly understood and their comprehension is further complicated by intrinsic differences among neuronal types, experimental conditions and the developmental stage of neurons [57].

During neuronal circuit assembly, GABA signaling precedes and promotes the formation of glutamatergic synapses [58]. The sequential development of GABA- and glutamate-mediated connections is independent on the arrival of afferent inputs but is related to the degree of maturation of targeted cells including changes in dendritic length, in somatic size and capacitance [58]. While functional GABAergic synapses require the presence of small apical dendrites in stratum radiatum of the hippocampus, glutamatergic connections require the presence of dendrites in stratum lacunosum moleculare.

The refinement of GABAergic connections and their translation into a potent inhibitory network is a protracted process which extends well beyond the first two postnatal weeks into the adolescent period and is regulated by neuronal activity and experience. In the visual cortex, for instance, experience-dependent regulation of the GABAergic innervation controls the onset of critical periods [59] during which neuronal circuits display a heightened sensitivity to environmental stimuli and are greatly shaped by sensory experience. Thus, a delayed and an accelerated onset in visual plasticity can be obtained by negatively or positively interfering with the GABAergic function, respectively [59]. GABA signaling itself would be responsible for the development of inhibitory connections as demonstrated by the observation that, knocking down GAD67 in basket interneurons severely impairs GABAergic innervation [20]. These effects may be attributed to the activity-dependent reduction in GABA synthesis and signaling following down regulation of GAD67 levels and/or enzyme activity [20].

To be highly efficient, synaptic transmission requires the presence of clustered postsynaptic receptors localized in precise apposition to presynaptic release sites. At inhibitory connections, this task is accomplished by gephyrin, a tubulin-binding protein which traps glycine and $GABA_A$ receptors in the right place anchoring them to the cytoskeleton [60].

Interestingly, a recent study has demonstrated that gephyrin directly interacts with adhesion molecules of the NLGs family [61] which in turn bind to their presynaptic partners NRXNs to regulate transmitter release (Figure 3).

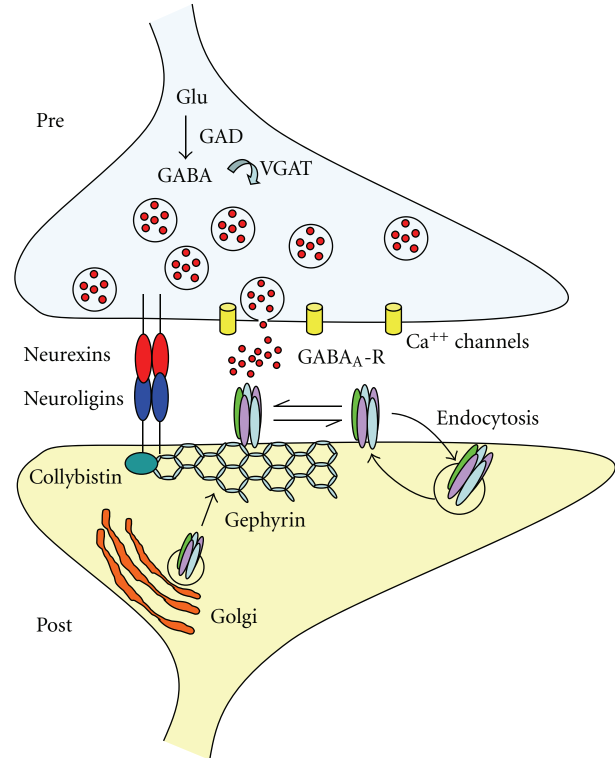


Figure 3: Structural organization of GABAergic synapses. The postsynaptic organization comprises a large number of proteins that allow the correct targeting, clustering and stabilization of $GABA_A$ receptors. Among them gephyrin forms hexagonal lattices that trap $GABA_A$ receptors in precise apposition to presynaptic release sites. Cell adhesion molecules of the neuroligin-neurexin families bridge the cleft and ensure transsynaptic signaling, essential for the maintenance of a proper E/I balance.

Therefore, gephyrin plays a key role not only in stabilizing $GABA_A$ receptors but also in regulating transsynaptic signaling and in maintaining an appropriate E/I balance. The NLG-NRXN complexes possess a potent "synaptogenic" or synapses organizing activities as demonstrated by their ability to induce presynaptic differentiation of contacting neuritis when expressed in heterologous nonneuronal cells. Postsynaptic NLGs promote the assembly of functional presynaptic specializations in axons while presynaptic NRXNs recruit postsynaptic scaffolding proteins and neurotransmitter receptors in dendrites *via* their interaction with NLGs [62]. By functionally coupling synaptic calcium channels with the release machinery, NRXNs are thought to play an essential role in calcium-triggered neurotransmitters release [63]. The NLGs family comprises five different genes (NLG1-NLG5 with various splice variants), which form homodimers through the extracellular domain. Among these, NLG2 is preferentially associated with GABAergic synapses, while NLG1 with glutamatergic synapses [64, 65]. The NRXN family includes α- and β-NRXN. Initially, β-NRXN was considered the main partner for NLG, but recently, also α-NRXN was found to bind NLG [66]. Unlike β-NRXN that participates in the formation of both glutamatergic and GABAergic synapses, α-NRXN seems to

be specific for GABAergic synapses [67]. Therefore, it is clear that within a neuronal network, the NLG-NRXN interaction controls the formation of both glutamatergic and GABAergic synapses [68]. At inhibitory synapses, $GABA_A$ receptors are firstly assembled in the endoplasmic reticulum from appropriate subunits and then delivered to the plasma membrane. Targeting and clustering $GABA_A$ receptors at synaptic and extrasynaptic sites is dynamically regulated by neuronal activity [69] and requires the precise interplay of various proteins and active transport processes along the cytoskeleton [60, 70].

Disrupting endogenous gephyrin with selective antibodies led to a reduction of $GABA_A$ receptor clusters [71], an effect that was associated with a decrease in the density and size of NLG2 clusters and with a loss of GABAergic innervation (Kasap, personal communication). Thus, pair recordings from interconnected cells demonstrated that, respect to controls, neurons transfected with recombinant antibodies against gephyrin exhibited a lower probability of GABA release. This reduction likely involves NLG2 which is preferentially concentrated at inhibitory synapses and directly binds gephyrin through a conserved cytoplasmatic domain [61]. Similarly, at glutamatergic synapses, the NLG-NRXN complex has been shown to act as a coordinator between postsynaptic and presynaptic sites [72]. Hence, overexpressing the glutamatergic scaffold protein PSD-95 on the postsynaptic site enhanced the probability of glutamate release *via* a retrograde modulation of neurotransmitter release which probably involves the NLG-NRXN complex. From the reported data, it is not surprising that single mutations in genes encoding for adhesion molecules belonging to the NLG-NRXN families, such as those found in few cases of ASDs [73], lead to defective architectural structuring of synaptic connections, molecular assembly of synapses and an E/I unbalance.

As outlined in the next section, the use of animal models of ASDs has enabled to investigate the mechanistic basis of the E/I imbalance for a range of neurodevelopmental disorders.

4. Altered GABAergic Signaling in Animal Models of ASDs

A dysfunction of GABAergic signaling mediates autism-like stereotypes in the majority of animal models of ASDs obtained by experimentally manipulating candidate genes for autism susceptibility or environmental risk factors. The characteristic ASDs phenotype is often associated with either a loss or a gain of the GABAergic function. Consistent with postmortem studies from brain tissues obtained from ASDs patients [74] alterations in GABA synthesising enzymes GAD65 and GAD67, in GABA release, in the expression of particular subtypes of $GABA_A$ receptors have been described.

A presynaptic reduction in glutamic acid decarboxylase 1 (*Gad1*) and glutamic acid decarboxylase 2 (*Gad2*) mRNA encoding for GAD67 and GAD65, respectively, has been recently found in mice lacking the *Mecp2* gene in GABA releasing neurons (*Viaat-Mecp2*$^{-/y}$, [75]). Mutations in the X-linked *Mecp2* gene, which encodes the transcriptional regulator methyl-CpG-binding protein 2 (MeCP2), cause the majority of Rett syndrome cases [76–78] which is characterized by an apparently normal early development followed by loss of language skill, motor abnormalities, cognitive deficits, stereotyped behavior, respiratory dysrhythmias, and seizures leading sometimes to premature death. *Viaat-Mecp2*$^{-/y}$ mice exhibit a significant reduction in amplitude (but not in frequency) of miniature inhibitory postsynaptic currents (mIPSCs) an effect which occurs in the absence of any alteration in amplitude or frequency of miniature excitatory postsynaptic currents (mEPSCs), indicating that MeCP2 deficiency in GABAergic neurons has a cell-autonomous impact on quantal release from glutamatergic neurons [75]. The reduction in GABA content and inhibitory neurotransmission affects synaptic plasticity processes as suggested by the impairment of long-term potentiation (LTP) induced by theta burst stimulation of Schaffer collateral [75]. Previous electrophysiological studies using *Mecp2* null mice, revealed a significant reduction in spontaneous firing associated with a decrease in amplitude of mEPSCs in layer 5 pyramidal neurons as compared to WT control animals at early presymptomatic and symptomatic stages [79]. In the hippocampus of *Mecp2* null mice, the diminished level of basal excitatory drive has been shown to contribute, at the network level, to slow down spontaneous rhythmic field potentials activity, generated by the interplay between excitation and inhibition [80]. This condition paradoxically makes the hippocampal network overresponsive to excitatory stimuli.

An imbalance between excitation and inhibition has been found also in individuals affected by Tuberous sclerosis, a genetic multisystem disorder characterized by widespread hamartomas in several organs, including the brain, heart, skin, eyes, kidney, lung, and liver [81]. Tuberous sclerosis patients exhibit a variety of neurological disorders including epilepsy and autism-like disorders. The affected genes are *Tsc1* and *Tsc2* encoding hamartin and tuberin, respectively. The hamartin-tuberin complex inhibits the mammalian-target-of-rapamycin pathway that controls cell growth and proliferation [81].

Interestingly, a loss of GABAergic function accounts for the hyper excitability observed in an animal model of fragile X syndrome (FXS), a common inherited cause of mental retardation with language deficits, hyperactivity, autistic behavior and seizures. FXS is caused by a trinucleotide expansion of fragile X mental retardation 1 (*fmr1*) gene which prevents the expression of the encoded protein called Fragile X mental retardation protein (FMRP, [82]). As the *Mecp2* gene, the *fmr1* gene is located in chromosome X (Xq27.3). The lack of FMRP in animal models of FXS (the *Fmr1*-null mouse) leads to an E/I imbalance in favor of excitation. Among the factors contributing to enhance cell excitability in *Fmr1* KO animals an impairment of GABAergic circuitry [83] and a decreased expression of $GABA_A$ receptor subunits have been reported [84–87]. In subicular neurons, for example, a down regulation of $GABA_A$-mediated tonic (but not phasic) inhibition associated with a reduced expression of $\alpha 5$ and δ $GABA_A$ receptors subunits

has been found [88]. These alterations may contribute to deficits in cognitive functions and to epileptic activity observed in FXS patients. In contrast, electrophysiological recordings from spiny neurons in the striatum, involved in motor control and in specific aspects of cognition and motivation, have revealed a selective increase in frequency of sIPSCs and mIPSCs, probably secondary to an enhanced probability of transmitter release from GABAergic terminals, suggesting that modifications in GABAergic function in *Fmr1* KO mice are region-specific [89].

Relevant inhibitory synaptic abnormalities (involving both phasic and tonic $GABA_A$-mediated inhibition), which may contribute to the abnormal social behavior of *Fmr1* null mice, are present in the basolateral nucleus of the amygdala [90], which regulates fear and anxiety behaviors.

Linkage and association studies have revealed that the chromosomal region 15q11-q13 is strongly implicated in ASDs [91]. Maternal duplications of this region remain one of the most common cytogenetic abnormalities found in cases of idiopathic ASDs, which account for 1-2% of cases. Deletion of this region results in either Angelman or Prader-Willi syndrome, depending from which parent the deletion has been inherited [92]. Interestingly, within this chromosomal region, exists a gene cluster of $GABA_A$ receptors, *G*abrb*3*, *G*abra*5*, and *G*abrg*3*, encoding for $\beta 3$, $\alpha 5$, and $\gamma 3$ subunits, respectively. $GABA_A$ receptors are hetero-oligomeric proteins spanning the membrane to form anion-permeable channels. Assembled from eight classes of subunits exhibiting different degrees of homology a large variety of functional receptors with different biophysical and pharmacological properties are expressed in mammalian brain. $GABA_A$ receptors play a crucial role in proliferation, migration, and differentiation of precursor cells thus contributing to the establishment of neuronal circuits [93]. A developmental deficit of $GABA_A$ receptors function would affect neurogenesis and maturation of neuronal network. Among different $GABA_A$ receptor genes, the targeted deletion of *Gabrb3* gene encoding for the $\beta 3$ subunit, which is highly expressed during brain development [94], leads to abnormalities in social behavior, cognitive deficits, motor stereotypes and seizures, reminiscent of the ASDs phenotype [56, 92, 95, 96].

Other mutations that affect the GABAergic system concern the homeobox genes *Dlx1* and *Dlx2*, involved in the development of most telencephalic GABAergic neurons [97]. Interestingly, the human locus with the highest LOD score for autism susceptibility (D2S2188 on chromosome 2q) maps very close to the gene encoding for the GABA synthesized enzyme GAD65 and to *Dlx1* and *Dlx2.* Furthermore, the autism susceptibility locus D7S477 on chromosome 7q maps within about six megabases of *Dlx5* and *Dlx6* which are implicated in the regulation of forebrain GABAergic neurons [98]. This region hosts the gene encoding for Reelin, a protein expressed in cortical GABAergic neurons [99]. Reelin is a signaling protein that plays a pivotal role in the migration of several neuronal cell types and in the development of neuronal connections [100, 101]. Reeler mice, devoid of Reelin, exhibit cytoarchitectonic alterations in their brain similar to those found in autistic patients [102] associated with decrease GABA turnover [103].

Interestingly, the removal of the homeobox containing transcription factors Engrailed-2 (*EN2*), known to be involved in the regionalization pattering and neuronal differentiation of the midbrain and hindbrain [104] in mice (*En2*−/−*mice)* leads to behavioral abnormalities similar to those observed in ASDs patients [105]. In addition, these mice exhibit a reduced expression of parvalbumin and somatostatin positive interneurons in the hippocampus, an effect associated with an increased susceptibility to seizures [105, Table 1].

While the majority of animal models so far examined exhibits a loss of GABAergic function, mice carrying the human R415C mutation in the *Nlgn3* gene display a gain of function. Neuroligins (NLGs) are specialized cell adhesion molecules that functionally couple the postsynaptic densities with the transmitter release machinery by forming transsynaptic complexes with their presynaptic-binding partners, neurexins [73]. NLG3 R451C KI mice bear a striking phenotype with mimics in many aspects that found in ASDs patients ([106] but see [107]). Functional characterization of these mice has revealed (in contrast with NLG3 KO mice) a loss of NLG3 in the forebrain associated with impaired social interactions and a 50% increase in the frequency of spontaneous inhibitory events with apparent no effects on excitatory synaptic transmission [106]. Interestingly, in NLG3 R451C KI mice, the gain of function is accompanied with a significant increase in the level of the vesicular transporter for GABA, VGAT, and gephyrin, a postsynaptic scaffolding protein, crucial for recruiting and maintaining neurotransmitter receptors in the right place and for ensuring a correct E/I balance. Whether the increased release of GABA selectively affects only a subset of GABAergic interneurons is still unclear. In addition, this animal model exhibits an asymmetric reduction of parvalbumin-positive basket cells across the two hemispheres [108]. However, immunocytochemical data from postmortem material obtained from ASDs patients have revealed an increased density of calbindin-, calretinin-, and parvalbumin-positive interneurons in the hippocampus [23], a condition that would alter neuronal signaling and synchronization leading to cognitive dysfunctions [109]. The enhanced GABAergic innervation may cause a compensatory downregulation of $GABA_A$ receptors. The reduction in benzodiazepine-binding sites on $GABA_A$ receptors observed in the hippocampus of autistic patients supports this hypothesis [110].

Among autism risk factors, prenatal or neonatal environmental challenges, including early exposure to valproic acid (VPA), a histone deacetylases inhibitor, are widely used as animal models of ASDs [111]. The VPA model has been developed on the basis of the observation that treatment of epilepsy or bipolar disorders in pregnant women (20–24 days after conception) with VPA leads to an increased incidence of ASDs in their children [112]. A unifying hypothesis where the core pathology of the autistic brain consists in hyper-functionality and excessive neuronal processing in local neuronal microcircuits in prefrontal, somatosensory cortex, and amygdala, leading to social and environmental withdrawal has been proposed [113, 114]. Interestingly, as the neuroligin-3 model, the VPA model of ASDs exhibits an

Table 1: Main alterations of GABAergic signaling present in different animal models of ASDs. For the Rett syndrome, different genotypes are expressed in brackets.

Mouse model	Alterations in GABAergic signaling	Ref.
Mecp 2-KO (Rett syndrome)	Reduced levels of GAD65 and GAD67 (*Viaat-Mecp2*$^{-/y}$)	[75]
	Reduced inhibitory quantal size in layer 2/3 pyramidal neurons of the somatosensory cortex	
	The E/I balance is shifted to favor inhibition over excitation in cortical networks (*Mecp2*$^{2lox/x}$, *Nestin-Cre*)	[79]
	Reduced frequency of IPSC-based spontaneous rhythmic field potentials in the hippocampus (*Mecp2*$^{tm1.1Bird}$)	[80]
Fmr 1-KO (X fragile)	Down regulation of GABAA-mediated tonic inhibition in the subiculum	[88]
	Reduced expression of $\alpha 5$ and δ GABAA receptor subunits in the subiculum	
	Increased frequency of sIPSCs and mIPSCs in the striatum	[89]
	Reduction in amplitude and frequency of sIPSCs and mIPSCs	[90]
	Reduced GABAA-mediated tonic inhibition	
	Reduced GABAergic innervation in the amygdala	[84–87]
	Reduced expression of GABAA receptor subunits	
Gabrb 3 KO	The E/I balance is shifted to favor excitation over inhibition in cortical networks (EEG recordings)	[56]
Dlx1/Dlx2 KO	Abnormal cell migration	
	Reduction in the number of GABAergic interneurons in the cortex, olfactory bulb and hippocampus	[97]
Reln-KO	Reduced level of GAD67	[103]
	Decreased GABA turnover	
En2-KO	Reduced expression of parvalbumin- and somatostatin-	
	positive GABAergic interneurons in the hippocampus	[115]
	Increased susceptibility to seizures	
Nlg3 R451C KI	Increased frequency of mIPSC	
	Increased level of VGAT and gephyrin	[106]
	Asymmetric reduction of PV positive basket cells across cortical hemispheres	[108]
valproic acid	The E/I balance is shifted to favor excitation over inhibition in the lateral amygdala (multi electrode arrays)	[114]
	Asymmetric reduction of PV positive basket cells across cortical hemispheres	[108]

asymmetric reduction of parvalbumin-positive cells across the two hemispheres [108]. The disruption of inhibitory circuits may delay critical periods in specific ASDs brain regions [59], thus perturbing γ-oscillations implicated in high cognitive functions.

5. Future Perspectives

Although much more work is required to understand the cellular and molecular mechanisms regulating the E/I balance at synapses, it is clear from the reviewed data that GABAergic signaling plays a key role in the construction of neuronal networks and that disruption of GABAergic circuits accounts for several neurodevelopmental disorders including ASDs. A significant progress has been made in characterizing genes involved in synapses formation and maintenance but their role in the organization of neuronal circuits is still limited. From a clinical perspective, a challenged task will be to identify, in animal models of ASDs, the cellular substrates of microcircuits implicated in different cognitive and behavioral deficits associated with ASDs. This can be accomplished by using new optogenetic tools that would allow to selectively activate or silence specific interneuronal populations and to study their functional consequences [116]. With this technique, GFP fusions of channelrhodopsin-related proteins and halorhodopsin, can be delivered into the brain *via* viral infection. In response to different wavelengths of light, label cells and axons can be either depolarized (in the case of channelrhodopsin, [117]) or hyperpolarized (in the case of halorhodopsin), thus allowing to switch on and off selective groups of genetically targeted interneurons and to study the neural basis of different behaviors [118]. This will allow better understanding the mechanistic bases of ASDs and to develop new selectively targeted therapeutic tools for most effective interventions.

Acknowledgments

This work was supported by a grant from Ministero Istruzione Universita' e Ricerca (MIUR) to E. Cherubini. The authors wish to thank the colleagues who contributed to some of the original work reported in this paper and all members of the laboratory for useful discussions.

References

[1] American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders (DMS-IV-TR)*, American Psychiatric Association, Washington, DC, USA, 2000.

[2] S. E. Levy, D. S. Mandell, and R. T. Schultz, "Autism," *The Lancet*, vol. 374, no. 9701, pp. 1627–1638, 2009.

[3] K. Garber, "Autism's cause may reside in abnormalities at the synapse," *Science*, vol. 317, no. 5835, pp. 190–191, 2007.

[4] D. H. Geschwind and P. Levitt, "Autism spectrum disorders: developmental disconnection syndromes," *Current Opinion in Neurobiology*, vol. 17, no. 1, pp. 103–111, 2007.

[5] B. S. Abrahams and D. H. Geschwind, "Advances in autism genetics: on the threshold of a new neurobiology," *Nature Reviews Genetics*, vol. 9, no. 5, pp. 341–355, 2008.

[6] P. Szatmari, A. D. Paterson, L. Zwaigenbaum et al., "Mapping autism risk loci using genetic linkage and chromosomal rearrangements," *Nature Genetics*, vol. 39, no. 3, pp. 319–328, 2007.

[7] H. G. Kim, S. Kishikawa, A. W. Higgins et al., "Disruption of neurexin 1 associated with autism spectrum disorder," *American Journal of Human Genetics*, vol. 82, no. 1, pp. 199–207, 2008.

[8] S. Jamain, H. Quach, C. Betancur et al., "Mutations of the X-linked genes encoding Nlgns NLGN3 and NLGN4 are associated with autism," *Nature Genetics*, vol. 34, pp. 27–29, 2003.

[9] F. Laumonnier, F. Bonnet-Brilhault, M. Gomot et al., "X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family," *American Journal of Human Genetics*, vol. 74, no. 3, pp. 552–557, 2004.

[10] J. Yan, G. Oliveira, A. Coutinho et al., "Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients," *Molecular Psychiatry*, vol. 10, no. 4, pp. 329–332, 2005.

[11] C. M. Durand, C. Betancur, T. M. Boeckers et al., "Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders," *Nature Genetics*, vol. 39, no. 1, pp. 25–27, 2007.

[12] G. A. Ascoli, L. Alonso-Nanclares, S. A. Anderson et al., "Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex," *Nature Reviews Neuroscience*, vol. 9, no. 7, pp. 557–568, 2008.

[13] Z. J. Huang, G. Di Cristo, and F. Ango, "Development of GABA innervation in the cerebral and cerebellar cortices," *Nature Reviews Neuroscience*, vol. 8, no. 9, pp. 673–686, 2007.

[14] R. Miles, K. Tóth, A. I. Gulyás, N. Hájos, and T. F. Freund, "Differences between somatic and dendritic inhibition in the hippocampus," *Neuron*, vol. 16, no. 4, pp. 815–823, 1996.

[15] P. Somogyi and T. Klausberger, "Defined types of cortical interneurone structure space and spike timing in the hippocampus," *Journal of Physiology*, vol. 562, no. 1, pp. 9–26, 2005.

[16] D. F. Owens and A. R. Kriegstein, "Is there more to GABA than synaptic inhibition?" *Nature Reviews Neuroscience*, vol. 3, no. 9, pp. 715–727, 2002.

[17] G. Nagappan and B. Lu, "Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications," *Trends in Neurosciences*, vol. 28, no. 9, pp. 464–471, 2005.

[18] G. Di Cristo, C. Wu, B. Chattopadhyaya et al., "Subcellular domain-restricted GABAergic innervation in primary visual cortex in the absence of sensory and thalamic inputs," *Nature Neuroscience*, vol. 7, no. 11, pp. 1184–1186, 2004.

[19] B. Chattopadhyaya, G. Di Cristo, H. Higashiyama et al., "Experience and activity-dependent maturation of perisomatic GABAergic innervation in primary visual cortex during a postnatal critical period," *Journal of Neuroscience*, vol. 24, no. 43, pp. 9598–9611, 2004.

[20] B. Chattopadhyaya, G. Di Cristo, C. Z. Wu et al., "GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex," *Neuron*, vol. 54, no. 6, pp. 889–903, 2007.

[21] J. L. R. Rubenstein and M. M. Merzenich, "Model of autism: increased ratio of excitation/inhibition in key neural systems," *Genes, Brain and Behavior*, vol. 2, no. 5, pp. 255–267, 2003.

[22] M. F. Casanova, D. P. Buxhoeveden, A. E. Switala, and E. Roy, "Minicolumnar pathology in autism," *Neurology*, vol. 58, no. 3, pp. 428–432, 2002.

[23] Y. A. Lawrence, T. L. Kemper, M. L. Bauman, and G. J. Blatt, "Parvalbumin-, calbindin-, and calretinin-immunoreactive hippocampal interneuron density in autism," *Acta Neurologica Scandinavica*, vol. 121, no. 2, pp. 99–108, 2010.

[24] S. H. Fatemi, A. R. Halt, J. M. Stary, R. Kanodia, S. C. Schulz, and G. R. Realmuto, "Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices," *Biological Psychiatry*, vol. 52, no. 8, pp. 805–810, 2002.

[25] J. Yip, J. J. Soghomonian, and G. J. Blatt, "Decreased GAD67 mRNA levels in cerebellar Purkinje cells in autism: pathophysiological implications," *Acta Neuropathologica*, vol. 113, no. 5, pp. 559–568, 2007.

[26] J. Yip, J. J. Soghomonian, and G. J. Blatt, "Increased GAD67 mRNA expression in cerebellar interneurons in autism: implications for Purkinje cell dysfunction," *Journal of Neuroscience Research*, vol. 86, no. 3, pp. 525–530, 2008.

[27] J. Yip, J. J. Soghomonian, and G. J. Blatt, "Decreased GAD65 mRNA levels in select subpopulations of neurons in the cerebellar dentate nuclei in autism: an in situ hybridization study," *Autism Research*, vol. 2, no. 1, pp. 50–59, 2009.

[28] E. H. Cook, R. Y. Courchesne, N. J. Cox et al., "Linkage-disequilibrium mapping of autistic disorder, with 15q11-13 markers," *American Journal of Human Genetics*, vol. 62, no. 5, pp. 1077–1083, 1998.

[29] Y. Shao, M. L. Cuccaro, E. R. Hauser et al., "Fine mapping of autistic disorder to chromosome 15q11-q13 by use of phenotypic subtypes," *American Journal of Human Genetics*, vol. 72, no. 3, pp. 539–548, 2003.

[30] A. L. Oblak, T. T. Gibbs, and G. J. Blatt, "Decreased GABA-B receptors in the cingulated cortex and fusiform gyrus in Autism," *Journal of Neurochemistry*, vol. 114, pp. 1414–1423, 2010.

[31] A. Brooks-Kayal, "Epilepsy and autism spectrum disorders: are there common developmental mechanisms?" *Brain and Development*, vol. 32, pp. 731–738, 2010.

[32] C. Allene and R. Cossart, "Early NMDA receptor-driven waves of activity in the developing neocortex: physiological or pathological network oscillations?" *Journal of Physiology*, vol. 588, no. 1, pp. 83–91, 2010.

[33] H. Komuro and P. Rakic, "Intracellular Ca2+ fluctuations modulate the rate of neuronal migration," *Neuron*, vol. 17, no. 2, pp. 275–285, 1996.

[34] R. Tyzio, R. Cossart, I. Khalilov et al., "Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery," *Science*, vol. 314, pp. 1788–1792, 2006.

[35] H. Arumugam, X. Liu, P. J. Colombo, R. A. Corriveau, and A. B. Belousov, "NMDA receptors regulate developmental gap junction uncoupling via CREB signaling," *Nature Neuroscience*, vol. 8, no. 12, pp. 1720–1726, 2005.

[36] Y. Ben-Ari, E. Cherubini, R. Corradetti, and J. L. Gaiarsa, "Giant synaptic potentials in immature rat CA3 hippocampal neurones," *Journal of Physiology*, vol. 416, pp. 303–325, 1989.

[37] O. Garaschuk, J. Linn, J. Eilers, and A. Konnerth, "Large-scale oscillatory calcium waves in the immature cortex," *Nature Neuroscience*, vol. 3, no. 5, pp. 452–459, 2000.

[38] J. W. Yang, I. L. Hanganu-Opatz, J. J. Sun, and H. J. Luhmann, "Three patterns of oscillatory activity differentially synchronize developing neocortical networks in vivo," *Journal of Neuroscience*, vol. 29, no. 28, pp. 9011–9025, 2009.

[39] J. Stockard-Pope, S. S. Werner, and R. G. Bickford, *Atlas of Neonatal Electroencephalography*, Raven Press, New York, NY, USA, 2nd edition, 1992.

[40] G. M. Durand, Y. Kovalchuk, and A. Konnerth, "Long-term potentiation and functional synapse induction in developing hippocampus," *Nature*, vol. 381, no. 6577, pp. 71–75, 1996.

[41] S. S. Kumar and J. R. Huguenard, "Pathway-specific differences in subunit composition of synaptic NMDA receptors on pyramidal neurons in neocortex," *Journal of Neuroscience*, vol. 23, no. 31, pp. 10074–10083, 2003.

[42] Y. Ben-Ari, E. Cherubini, and K. Krnjevic, "Changes in voltage dependence of NMDA currents during development," *Neuroscience Letters*, vol. 94, no. 1-2, pp. 88–92, 1988.

[43] E. Cherubini, J. L. Gaiarsa, and Y. Ben-Ari, "GABA: an excitatory transmitter in early postnatal life," *Trends in Neurosciences*, vol. 14, no. 12, pp. 515–519, 1991.

[44] M. Demarque, A. Represa, H. E. Becq, I. Khalilov, Y. Ben-Ari, and L. Aniksztejn, "Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation," *Neuron*, vol. 36, no. 6, pp. 1051–1061, 2002.

[45] P. Bonifazi, M. Goldin, M. A. Picardo et al., "GABAergic hub neurons orchestrate synchrony in developing hippocampal networks," *Science*, vol. 326, no. 5958, pp. 1419–1424, 2009.

[46] P. Blaesse, M. S. Airaksinen, C. Rivera, and K. Kaila, "Cation-chloride cotransporters and neuronal function," *Neuron*, vol. 61, no. 6, pp. 820–838, 2009.

[47] L. Cancedda, H. Fiumelli, K. Chen, and MU. M. Poo, "Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo," *Journal of Neuroscience*, vol. 27, no. 19, pp. 5224–5235, 2007.

[48] D. D. Wang and A. R. Kriegstein, "GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation," *Journal of Neuroscience*, vol. 28, no. 21, pp. 5547–5558, 2008.

[49] A. M. Kasyanov, V. F. Safiulina, L. L. Voronin, and E. Cherubini, "GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 11, pp. 3967–3972, 2004.

[50] M. H. Mohajerani, S. Sivakumaran, P. Zacchi, P. Aguilera, and E. Cherubini, "Correlated network activity enhances synaptic efficacy via BDNF and the ERK pathway at immature CA3-CA1 connections in the hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 32, pp. 13176–13181, 2007.

[51] L. L. Voronin and E. Cherubini, "'Deaf, mute and whispering' silent synapses: their role in synaptic plasticity," *Journal of Physiology*, vol. 557, no. 1, pp. 3–12, 2004.

[52] G. G. Turrigiano and S. B. Nelson, "Homeostatic plasticity in the developing nervous system," *Nature Reviews Neuroscience*, vol. 5, no. 2, pp. 97–107, 2004.

[53] M. B. Ramocki and H. Y. Zoghbi, "Failure of neuronal homeostasis results in common neuropsychiatric phenotypes," *Nature*, vol. 455, no. 7215, pp. 912–918, 2008.

[54] Y. Ben-Ari, J. L. Gaiarsa, R. Tyzio, and R. Khazipov, "GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations," *Physiological Reviews*, vol. 87, no. 4, pp. 1215–1284, 2007.

[55] J. L. Noebels, "The biology of epilepsy genes," *Annual Review of Neuroscience*, vol. 26, pp. 599–625, 2003.

[56] T. M. DeLorey, A. Handforth, S. G. Anagnostaras et al., "Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome," *Journal of Neurosciences*, vol. 18, pp. 8505–8514, 1998.

[57] J. Burrone and V. N. Murthy, "Synaptic gain control and homeostasis," *Current Opinion in Neurobiology*, vol. 13, no. 5, pp. 560–567, 2003.

[58] R. Tyzio, A. Represa, I. Jorquera, Y. Ben-Ari, H. Gozlan, and L. Aniksztejn, "The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite," *Journal of Neuroscience*, vol. 19, no. 23, pp. 10372–10382, 1999.

[59] T. K. Hensch, "Critical period plasticity in local cortical circuits," *Nature Reviews Neuroscience*, vol. 6, no. 11, pp. 877–888, 2005.

[60] M. Kneussel and S. Loebrich, "Trafficking and synaptic anchoring of ionotropic inhibitory neurotransmitter receptors," *Biology of the Cell*, vol. 99, no. 6, pp. 297–309, 2007.

[61] A. Poulopoulos, G. Aramuni, G. Meyer et al., "Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin," *Neuron*, vol. 63, no. 5, pp. 628–642, 2009.

[62] Z. J. Huang and P. Scheiffele, "GABA and neuroligin signaling: linking synaptic activity and adhesion in inhibitory synapse development," *Current Opinion in Neurobiology*, vol. 18, no. 1, pp. 77–83, 2008.

[63] M. Missier, W. Zhang, A. Rohlmann et al., "α-neurexins couple Ca2+ channels to synaptic vesicle exocytosis," *Nature*, vol. 423, no. 6943, pp. 939–948, 2003.

[64] J. Y. Song, K. Ichtchenko, T. C. Südhof, and N. Brose, "Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 1100–1105, 1999.

[65] F. Varoqueaux, S. Jamain, and N. Brose, "Neuroligin 2 is exclusively localized to inhibitory synapses," *European Journal of Cell Biology*, vol. 83, no. 9, pp. 449–456, 2004.

[66] A. A. Boucard, A. A. Chubykin, D. Comoletti, P. Taylor, and T. C. Südhof, "A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to α- and β-neurexins," *Neuron*, vol. 48, no. 2, pp. 229–236, 2005.

[67] Y. Kang, X. Zhang, F. Dobie, H. Wu, and A. M. Craig, "Induction of GABAergic postsynaptic differentiation by α-neurexins," *Journal of Biological Chemistry*, vol. 283, no. 4, pp. 2323–2334, 2008.

[68] A. M. Craig and Y. Kang, "Neurexin-neuroligin signaling in synapse development," *Current Opinion in Neurobiology*, vol. 17, no. 1, pp. 43–52, 2007.

[69] C. Hanus, C. Vannier, and A. Triller, "Intracellular association of glycine receptor with gephyrin increases its plasma membrane accumulation rate," *Journal of Neuroscience*, vol. 24, no. 5, pp. 1119–1128, 2004.

[70] V. Tretter, T. C. Jacob, J. Mukherjee, J. M. Fritschy, M. N. Pangalos, and S. J. Moss, "The clustering of GABA receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor $\alpha 2$ subunits to gephyrin," *Journal of Neuroscience*, vol. 28, no. 6, pp. 1356–1365, 2008.

[71] I. Marchionni, Z. Kasap, J. W. Mozrzymas, W. Sieghart, E. Cherubini, and P. Zacchi, "New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture," *Neuroscience*, vol. 164, no. 2, pp. 552–562, 2009.

[72] K. Futai, M. J. Kim, T. Hashikawa, P. Scheiffele, M. Sheng, and Y. Hayashi, "Retrograde modulation of presynaptic release probability through signaling mediated by PSD-95-neuroligin," *Nature Neuroscience*, vol. 10, no. 2, pp. 186–195, 2007.

[73] T. C. Südhof, "Neuroligins and neurexins link synaptic function to cognitive disease," *Nature*, vol. 455, no. 7215, pp. 903–911, 2008.

[74] I. A. J. van Kooten, P. R. Hof, H. van Engeland, H. W. M. Steinbusch, P. H. Patterson, and C. Schmitz, "Autism: neuropathology, alterations of the GABAergic system, and animal models," *International Review of Neurobiology*, vol. 71, pp. 1–26, 2005.

[75] H. T. Chao, H. Chen, R. C. Samaco et al., "Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes," *Nature*, vol. 468, pp. 263–269, 2010.

[76] J. Guy, B. Hendrich, M. Holmes, J. E. Martin, and A. Bird, "A mouse Mecp2-null mutation causes neurological symptoms that mimic rett syndrome," *Nature Genetics*, vol. 27, no. 3, pp. 322–326, 2001.

[77] R. Z. Chen, S. Akbarian, M. Tudor, and R. Jaenisch, "Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice," *Nature Genetics*, vol. 27, no. 3, pp. 327–331, 2001.

[78] M. D. Shahbazian, J. I. Young, L. A. Yuva-Paylor et al., "Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3," *Neuron*, vol. 35, no. 2, pp. 243–254, 2002.

[79] V. S. Dani, Q. Chang, A. Maffei, G. G. Turrigiano, R. Jaenisch, and S. B. Nelson, "Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett Syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 35, pp. 12560–12565, 2005.

[80] L. Zhang, J. He, D. G. M. Jugloff, and J. H. Eubanks, "The MeCP2-null mouse hippocampus displays altered basal inhibitory rhythms and is prone to hyperexcitability," *Hippocampus*, vol. 18, no. 3, pp. 294–309, 2008.

[81] P. Curatolo, R. Bombardieri, and S. Jozwiak, "Tuberous sclerosis," *The Lancet*, vol. 372, no. 9639, pp. 657–668, 2008.

[82] B. A. Oostra and P. Chiurazzi, "The fragile X gene and its function," *Clinical Genetics*, vol. 60, no. 6, pp. 399–408, 2001.

[83] L. Selby, C. Zhang, and Q. Q. Sun, "Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein," *Neuroscience Letters*, vol. 412, no. 3, pp. 227–232, 2007.

[84] A. El Idrissi, X. H. Ding, J. Scalia, E. Trenkner, W. T. Brown, and C. Dobkin, "Decreased GABA receptor expression in the seizure-prone fragile X mouse," *Neuroscience Letters*, vol. 377, no. 3, pp. 141–146, 2005.

[85] C. D'Hulst, N. De Geest, S. P. Reeve et al., "Decreased expression of the GABA receptor in fragile X syndrome," *Brain Research*, vol. 1121, no. 1, pp. 238–245, 2006.

[86] I. Gantois, J. Vandesompele, F. Speleman et al., "Expression profiling suggests underexpression of the GABA receptor subunit δ in the fragile X knockout mouse model," *Neurobiology of Disease*, vol. 21, no. 2, pp. 346–357, 2006.

[87] D. C. Adusei, L. K. K. Pacey, D. Chen, and D. R. Hampson, "Early developmental alterations in GABAergic protein expression in fragile X knockout mice," *Neuropharmacology*, vol. 59, no. 3, pp. 167–171, 2010.

[88] G. Curia, T. Papouin, P. Séguéla, and M. Avoli, "Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome," *Cerebral Cortex*, vol. 19, no. 7, pp. 1515–1520, 2009.

[89] D. Centonze, S. Rossi, V. Mercaldo et al., "Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome," *Biological Psychiatry*, vol. 63, no. 10, pp. 963–973, 2008.

[90] J. L. Olmos-Serrano, S. M. Paluszkiewicz, B. S. Martin, W. E. Kaufmann, J. G. Corbin, and M. M. Huntsman, "Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome," *The Journal of Neuroscience*, vol. 30, pp. 9929–9938, 2010.

[91] Y. Shao, M. L. Cuccaro, E. R. Hauser et al., "Fine mapping of autistic disorder to chromosome 15q11-q13 by use of phenotypic subtypes," *American Journal of Human Genetics*, vol. 72, no. 3, pp. 539–548, 2003.

[92] T. M. DeLorey, P. Sahbaie, E. Hashemi, G. E. Homanics, and J. D. Clark, "Gabrb3 gene deficient mice exhibit impaired social and exploratory behaviors, deficits in non-selective attention and hypoplasia of cerebellar vermal lobules: a potential model of autism spectrum disorder," *Behavioural Brain Research*, vol. 187, no. 2, pp. 207–220, 2008.

[93] J. L. Barker, T. Behar, Y. X. Li et al., "GABAergic cells and signals in CNS development," *Perspectives on Developmental Neurobiology*, vol. 5, no. 2-3, pp. 305–322, 1998.

[94] D. J. Laurie, W. Wisden, and P. H. Seeburg, "The distribution of thirteen GABA(A) receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development," *Journal of Neuroscience*, vol. 12, no. 11, pp. 4151–4172, 1992.

[95] G. E. Homanics, T. M. DeLorey, L. L. Firestone et al., "Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior," *Proceedings of the National Academy of Sciences of USA*, vol. 94, pp. 4143–4148, 1997.

[96] T. M. DeLorey, "GABRB3 gene deficient ice: a potential model of autism spectrum disorder," *International Review of Neurobiology*, vol. 71, pp. 359–382, 2005.

[97] O. Marín and J. L. R. Rubenstein, "A long, remarkable journey: tangential migration in the telencephalon," *Nature Reviews Neuroscience*, vol. 2, no. 11, pp. 780–790, 2001.

[98] T. Stühmer, S. A. Anderson, M. Ekker, and J. L. R. Rubenstein, "Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression," *Development*, vol. 129, no. 1, pp. 245–252, 2002.

[99] S. Alcántara, M. Ruiz, G. D'Arcangelo et al., "Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse," *Journal of Neuroscience*, vol. 18, no. 19, pp. 7779–7799, 1998.

[100] A. M. Goffinet, "Events governing organization of post migratory neurons: studies on brain development in normal and reeler mice," *Brain Research*, vol. 319, pp. 261–296, 1984.

[101] J. A. Del Río, B. Heimrich, V. Borrell et al., "A role for Cajal-retzius cells and reelin in the development of hippocampal connections," *Nature*, vol. 385, no. 6611, pp. 70–74, 1997.

[102] A. Bailey, P. Luthert, A. Dean et al., "A clinicopathological study of autism," *Brain*, vol. 121, no. 5, pp. 889–905, 1998.

[103] G. Carboni, P. Tueting, L. Tremolizzo et al., "Enhanced dizocilpine efficacy in heterozygous reeler mice relates to GABA turnover downregulation," *Neuropharmacology*, vol. 46, no. 8, pp. 1070–1081, 2004.

[104] K. Herrup, C. Murcia, F. Gulden, B. Kuemerle, and N. Bilovocky, "The genetics of early cerebellar development: networks not pathways," *Progress in Brain Research*, vol. 148, pp. 21–27, 2005.

[105] M. A. Cheh, J. H. Millonig, L. M. Roselli et al., "En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder," *Brain Research*, vol. 1116, no. 1, pp. 166–176, 2006.

[106] K. Tabuchi, J. Blundell, M. R. Etherton et al., "A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice," *Science*, vol. 318, no. 5847, pp. 71–76, 2007.

[107] K. K. Chadman, S. Gong, M. L. Scattoni et al., "Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice," *Autism Research*, vol. 1, no. 3, pp. 147–158, 2008.

[108] N. Gogolla, J. J. Leblanc, K. B. Quast, T. Südhof, M. Fagiolini, and T. K. Hensch, "Common circuit defect of excitatory-inhibitory balance in mouse models of autism," *Journal Neurodevelopmental Disorders*, vol. 1, pp. 172–181, 2009.

[109] R. C. Gutierrez, J. Hung, Y. Zhang, A. C. Kertesz, F. J. Espina, and M. A. Colicos, "Altered synchrony and connectivity in neuronal networks expressing an autism-related mutation of neuroligin 3," *Neuroscience*, vol. 162, no. 1, pp. 208–221, 2009.

[110] J. T. Guptill, A. B. Booker, T. T. Gibbs, T. L. Kemper, M. L. Bauman, and G. J. Blatt, "[3H]-flunitrazepam-labeled benzodiazepine binding sites in the hippocampal formation in autism: a multiple concentration autoradiographic study," *Journal of Autism and Developmental Disorders*, vol. 37, no. 5, pp. 911–920, 2007.

[111] G. C. Wagner, K. R. Reuhl, M. Cheh, P. McRae, and A. K. Halladay, "A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate," *Journal of Autism and Developmental Disorders*, vol. 36, no. 6, pp. 779–793, 2006.

[112] P. Gail Williams and J. H. Hersh, "A male with fetal valproate syndrome and autism," *Developmental Medicine and Child Neurology*, vol. 39, no. 9, pp. 632–634, 1997.

[113] H. Markram, T. Rinaldi, and K. Markram, "The intense world syndrome-an alternative hypothesis for autism," *Frontiers in Neuroscience*, vol. 1, pp. 77–96, 2007.

[114] K. Markram, T. Rinaldi, D. L. Mendola, C. Sandi, and H. Markram, "Abnormal fear conditioning and amygdala processing in an animal model of autism," *Neuropsychopharmacology*, vol. 33, no. 4, pp. 901–912, 2008.

[115] P. P. Tripathi, P. Sgadò, M. Scali et al., "Increased susceptibility to kainic acid-induced seizures in Engrailed-2 knockout mice," *Neuroscience*, vol. 159, no. 2, pp. 842–849, 2009.

[116] F. Zhang, L. P. Wang, M. Brauner et al., "Multimodal fast optical interrogation of neural circuitry," *Nature*, vol. 446, no. 7136, pp. 633–639, 2007.

[117] G. Nagel, T. Szellas, W. Huhn et al., "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 13940–13945, 2003.

[118] L. Petreanu, D. Huber, A. Sobczyk, and K. Svoboda, "Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections," *Nature Neuroscience*, vol. 10, no. 5, pp. 663–668, 2007.

Conclusions and future perspectives

To be highly efficient, synaptic transmission requires the presence of clustered postsynaptic receptors localized in precise apposition to presynaptic release sites. The molecular mechanisms which accomplish this level of organization along with the functional role of different synaptic proteins are not fully understood.

At inhibitory synapses, the interplay of different proteins including gephyrin, collybistin and neuroligin 2 and 3 seems to have a major role in recruiting and stabilizing receptors at GABAergic synapses. In the first study, I have demonstrated that the scaffold protein gephyrin not only stabilizes $GABA_A$ receptors at subsynaptic membranes but *via* a transynaptic action involving the neuroligin-neurexin complex regulates GABA release. This could be of particular relevance as it has been proposed that the interaction between scaffold and adhesion molecules of the neuroligin-neurexin families contributes to set the balance between excitation and inhibition in local networks. An altered E/I balance may account for several behavioral and cognitive deficits observed in patients affected by neuropsychiatric disorders, including ASDs. To date the etiology of ASDs remains largely unknown, however, recent genetic findings together with emerging anatomical and functional imaging studies suggest a potential unifying model in which the unbalance between excitation and inhibition may be responsible for high order association areas disconnection in the brain.

Taking advantage of the mouse model of autism carrying the human R451C mutation of the *Nlgn3* gene, found in two families of autistic patients I have observed an increased GABAergic but not glutamatergic signaling in the hippocampus already at early stages of postnatal development. The early alteration in the excitatory/inhibitory balance, may account for behavioral deficits observed in children with ASDs. Although my experiments have revealed postsynaptic $GABA_A$ receptors modifications associated with alterations in transmitter release, they have not addressed the mechanisms responsible for the observed phenotype, including the type of interneurons involved and networks function. To gain more details about the mechanisms responsible for the observed effects, we are planning to investigate:

1. The mechanisms underlying the gain-of-function phenotype;
2. The role of a subset of GABAergic interneurons in regulating the activity of local networks.

The mechanisms underlies the gain-of-function phenotype

In vitro experiments have demonstrated that the $NL3^{R451C}$ mutant shows defective trafficking with retention of up to 90 % of the NL3 protein in the

endoplasmic reticulum with only a 10 % delivered to the synapses (Tabuchi et al. 2007). At GABAergic and glutamatergic synapses, scaffold molecules such as gephyrin and PSD-95, certainly contribute to proper receptors targeting. However, their contribution to the localization of NL3 at GABAergic and glutamatergic synapses is still unknown. Co-immunoprecipitation experiments following ectopic expression of gephyrin, PSD-95 with a truncated versions of NL3 (NL3 lacking the PDZ-binding domain or NL3 lacking the gephyrin binding motif etc.) in HEK293 cells could shed light on the mechanisms of protein-protein interaction and cellular localization of NL3. The interaction of NL3 with collybistin will be also tested. Quantitative western blot and immunofluorescence approaches (both on brain tissues and on cultured hippocampal neurons) would allow to assess whether the truncated mutants and the $NL3^{R451C}$ mutation alters the expression/distribution of inhibitory proteins like gephyrin, NL2, collybistin and $GABA_A$ receptor subunits. A redistribution of NLs at inhibitory synapses may explain the gain of function found in the present experiments.
In addition, over-expression of NL3 mutants in hippocampal cell cultures obtained from NL3 KO mice may provide functional insights into the mechanisms of NL3 function.

The role of a subset of GABAergic interneurons in regulating the activity of local networks

From a clinical point of view, it would be extremely important to identify the dynamics of GABAergic microcircuits implicated in network abnormalities responsible for cognitive and behavioral deficits associated with ASDs. Post-mortem analysis of brain tissue from autistic patients, have revealed an increased expression of calbindin-, calretinin- and parvalbumin-positive GABAergic interneurons (Lawrence et al., 2010). In particular, several lines of evidences suggest a major role of parvalbumin positive (PV^+) interneurons in neuropsychiatric disorders including schizophrenia and ASDs. These interneurons not only contribute to synchronize neuronal ensembles but exert a powerful control on spike timing and synaptic integration. To unveil possible dysfunctions in local inhibitory circuits involving PV^+ interneurons, we plan to cross $NL3^{R451C}$ knock-in mice with GAD67-GFP mice (selectively expressing the EGFP in PV^+ interneurons). Pair recordings will be performed from interconnected PV^+ or PV^- basket cells and pyramidal cells in the CA1 hippocampal region. PV^+ and PV^- interneurons will be characterized on the basis of their different firing patters, membrane time constants, input resistances and on their ability to exhibit Depolarization-induced Suppression of Inhibition (DSI) upon membrane depolarization of their pyramidal target neuron. In contrast to PV^+, PV^- interneurons exhibit CB1-sensitive DSI (Glickfeld and Scanziani, 2006). The relative amount of excitation received by the PV^+ and PV^- basket cells will be

measured in control and upon repetitive stimulation of Schaffer collateral (in controls EPSCs evoked in PV^- cells respond poorly to the repetitive activation of a single pathway as compared to PV^+ cells). Changes in short-term plasticity may underline differences in the recruitment of the two types of interneurons during ongoing hippocampal activity. Furthermore, the magnitude of feed-back and feed-forward inhibition and their ability to integrate and summate consecutive inputs will be compared in the same way as in Glickfeld and Scanziani, (2006). In particular changes in feedback inhibition could be of interest for their importance in gamma oscillations (Wang, 2010).

EEG gamma (γ) oscillations have received particular attention because of their putative role in higher brain function (Bartos et al., 2007). In autistic patients, alterations of high cognitive functions including sensory integration, have been found to be associated with modifications of gamma-band oscillations in the electroencephalogram (Orekhova et al., 2012; Stroganova et al., 2012). Similarly, animal models of ASD show a decreased synchrony in the gamma frequency range (Gibson et al., 2008; Gandal et al., 2010). γ oscillations which are strongly dependent on excitatory/ inhibitory (E/I) balance (Wang, 2010) prevail in the hippocampus and in the somato-sensory cortex. Principal cells in the somato-sensory cortex receive excitatory inputs directly from the thalamus, but the same thalamic fibers innervate also fast spiking, PV^+ basket cells, which mediate the feed-forward inhibition. Thus, principal cells receive excitatory inputs directly from the thalamus and inhibitory ones, from PV^+ interneurons, that are excited by the same thalamic fibers (Agmon & Connors, 1991). Therefore, we plan to investigate whether also in our model, an altered E/I balance at the level of the local circuit involving layer IV spiny neurons may account for oscillatory dysfunction in the gamma frequency range.

Finally, in cultured hippocampal neurons from mice obtained by crossing $NL3^{R451C}$ knock-in with GAD67-GFP we will studied whether GABAergic innervation is segregated along the somato-dendritic compartment of a given cell. Segregation associated with a selective loss of perisomatic but not dendritic GABAergic synapses has been demonstrated in NL-2 KO mice, a condition that alters the E/I balance (Poulopoulos et al, 2009). To this aim, individual glutamatergic and GABAergic currents will be evoked in principal cells by directly un-caging glutamate and GABA, onto individual glutamatergic and GABAergic synapses. In this way the dendritic filtering will similarly affect both the GABAergic and the glutamatergic components. Synapses will be simultaneously visualized with VGLUT and VGAT, respectively, coupled to different fluorescent dyes. Individual neurons will be visualized by fluorescent dye injection through the recording pipette. Amplitudes and kinetics of glutamatergic and GABAergic currents recorded from different regions will be compared with those obtained from KO or WT animals.

References

Abbott, L.F., Regehr, W.G., Synaptic computation. *Nature* 2004; 431:796–803

Acsády, L., Kamondi, A., Sík, A., Freund, T., Buzsáki, G., GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *J Neurosci*. 1998; 18:3386-403

Agmon, A., Connors, B.W., Thalamocortical responses of mouse somatosensory barrel cortex in vitro. *Neuroscience.* 1991; 41:365-79.

Ali, A.B., Bannister, A.P., Thomson, A.M., IPSPs elicited in CA1 pyramidal cells by putative basket cells in slices of adult rat hippocampus. *Eur J Neurosci*. 1999; 11:1741-53

Alldred, M.J., Mulder-Rosi, J., Lingenfelter, S.E., Chen, G., Lüscher, B. Distinct gamma2 subunit domains mediate clustering and synaptic function of postsynaptic $GABA_A$ receptors and gephyrin. *J Neurosci*. 2005; 25:594-603.

Alle, H., Geiger, J.R., GABAergic spill-over transmission onto hippocampal mossy fiber boutons. *J Neurosci*. 2007; 27:942-50

Amaral, D.G., Witter, M.P., The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience* 1989; 31:571-91

Andjus, P. R., Stevic-Marinkovic, Z., Cherubini, E., Immunoglobulins from motoneurone disease patients enhance glutamate release from rat hippocampal neurones in culture. *J. Physiol*. 1997;504: 103–112.

American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders (DMS-IV-TR), American Psychiatric Association, Washington, DC, USA, 2000.

Astman, N., Gutnick, M.J., Fleidervish, I.A., Persistent sodium current in layer 5 neocortical neurons is primarily generated in the proximal axon. *J. Neurosci.* 2006; 26:3465–3473.

Atluri, P.P., Regehr, W.G., Delayed release of neurotransmitter from cerebellar granule cells. *J. Neurosci.* 1998; 18:8214-27.

Augustine, G.J., Charlton, M.P., Smith, S.J., Calcium action in synaptic transmitter release *Ann Rev Neurosci.* 1987; 10: 633–693

Barberis, A., Petrini, E.M., Mozrzymas, J.W., Impact of synaptic neurotransmitter concentration time course on the kinetics and pharmacological modulation of inhibitory synaptic currents. *Front Cell Neurosci.* 2011;5:6. Epub 2011 Jun 22

Barrett, E.F., Stevens, C.F., The kinetics of transmitter release at the frog neuromuscular junction. *J Physiol*. 1972; 227:691-70

Bartos, M., Vida, I., Jonas, P., Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. *Nat Rev Neurosci.* 2007; 8:45-56.

Bats, C., Groc, L., Choquet, D., The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 2007; 53:719-34.

Baude, A., Nusser, Z., Roberts, J.D., Mulvihill, E., McIlhinney, R.A., Somogyi, P., The metabotropic glutamate receptor (mGluR1α) is concentrated at peri-synaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* 1993; 11:771-87.

Baudouin, S.J., Gaudias, J., Gerharz, S., Hatstatt, L., Zhou, K., Punnakkal, P., Tanaka K.F., Spooren, W., Hen, R., De Zeeuw, C.I., Vogt, K., Scheiffele, P., Shared Synaptic Pathophysiology in Syndromic and Nonsyndromic Rodent Models of Autism. *Science* 2012 Sep 13. [Epub ahead of print]

Bausen, M., Fuhrmann, J.C., Betz, H., O'sullivan, G.A., The state of the actin cytoskeleton determines its association with gephyrin: role of Mena/VASP family members. *Mol Cell Neurosci.* 2006; 31:376-86.

Bear, M.F., Malenka, R.C., Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* 1994; 4:389-99.

Beato, M., The time course of transmitter at glycinergic synapses onto motoneurons. *J Neurosci.* 2008; 28:7412-25.

Béïque, J.C., Lin, D.T., Kang, M.G., Aizawa, H., Takamiya, K., Huganir, R.L., Synapse-specific regulation of AMPA receptor function by PSD-95. *Proc Natl Acad Sci USA.* 2006; 103:19535-40.

Bender, K.J., Trussell, L.O., Axon initial segment Ca^{2+} channels influence action potential generation and timing. *Neuron* 2009; 61:259-71.

Benson, D.L., Watkins, F.H., Steward, O., Banker, G., Characterization of GABAergic neurons in hippocampal cell cultures *J. Neurocytol.* 1994; 23:279–295.

Bhattacharyya, S., Biou, V., Xu, W., Schlüter, O., Malenka, R.C., A critical role for PSD-95/AKAP interactions in endocytosis of synaptic AMPA receptors. *Nat Neurosci.* 2009; 12:172-81.

Blatow, M., Caputi, A., Burnashev, N., Monyer, H., Rozov, A. Calcium buffer saturation underlies paired pulse facilitation in calbindin-D28k-containing terminals. *Neuron* 2003; 38:79–88.

Boehning, D., Snyder, S.H., Novel neural modulators. *Annu Rev Neurosci.* 2003; 26:105–131.

Borst, J.G.,Sakmann, B., Calcium influx and transmitter release in a fast CNS synapse. *Nature* 1996; 383:431-4.

Bourne, J.N., Harris, K.M., Balancing structure and function at hippocampal dendritic spines. *Annu Rev Neurosci.* 2008; 31:47-67

Brickley, S.G., Cull-Candy, S.G., Farrant, M., Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. *J Physiol*. 1996; 497:753-9.

Brickley, S.G., Mody, I., Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* 2012; 73:23-34.

Brose, N., Synaptogenic proteins and synaptic organizers: "many hands make light work". *Neuron* 2009; 61:650-2

Brünig, I., Scotti, E., Sidler, C., Fritschy, J.M., Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol.* 2002; 443:43-55.

Buhl, E., Whittington, M., Local circuits. Anderson. P., Morris, R., Amaral, D., Bliss, T., O'Keefe, J., editors.The hippocampus book. New York: Oxford University Press; pp.297-319.

Burgess, N., Becker, S., King, J.A., O'Keefe, J., Memory for events and their spatial context: models and experiments. *Philos Trans R Soc Lond Biol Sci.* 2001; 356:1493-503.

Buzsáki, G. & Draguhn, A., Neuronal oscillations in cortical networks. *Science* 2004; 304:1926–1929.

Buzsáki, G., Feed-forward inhibition in the hippocampal formation. *Prog Neurobiol.* 1984; 22:131-53.

Buzsáki, G., Kandel, A., Somadendritic backpropagation of action potentials in cortical pyramidal cells of the awake rat. *J Neurophysiol.* 1998; 79:1587-91.

Caraiscos, V.B., Elliott, E.M.,You-Ten, K.E., Cheng, V.Y., Belelli, D., Newell, J.G., Jackson, M.F., Lambert, J.J., Rosahl, T.W., Wafford, K.A., MacDonald, J.F., Orser, B.A., Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A.* 2004; 101:3662-7.

Carlin, R.K., Grab, D.J., Cohen, R.S., Siekevitz, P., Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J Cell Biol.* 1980; 86:831-45.

Charrier, C., Machado, P., Tweedie-Cullen, R.Y., Rutishauser, D., Mansuy, I.M., Triller, A., A crosstalk between β1 and β3 integrins controls glycine receptor and gephyrin trafficking at synapses. *Nat. Neurosci.* 2010; 13:1388-95.

Chen, L., Chetkovich, D.M., Petralia, R.S., Sweeney, N.T., Kawasaki, Y., Wenthold, R.J., Bredt, D.S., Nicoll, RA., Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 2000; 408:936-43.

Cherubini, E., Conti, F., Generating diversity at GABAergic synapses. *Trends Neurosci.* 2001;24:155-62.

Chih, B., Gollan, L., Scheiffele, P., Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. *Neuron* 2006; 51:171–178.

Clements, J.D., Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci*. 1996; 19:163-71.

Clements, J.D., Lester, R.A., Tong, G., Jahr, C.E., Westbrook, G.L., The time course of glutamate in the synaptic cleft. *Science* 1992; 258:1498-501.

Cobb, S.R., Buhl, E.H., Halasy, K., Paulsen, O. & Somogyi, P. Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature* 1995; 378: 75–78.

Collingridge, G.L., Bliss, T.V., Memories of NMDA receptors and LTP. *Trends Neurosci.* 1995; 18:54-6.

Collingridge, G.L., Singer, W., Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol Sci.* 1990; 11:290-6.

Colonnier, M., Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. *Brain Res.* 1968; 9:268-87

Comoletti, D., De Jaco, A., Jennings, L.L., Flynn, R.E., Gaietta, G., Tsigelny, I., Ellisman, M.H., Taylor, P., The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J Neurosci.* 2004; 24:4889-93.

Contractor, A., Mulle, C., Swanson, G.T., Kainate receptors coming of age: milestones of two decades of research. *Trends Neurosci.* 2011; 34:154-63.

Craig, A.M., Kang, Y., Neurexin-neuroligin signaling in synapse development. *Curr Opin Neurobiol.* 2007; 17:43-52.

Crestani, F., Keist, R., Fritschy, J.M., Benke, D., Vogt, K., Prut, L., Blüthmann, H., Möhler, H., Rudolph, U., Trace fear conditioning involves hippocampal alpha5 $GABA_A$ receptors. *Proc Natl Acad Sci U S A*. 2002; 99:8980-5.

Cull-Candy, S.G., Leszkiewicz, D.N., Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE.* 2004; 2004:16.

Dalva, M.B., McClelland, A.C., Kayser, M.S., Cell adhesion molecules: signalling functions at the synapse. *Nat Rev Neurosci.* 2007; 8:206-20.

Daoud, H., Bonnet-Brilhault, F., Vedrine, S., Demattei, M.V., Vourc'h, P., Bayou, N., Andres, C.R., Barthelemy, C., Laumonnier, F., Briault, S., Autism and non-syndromic mental retardation associated with a de novo mutation in the NLGN4 X gene promoter causing an increased expression level. *Biol. Psychiatry* 2009; 66: 906–910.

Debanne, D., Campanac, E., Bialowas, A., Carlier, E., Alcaraz, G., Axon physiology. *Physiol Rev.* 2011; 91:555-602.

Del Castillo, J., Katz, B., Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* 1954; 124: 574–585.

Del Castillo, J., Katz, B., Quantal component of the end-plate potential. *J.Physiol* 1954; 124:560-73.

Diamond, J.S., Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J Neurosci.* 2001; 21:8328-38.

Dickenson, A.H., Chapman, V., Green, G.M., The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. *Gen Pharmacol.* 1997; 28:633-8.

Dickinson-Nelson, A., Reese, T.S., Structural changes during transmitter release at synapses in the frog sympathetic ganglion. *J Neurosci* 1983; 3: 42–52.

Dingledine, R., Borges, K., Bowie, D., Traynelis, S.F., The glutamate receptor ion channels. *Pharmacol Rev.* 1999; 51:7-61.

Dittman, J.S., Kreitzer, A.C., Regehr, W.G., Interplay between facilitation, depression, and residual calcium at three presynaptic terminals. *J Neurosci* 2000; 20: 1374–1385.

Dobrunz, L. E., Stevens, C.F., Heterogeneity of release probability, facilitation, and depletion at central synapses. *Neuron* 1997; 18:995–1008.

Dodge, F.A., Rahamimoff, R., Co-operative action a calcium ions in transmitter release at the neuromuscular junction. *J Physiol* 1967; 193: 419–432.

Dresbach,T., Nawrotzki,R., Kremer, T., Schumacher,S., Quinones,D., , Jochen Kuhse, M., Kirsch, J., Molecular architecture of glycinergic synapses*. Histochem Cell Biol.* 2008; 130:617–633.

Du, J.L., Poo, M.M., Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. *Nature* 2004; 429:878–883.

Dunlap, K., Luebke, J.I., Turner, T.J., Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci.* 1995; 18:89-98.

Ehrlich, I. and Malinow, R. Postsynaptic density 95 controls AMPA receptor incorporation during long-term potentiation and experience-driven synaptic plasticity. *J. Neurosci.* 2004; 24:916–927.

Eichenbaum, H., Dudchenko, P., Wood, E., Shapiro, M., Tanila, H., The hippocampus, memory, and place cells: is it spatial memory or a memory space? *Neuron* 1999; 23:209-26.

Ekstrom, A.D., Kahana, M.J., Caplan, J.B., Fields, T.A., Isham, E.A., Newman, E.L., Fried, I., Cellular networks underlying human spatial navigation. *Nature* 2003; 425:184-8.

El-Husseini, A.E., Schnell, E., Chetkovich, D.M., Nicoll, R.A., Bredt, D.S., PSD95 involvement in maturation of excitatory synapses. *Science* 2000; 290:1364-8.

Elias, G.M., Funke, L., Stein, V., Grant, S.G., Bredt, D.S., Nicoll, R.A., Synapse-specific and developmentally regulated targeting of AMPA receptors by a family of MAGUK scaffolding proteins. *Neuron* 2006; 52:307-20.

Essrich, C., Lorez, M., Benson, J.A., Fritschy, J.M., Lüscher, B., Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat Neurosci.* 1998; 1:563-71.

Etherton, M.R., Tabuchi, K., Sharma, M., Ko, J., Südhof, T.C., An autism-associated point mutation in the neuroligin cytoplasmic tail selectively impairs AMPA receptor-mediated synaptic transmission in hippocampus. *EMBO J.* 2011; 30:2908-19.

Eyre, M.D., Renzi, M., Farrant , M., Nusser, Z., Setting the time course of inhibitory synaptic currents by mixing multiple $GABA_A$ receptor α subunit isoforms. *J Neurosci.* 2012; 32:5853-67.

Fagni, L., Chavis, P., Ango, F., Bockaert, J., Complex interactions between mGluRs, intracellular Ca2+ stores and ion channels in neurons. *Trends Neurosci.* 2000 ; 23:80-8.

Farrant, M., Nusser, Z., Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci.* 2005; 6:215-29.

Fatt, P., Katz, B., Spontaneous sub-threshold activity at motor nerve endings *J. Physiol.* 1952; 117:109.

Felmy, F., Neher, E., Schneggenburger, R., Probing the intracellular calcium sensitivity of transmitter release during synaptic facilitation. *Neuron* 2003; 37:801–811.

Feng, W., Zhang, M., Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. *Nat. Rev. Neurosci.* 2009; 10:87-99.

Feng, G., Tintrup, H., Kirsch, J., Nichol, M.C., Kuhse, J., Betz, H., Sanes, J.R., Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* 1998; 282:1321-4.

Fino, E., Yuste, R., Dense inhibitory connectivity in neocortex. *Neuron* 2011; 69:1188-203.

Fischer F, Kneussel M, Tintrup H, Haverkamp S, Rauen T, Betz H, Wässle H., Reduced synaptic clustering of GABA and glycine receptors in the retina of the gephyrin null mutant mouse. *J Comp Neurol*. 2000; 427:634-48.

Förster, E., Zhao, S., Frotscher, M., Laminating the hippocampus. *Nat Rev Neurosci.* 2006 ;7:259-67.

Forsythe, I.D., Tsujimoto, T., Barnes-Davies, M., Cuttle, M.F., Takahashi, T., Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. *Neuron* 1998; 20:797-807.

Foster, K.A., Crowley, J.J., Regehr, W.G., The influence of multivesicular release and postsynaptic receptor saturation on transmission at granule cell to Purkinje cell synapses. *J Neurosci.* 2005; 25:11655-65.

Foster, T.C., Fugger, H.N., Cunningham, S.G., Receptor blockade reveals a correspondence between hippocampal-dependent behavior and experience-dependent synaptic enhancement. *Brain Res.* 2000; 871:39-43.

Freund, T.F., Buzsáki, G., Interneurons of the hippocampus. *Hippocampus.* 1996; 6:347-470.

Freund, T.F., Hájos, N., Excitement reduces inhibition via endocannabinoids. *Neuron* 2003; 38:362-5.

Freund, T.F., Katona, I., Perisomatic inhibition. *Neuron* 2007; 56:33-42.

Fritschy, J.M., Harvey, R.J., Schwarz, G., Gephyrin: where do we stand, where do we go? *Trends Neurosci.* 2008; 31:257-64.

Fritschy, J.M., Panzanelli, P., Tyagarajan, S.K., Molecular and functional heterogeneity of GABAergic synapses. *Cell Mol Life Sci.* 2012; 69:2485-99.

Funke, L., Dakoji, S., Bredt, D.S., Membrane-associated guanylate kinases regulate adhesion and plasticity at cell junctions. *Annu. Rev. Biochem.* 2004; 74: 219–245.

Futai, K., Kim, M.J., Hashikawa, T., Scheiffele, P., Sheng, M., Hayashi, Y., Retrograde modulation of presynaptic release probability through signaling mediated by PSD-95-neuroligin. *Nat Neurosci.* 2007; 10:186-95.

Gandal, M.J., Edgar, J.C., Ehrlichman, R.S., Mehta, M., Roberts, T.P., Siegel, S.J., Validating γ oscillations and delayed auditory responses as translational biomarkers of autism. *Biol Psychiatry.* 2010; 68:1100-6.

Garber, K., Neuroscience. Autism's cause may reside in abnormalities at the synapse. *Science* 2007; 317:190-1.

Garthwaite, J., Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci.* 2008; 27: 2783–2802.

Gibson, J.R., Bartley, A.F., Hays, S.A., Huber, K.M., Imbalance of neocortical excitation and inhibition and altered UP states reflect network hyperexcitability in the mouse model of fragile X syndrome. *J Neurophysiol.* 2008 ; 100:2615-26.

Giesemann, T., Schwarz, G., Nawrotzki, R., Berhörster, K., Rothkegel, M., Schlüter, K., Schrader, N., Schindelin, H., Mendel, R.R., Kirsch, J., Jockusch, B.M., Complex formation between the postsynaptic scaffolding protein gephyrin, profilin, and Mena: a possible link to the microfilament system. *J Neurosci.* 2003; 23:8330-9.

Gingrich, K.J., Roberts, W.A., Kass, R.S., Dependence of the $GABA_A$ receptor gating kinetics on the alpha-subunit isoform: implications for structure-function relations and synaptic transmission. *J Physiol.* 1995 ; 489:529-43.

Glickfeld, L.L., Roberts, J.D., Somogyi, P., Scanziani, M., Interneurons hyperpolarize pyramidal cells along their entire somatodendritic axis. *Nat Neurosci.* 2009; 12:21-3.

Glickfeld, L.L., Scanziani, M., Distinct timing in the activity of cannabinoid-sensitive and cannabinoid-insensitive basket cells. *Nat Neurosci.* 2006; 9:807-15.

Glykys, J., Mann, E.O., Mody, I., Which $GABA_A$ receptor subunits are necessary for tonic inhibition in the hippocampus? *J Neurosci.* 2008; 28:1421-6.

Glykys, J., Mody, I., Activation of $GABA_A$ receptors: views from outside the synaptic cleft. *Neuron* 2007; 56:763-70.

Glykys, J., Mody,I., The main source of ambient GABA responsible for tonic inhibition in the mouse hippocampus. *J Physiol.* 2007; 582:1163-78.

Goda, Y., Stevens, C.F., Two components of transmitter release at a central synapse. *Proc Natl Acad Sci U S A.* 1994; 91:12942-6.

Gottmann, K., Transsynaptic modulation of the synaptic vesicle cycle by cell-adhesion molecules. *J Neurosci Res.* 2008; 86:223-32.

Graf, E.R., Zhang, X., Jin, S.X., Linhoff, M.W., Craig, A.M., Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 2004; 119:1013-1026.

Gray, E. G., Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. *Nature* 1959; 183:1592 – 1593.

Griguoli, M., Maul, A., Nguyen, C., Giorgetti, A., Carloni, P., Cherubini, E., Nicotine blocks the hyperpolarization-activated current Ih and severely impairs the oscillatory behavior of oriens-lacunosum moleculare interneurons. *J Neurosci.* 2010; 30:10773-83.

Gulyás, A.I., Tóth, K., McBain, C.J., Freund, T.F., Stratum radiatum giant cells: a type of principal cell in the rat hippocampus. *Eur J Neurosci.* 1998; 10:3813-22.

Habets, R.L., Borst, J.G., Post-tetanic potentiation in the rat calyx of Held synapse. *J Physiol* 2005; 564:173–187.

Hagler, D.J., Goda, Y., Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. *J Neurophysiol* 2001; 85: 2324–2334

Han, Z.S., Buhl, E.H., Lörinczi, Z., Somogyi, P., A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. *Eur J Neurosci.* 1993; 5:395-410

Hefft, S., Jonas, P., Asynchronous GABA release generates long-lasting inhibition at a hippocampal interneuron-principal neuron synapse. *Nat Neurosci.* 2005; 8:1319-28

Hochner, B., Parnas, H., Parnas, I., Effects of intra-axonal injection of Ca^{2+} buffers on evoked release and on facilitation in the crayfish neuromuscular junction. *Neurosci Lett* 1991; 125: 215–218.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W., Heinemann, S., Cloning by functional expression of a member of the glutamate receptor family. *Nature* 1989; 342:643-8.

Hubbard, J., repetitive stimulation at the mammalian neuromuscular junction and the mobilization of transmitter *J Physiol.* 1963; 169:641-62.

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T.W., Südhof, T.C., Binding of neuroligins to PSD-95. *Science* 1997; 277: 1511-5.

Jacob, T.C., Bogdanov, Y.D., Magnus, C., Saliba, R.S., Kittler, J.T., Haydon, P.G., Moss, S.J., Gephyrin regulates the cell surface dynamics of synaptic $GABA_A$ receptors. *J Neurosci.* 2005; 25:10469-78.

Jamain, S., Radyushkin, K., Hammerschmidt, K., Granon, S., Boretius, S., Varoqueaux, F., Ramanantsoa, N., Gallego, J., Ronnenberg, A., Winter, D., Frahm, J., Fischer, J., Bourgeron, T., Ehrenreich, H., Brose, N., Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. *Proc Natl Acad Sci U S A.* 2008; 105:1710-5.

Jang, I.S., Nakamura, M., Ito, Y., Akaike, N., Presynaptic $GABA_A$ receptors facilitate spontaneous glutamate release from presynaptic terminals on mechanically dissociated rat CA3 pyramidal neurons. *Neuroscience* 2006; 138:25-35.

Johnston, J., Griffin, S.J., Baker, C., Skrzypiec, A., Chernova, T., Forsythe, I.D., Initial segment Kv2.2 channels mediate a slow delayed rectifier and maintain high frequency action potential firing in medial nucleus of the trapezoid body neurons. *J Physiol* 2008; 586: 3493–3509.

Jonas, P., Major, G., Sakmann, B., Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. *J Physiol.* 1993; 472:615-63.

Jonas, P., Burnashev, N., Molecular mechanisms controlling calcium entry through AMPA-type glutamate receptor channels. *Neuron* 1995; 15:987-90.

Jones, M.V., Westbrook, G.L., The impact of receptor desensitization on fast synaptic transmission. *Trends Neurosci.* 1996; 19:96-101.

Kalscheuer, V.M., Musante, L., Fang, C., Hoffmann, K., Fuchs, C., Carta, E., Deas, E., Venkateswarlu, K., Menzel, C., Ullmann, R., Tommerup, N., Dalprà, L., Tzschach, A., Selicorni, A., Lüscher, B., Ropers, H.H., Harvey, K., Harvey, R.J.A balanced chromosomal translocation disrupting ARHGEF9 is associated with epilepsy, anxiety, aggression, and mental retardation. *Hum Mutat.* 2009; 30:61-8.

Kaneda, M., Farrant, M., Cull-Candy, S.G., Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. *J Physiol.* 1995; 485:419-35.

Kasugai, Y., Swinny, J.D., Roberts, J.D., Dalezios, Y., Fukazawa, Y., Sieghart, W., Shigemoto, R., Somogyi P., Quantitative localisation of synaptic and extrasynaptic GABAA receptor subunits on hippocampal pyramidal cells by freeze-fracture replica immunolabelling. *Eur J Neurosci.* 2010; 32:1868-88.

Katz, B., Miledi, R., Further study of the role of calcium in synaptic transmission. *J Physiol* 1970; 207: 789–801.

Katz, B., Miledi, R., The role of calcium in neuromuscular facilitation. *J Physiol* 1968; 195:481–492.

Katz, B., The Release of Neural Transmitter Substances, Liverpool University Press, 1969.

Keros, S., Hablitz, J.J., Subtype-specific GABA transporter antagonists synergistically modulate phasic and tonic $GABA_A$ conductances in rat neocortex. *J Neurophysiol.* 2005; 94:2073-85.

Kim, E., Sheng, M., PDZ domain proteins of synapses. *Nat Rev Neurosci.* 2004; 5:771-81.

Kim, J., Jung, S.Y., Lee, Y.K., Park, S., Choi, J.S., Lee, C.J., Kim, H.S., Choi, Y.B., Scheiffele, P., Bailey, C.H., Kandel, E.R., Kim, J.H., Neuroligin-1 is required for normal expression of LTP and associative fear memory in the amygdala of adult animals. Proc Natl Acad Sci U S A. 2008; 105:9087-92.

Kim, J.J., Baxter, M.G., Multiple brain-memory systems: the whole does not equal the sum of its parts. *Trends Neurosci.* 2001;24:324-30.

Kins, S., Betz, H., Kirsch, J., Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat Neurosci.* 2000; 3:22-9.

Kirsch, J., Betz, H., The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton. *J Neurosci.* 1995; 15:4148-56.

Klausberger, T., Somogyi, P., Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* 2008; 321:53-7.

Kneussel, M., Brandstätter, J.H., Gasnier, B., Feng, G., Sanes, J.R., Betz, H., Gephyrin-independent clustering of postsynaptic $GABA_A$ receptor subtypes. *Mol Cell Neurosci.* 2001; 17:973-82.

Kneussel, M., Brandstätter, J.H., Laube, B., Stahl, S., Müller, U., Betz, H., Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. *J Neurosci.* 1999;19:9289-97.

Kneussel, M., Betz, H., Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. *Trends Neurosci.* 2000; 23:429-35.

Kole, M.H., Letzkus, J.J., Stuart, G.J., Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. *Neuron* 2007; 55: 633–647.

Korn, H., Faber, D.S., Quantal analysis and synaptic efficacy in the CNS *Trends Neurosci.* 1991; 14:439–445.

Kornau, H.C., Seeburg, P.H., Kennedy, M.B., Interaction of ion channels and receptors with PDZ domain proteins. *Curr Opin Neurobiol.* 1997; 7:368-73.

Korogod, N., Lou, X., Schneggenburger, R., Presynaptic Ca^{2+} requirements anddevelopmental regulation of post-tetanic potentiation at the calyx of Held *J Neurosci* 2005; 25: 5127–5137.

Kralic, J.E., Sidler, C., Parpan, F., Homanics, G.E., Morrow, A.L., Fritschy, J.M., Compensatory alteration of inhibitory synaptic circuits in cerebellum and thalamus of gamma-aminobutyric acid type A receptor alpha1 subunit knockout mice. *J Comp Neurol.* 2006; 495:408-21.

Kruk, P.J., Korn, H., Faber, D.S., The effects of geometrical parameters on synaptic transmission: a Monte Carlo simulation study. *Biophys. J.* 1996;73: 2874–2890.

Kullmann, D.M., Ruiz, A., Rusakov, D.M., Scott, R., Semyanov, A., Walker, M.C., Presynaptic, extrasynaptic and axonal GABAA receptors in the CNS: where and why? *Prog Biophys Mol Biol.* 2005; 87:33-46.

Kuno, M., Quantal components of excitatory synaptic potentials in motoneurons. *J. Physiol* 1964; 175: 81-98.

Kwok, S.C., Buckley, M.J., Fornix transection impairs exploration but not locomotion in ambulatory macaque monkeys. *Hippocampus* 2006; 16:655-63.

Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M.P., Raynaud, M., Ronce, N., Lemonnier, E., Calvas, P., Laudier, B., Chelly, J., Fryns, J.P., Ropers, H.H., Hamel, B.C., Andres, C., Barthélémy, C., Moraine, C., Briault, S., X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet.* 2004; 74:552-7.

Lawrence, Y.A., Kemper, T.L., Bauman, M.L., Blatt, G.J., Parvalbumin-, calbindin- and calretinin-immunoreactive hippocampal interneuron density in autism. *Acta Neurol Scand.* 2010; 121: 99–108.

Lawson-Yuen, A., Saldivar, J.S., Sommer, S., Picker, J., Familial deletion within NLGN4 associated with autism and Tourette syndrome. *Eur J Hum Genet.* 2008 ; 16:614-8.

Lester R.A., Jahr C.E., NMDA channel behavior depends on agonist affinity. *J Neurosci.* 1992; 12:635-43.

Lévi S, Logan SM, Tovar KR, Craig AM. Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J Neurosci.* 2004; 24:207-17.

Lévi, S., Logan, S.M., Tovar, K.R., Craig, A.M., Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J Neurosci*. 2004; 24:207-17.

Levinson, J.N., Chéry, N., Huang, K., Wong, T.P., Gerrow, K., Kang, R., Prange, O., Wang, Y.T., El-Husseini, A., Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem.* 2005; 280:17312-9.

Li, R.W., Yu, W., Christie, S., Miralles, C.P., Bai, J., Loturco, J.J., De Blas, A.L., Disruption of postsynaptic GABA receptor clusters leads to decreased GABAergic innervation of pyramidal neurons. *J Neurochem.* 2005; 95:756-70.

Lin, Y.Q., Brain, K.L., Bennett, M.R., Calcium in sympathetic boutons of rat superior cervical ganglion during facilitation, augmentation and potentiation *J. Auton. Nerv. Syst.* 1998; 73:26–37.

Llano, I., Leresche, N., Marty, A., Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. *Neuron* 1991; 6:565–574.

Llinás, R., Steinberg, I.Z., Walton, K., Relationship between presynaptic calciumcurrent and postsynaptic potential in squid giant synapse. *Biophys J.* 1981; 33:323-51.

Lu, T., Trussell, L.O., Inhibitory transmission mediated by asynchronous transmitter release. *Neuron* 2000; 26: 683–694.

Luscher, B., Fuchs, T., Kilpatrick, C.L., $GABA_A$ receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* 2011; 70:385-409.

Lüscher, B., Keller, C.A., Regulation of GABAA receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther*. 2004; 102:195-221.

Maas, C., Tagnaouti, N., Loebrich, S., Behrend, B., Lappe-Siefke, C., Kneussel, M., Neuronal cotransport of glycine receptor and the scaffold protein gephyrin. *J Cell Biol.* 2006 ;172:441-51.

Magistretti, J., Alonso, A., Fine gating properties of channels responsible for persistent sodium current generation in entorhinal cortex neurons. *J Gen Physiol* 2002; 120: 855–873.

Mammoto, A., Sasaki, T., Asakura, T., Hotta, I., Imamura, H., Takahashi K., Matsuura, Y., Shirao, T., Takai, Y., Interactions of drebrin and gephyrin with profilin. *Biochem Biophys Res Commun.* 1998; 243:86-9.

Marchionni, I., Kasap, Z., Mozrzymas, J.W., Sieghart, W., Cherubini, E., Zacchi, P., New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture. *Neuroscience* 2009 ; 164:552-62.

McBain, C.J., Mayer, M.L. N-methyl-D-aspartic acid receptor structure and function. *Physiol Rev.* 1994; 74:723-60.

Meyer, G., Kirsch, J., Betz, H., Langosch, D., Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron* 1995; 15:563-72.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsa, M.F., Morris, R.G., Morrison, J.H., O'Dell, T.J., Grant, S.G., Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 1998; 396:433-9.

Miles, R., Wong, R.K., Excitatory synaptic interactions between CA3 neurones in the guinea-pig hippocampus. *J Physiol.* 1986; 373:397-418.

Milner, B., Squire, L.R., Kandel, E.R., Cognitive neuroscience and the study of memory. *Neuron* 1998; 20:445-68.

Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R.E., Gottmann, K., Sudhof, T.C., α-Neurexins couple Ca^{2+} channels to synaptic vesicle exocytosis. *Nature* 2003; 423:939-948.

Mitchell, S.J. & Silver, R.A. Shunting inhibition modulates neuronal gain during synaptic excitation. *Neuron* 2003; 38: 433–445.

Mochida, S., Few, A.P., Scheuer, T., Catterall , W.A. Regulation of presynaptic $Ca_{(V)}$2.1 channels by Ca^{2+} sensor proteins mediates short-term synaptic plasticity. *Neuron* 2008; 57:210–216.

Momiyama, A., Silver, R.A., Hausser, M., Notomi, T., Wu, Y., Shigemoto, R., Cull-Candy, S.G., The density of AMPA receptors activated by a transmitter quantum at the climbing fibre-Purkinje cell synapse in immature rats. *J. Physiol*. 2003 ; 549:75-92.

Mozrzymas, J.W., Barberis, A., Mercik, K., Zarnowska, E.D., Binding sites, singly bound states, and conformation coupling shape GABA-evoked currents *J Neurophysiol.* 2003; 89:871-83.

Muller, M., Felmy, F., Schwaller, B., Schneggenburger, R., Parvalbumin is a mobile presynaptic Ca^{2+} buffer in the calyx of held that accelerates the decay of Ca^{2+} and short-term facilitation*. J Neurosci* 2007; 27:2261–2271.

Nakanishi, S., Masu, M., Molecular diversity and functions of glutamate receptors. *Annu Rev Biophys Biomol Struct.* 1994; 23:319-48.

Nam, C.I., Chen, L., Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci U S A.* 2005 ;102:6137-42.

Narita, K., Akita, T., Hachisuka, J., Huang, S., Ochi, K., Kuba, K., Functional coupling of Ca^{2+} channels to ryanodine receptors at presynaptic terminals.Amplification of exocytosis and plasticity. *J Gen Physiol* 2000; 115: 519–532.

Nestler, E.J., Hyman, S.E., Animal models of neuropsychiatric disorders. *Nat Neurosci.* 2010; 13:1161-9.

Newpher, T.M., Ehlers, M.D., Glutamate receptor dynamics in dendritic microdomains. *Neuron* 2008; 58:472-97.

Nguyen, L., Malgrange, B., Breuskin, I., Bettendorff, L., Moonen, G., Belachew, S., Rigo, J.M., Autocrine/paracrine activation of the $GABA_A$ receptor inhibits the proliferation of neurogenic polysialylated neural cell adhesion molecule-positive (PSA-NCAM+) precursor cells from postnatal striatum. *J Neurosci.* 2003; 23:3278-94.

Nicoll, R.A., Tomita, S., Bredt, D.S., Auxiliary subunits assist AMPA-type glutamate receptors. *Science* 2006; 311:1253-6.

Nusser, Z., Mody, I., Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol*. 2002; 877:2624-8.

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J.M., Somogyi, P., Differential synaptic localization of two major gamma-aminobutyric acid type A receptor alpha subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci U S A*. 1996; 93:11939-44.

Ogiwara, I., Miyamoto, H., Morita, N., Atapour, N., Mazaki, E., Inoue, I., Takeuchi, T., Itohara, S., Yanagawa, Y., Obata, K., Furuichi, T., Hensch, T.K., Yamakawa, K., Na(v)1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an Scn1a gene mutation. *J Neurosci* 2007; 27:5903–5914.

Olivera, B.M., Miljanich, G.P., Ramachandran, J., Adams, M.E.,Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. *Annu Rev Biochem*. 1994; 63:823-67.

Orekhova, E.V., Tsetlin, M.M., Butorina, A.V., Novikova, S.I., Gratchev, V.V., Sokolov, P.A., Elam, M., Stroganova, T.A., Auditory cortex responses to clicks and sensory modulation difficulties in children with autism spectrum disorders (ASDs). *PLoS One* 2012; 7:e39906. Epub 2012 Jun 29.

Overstreet, L.S., Westbrook, G.L., Synapse density regulates independence at unitary inhibitory synapses. *J Neurosci.* 2003; 23:2618-26.

Paarmann, I., Schmitt, B., Meyer, B., Karas, M., Betz, H., Mass spectrometric analysis of glycine receptor-associated gephyrin splice variants. *J Biol Chem.* 2006; 281:34918-25

Pan, Z., Selyanko, A.A., Hadley, J.K., Brown, D.A., Dixon, J.E., McKinnon, D. Alternative splicing of KCNQ2 potassium channel transcripts contributes to the functional diversity of M-currents. *J Physiol* 2001; 531: 347–358.

Paoletti, P., Molecular basis of NMDA receptor functional diversity. *Eur J Neurosci.* 2011; 33:1351-65.

Parsons, C.G., Quack, G., Bresink, I., Baran, L., Przegalinski, E., Kostowski, W., Krzascik, P., Hartmann, S., Danysz, W., Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists

in vitro with anticonvulsive and motor impairment activity in vivo. *Neuropharmacology* 1995; 34:1239-58.

Perrais, D., Ropert N., Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. *J Neurosci.* 1999 ;19:578-88

Petilla Interneuron Nomenclature Group, Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat Rev Neurosci.* 2008; 9:557-68.

Petralia, R.S., Sans, N., Wang, Y.X., Wenthold, R.J., Ontogeny of postsynaptic density proteins at glutamatergic synapses. *Mol Cell Neurosci.* 2005; 29:436-52.

Pfeiffer, F., Graham, D., Betz, H., Purification by affinity chromatography of the glycine receptor of rat spinal cord. *J Biol Chem.* 1982; 257:9389-93

Pitler, T.A., Alger, B.E., Postsynaptic spike firing reduces synaptic $GABA_A$ responses in hippocampal pyramidal cells. *J Neurosci.* 1992; 12:4122–4132.

Pizzarelli, R., Cherubini, E., Alterations of GABAergic signaling in autism spectrum disorders *Neural Plast* 2011; 2011:297153

Poncer, J.C., McKinney, R.A., Gähwiler, B.H., Thompson, S.M., Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron* 1997; 18:463-72.

Pouille, F., Scanziani, M., Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. *Science* 2001; 293:1159-63.

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K., Jedlicka, P., Schwarzacher, S.W., Betz, H., Harvey, R.J., Brose, N., Zhang, W., Varoqueaux, F., Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* 2009; 63:628-42.

Prange, O., Wong, T.P., Gerrow, K., Wang, Y.T., El-Husseini, A., A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *Proc Natl Acad Sci U S A*. 2004; 101:13915-20.

Raastad, M., Extracellular activation of unitary excitatory synapses between hippocampal CA3 and CA1 pyramidal cells. *Eur J Neurosci.* 1995; 7:1882-8.

Radyushkin, K., Hammerschmidt, K., Boretius, S., Varoqueaux, F., El-Kordi, A., Ronnenberg, A., Winter, D., Frahm, J., Fischer, J., Brose, N., Ehrenreich, H.,

Neuroligin-3-deficient mice: model of a monogenic heritable form of autism with an olfactory deficit. *Genes Brain Behav.* 2009; 8:416-25.

Rahamimoff, R., Yaari, Y., Delayed release of transmitter at the frog neuromuscular junction. *J Physiol.* 1973; 228:241-57.

Raman, I.M., Bean, B.P., Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons. *J Neurosci* 1997; 17: 4517–4526.

Redman, S.J., Faber, D.S Adapting Quantal Analysis to Central Synapses *J. Neurosci. Methods* 2003; 2:103-206.

Regehr, W.G., Delaney, K.R., Tank, D.W., The role of presynaptic calcium in short-term enhancement at the hippocampal mossy fiber synapse. *J Neurosci* 1994; 14:523–537.

Roche, K.W., Standley, S., McCallum, J., Dune, L.C., Ehlers, M.D., Wenthold, R.J., Molecular determinants of NMDA receptor internalization. *Nat Neurosci.* 2001; 4:794-802.

Rossmann, M., Sukumaran, M., Penn, A.C., Veprintsev, D.B., Babu, M.M., Greger, I.H.,Subunit-selective N-terminal domain associations organize the formation of AMPA receptor heteromers. *EMBO J.* 2011; 30:959-71.

Rozov, A., Burnashev, N., Polyamine-dependent facilitation of postsynaptic AMPA receptors counteracts paired-pulse depression. *Nature* 1999; 401:594-8.

Ruiz, A., Fabian-Fine, R., Scott, R., Walker, M.C., Rusakov, D.A,, Kullmann, D.M., GABAA receptors at hippocampal mossy fibers. *Neuron* 2003; 39:961-73.

Sabatini, B.L., Regehr, W.G., Timing of neurotransmission at fast synapses in the mammalian brain. *Nature* 1996; 384: 170–172.

Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R.J., Harvey, K., Complex role of collybistin and gephyrin in GABAA receptor clustering. *J Biol Chem.* 2010; 285:29623-31.

Sala, C., Piëch, V., Wilson, N.R., Passafaro, M., Liu, G., Sheng, M., Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 2001; 31:115-30.

Sassoè-Pognetto, M., Kirsch, J., Grünert, U., Greferath, U., Fritschy, J.M., Möhler, H., Betz, H., Wässle, H., Colocalization of gephyrin and GABAA-receptor subunits in the rat retina. *J Comp Neurol*. 1995; 357:1-14.

Sassoè-Pognetto, M., Panzanelli, P., Sieghart, W., Fritschy, J.M., Colocalization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. *J Comp Neurol.* 2000; 420:481-98.

Sassoè-Pognetto, M., Fritschy, J.M.,Mini-review: gephyrin, a major postsynaptic protein of GABAergic synapses. *Eur J Neurosci.* 2000; 12:2205-10.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., Serafini, T.,: Neuroligin expressed in non-neuronal cells triggers presynaptic development in contacting axons. *Cell* 2000; 101:657-669.

Schnell, E., Sizemore, M., Karimzadegan, S., Chen, L., Bredt, D.S., Nicoll, R.A., Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number.*Proc Natl Acad Sci U S A*. 2002; 99:13902-7.

Schweizer, C., Balsiger, S., Bluethmann, H., Mansuy, I.M., Fritschy, J.M., Mohler, H., Lüscher, B.,The gamma 2 subunit of $GABA_A$ receptors is required for maintenance of receptors at mature synapses. *Mol Cell Neurosci.* 2003; 24:442-50.

Scimemi, A., Beato, M., Determining the neurotransmitter concentration profile at active synapses. *Mol Neurobiol.* 2009; 40:289-306.

Scoville, W.B., Milner, B., Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry.* 1957; 20:11-21.

Seeburg P.H., The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* 1993; 16:359-65.

Shah, M.M., Migliore, M., Valencia, I., Cooper, E.C., Brown, D.A., Functional significance of axonal Kv7 channels in hippocampal pyramidal neurons. *Proc Natl Acad Sci USA* 2008; 105: 7869–7874.

Sheng, M., Kim E., The postsynaptic organization of synapses. *Cold Spring Harb Perspect Biol.* 2011; 3.

Sheng, M., Hoogenraad, C.C., The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem*. 2007; 76:823-47.

Shepherd, J.D., Huganir, R.L., The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol*. 2007; 23:613-43.

Shields, C.R., Tran, M.N., Wong, R.O., Lukasiewicz, P.D., Distinct ionotropic GABA receptors mediate presynaptic and postsynaptic inhibition in retinal bipolar cells. *J Neurosci*. 2000; 20:2673-82.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P.J., Neki, A., Abe, T., Nakanishi, S., Mizuno, N., Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J Neurosci.* 1997; 17:7503-22.

Shigemoto, R., Kulik, A., Roberts, J.D., Ohishi, H., Nusser, Z., Kaneko, T., Somogyi, P., Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. *Nature* 1996; 381:523-5.

Siekevitz, P., The postsynaptic density: a possible role in long-lasting effects in the central nervous system. *Proc Natl Acad Sci U S A*. 1985; 82:3494-8.

Sipilä, S.T., Voipio, J., Kaila, K., GAT-1 acts to limit a tonic $GABA_A$ current in rat CA3 pyramidal neurons at birth. *Eur J Neurosci.* 2007; 25:717-22.

Sippy, T., Cruz-Martin, A. , Jeromin, A., Schweizer, F.E., Acute changes in short-term plasticity at synapses with elevated levels of neuronal calcium sensor-1. *Nature Neurosciences* 2003; 6:1031–1038.

Stan, A., Pielarski, K.N., Brigadski, T., Wittenmayer, N., Fedorchenko, O., Gohla, A., Lessmann, V., Dresbach, T., Gottmann, K., Essential cooperation of N-cadherin and neuroligin-1 in the trans-synaptic control of vesicle accumulation. *Proc Natl Acad Sci U S A*. 2010; 1077:11116-21.

Stein, V. Postsynaptic density-95 mimics and occludes hippocampal long-term potentiation and enhances long-term depression. *J. Neurosci.* 2003; 23:5503–5506.

Stell, B.M., Mody, I., Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons. *J Neurosci.* 2002; 22:RC223.

Stell, B.M., Rostaing, P., Triller, A., Marty, A., Activation of presynaptic $GABA_A$ receptors induces glutamate release from parallel fiber synapses. *J Neurosci.* 2007; 27:9022-31.

Stevens, C.F., Wesseling, J.F. Augmentation is a potentiation of the exocytotic process. *Neuron* 1999; 22:139–146.

Stroganova, T.A., Nygren, G., Tsetlin, M.M., Posikera, I.N., Gillberg, C., Elam, M., Orekhova, E.V., Abnormal EEG lateralization in boys with autism. *Clin Neurophysiol*. 2007; 118:1842-54.

Stuart, G., Sakmann, B., Amplification of EPSPs by axosomatic sodium channels in neocortical pyramidal neurons. *Neuron* 1995; 15:1065–1076.

Studer, R., von Boehmer, L., Haenggi, T., Schweizer, C., Benke, D., Rudolph, U., Fritschy, J.M., Alteration of GABAergic synapses and gephyrin clusters in the thalamic reticular nucleus of GABAA receptor alpha3 subunit-null mice. *Eur J Neurosci.* 2006; 24:1307-15.

Sudhof, T.C., The synaptic vesicle cycle. *Annu Rev Neurosci.* 2004; 27:509-47.

Südhof, T.C., Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* 2008; 455:903-11.

Sullivan, J.M., A simple depletion model of the readily releasable pool of synaptic vesicles cannot account for paired-pulse depression *J. Neurophysiol.* 2007; 97: 948–950.

Sun, H.Y., Lyons, S.A. & Dobrunz, L.E. Mechanisms of target-cell specific short-term plasticity at Schaffer collateral synapses onto interneurones versus pyramidal cells in juvenile rats. *J. Physiol.* 2005*;* 568:815–840.

Svoboda, K., Helmchen, F., Denk, W., Tank, D.W., Spread of dendritic excitation in layer 2/3 pyramidal neurons in rat barrel cortex in vivo. *Nat Neurosci.* 1999; 2:65-73.

Swulius, M.T., Kubota, Y., Forest, A., Waxham, M.N., Structure and composition of the postsynaptic density during development. *J Comp Neurol.* 2010; 518:4243-60.

Szabadics, J., Varga, C., Molnár, G., Oláh, S., Barzó, P., Tamás, G., Excitatory effect of GABAergic axo-axonic cells in cortical microcircuits. *Science* 2006; 311:233-5.

Tabuchi, K., Blundell, J., Etherton, M.R., Hammer, R.E., Liu, X., Powell, C.M., Südhof, T.C., A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 2007; 318:71-6.

Tamás, G., Somogyi, P., Buhl, E.H., Differentially interconnected networks of GABAergic interneurons in the visual cortex of the cat. *J Neurosci.* 1998; 18:4255-70.

Tang, Y., Zucker, R.S., Mitochondrial involvement in post-tetanic potentiation of synaptic transmission *Neuron* 1997; 18:483–491.

Thomas, P., Mortensen, M., Hosie, A.M., Smart, T.G., Dynamic mobility of functional GABAA receptors at inhibitory synapses. *Nat Neurosci.* 2005; 8:889-97.

Traynelis, S.F., Silver, R.A., Cull-Candy, S.G., Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. *Neuron* 1993; 11:279-89.

Traub, R.D., Miles, R., Multiple modes of neuronal population activity emerge after modifying specific synapses in a model of the CA3 region of the hippocampus. *Ann N Y Acad Sci.* 1991; 627:277-90.

Tretter, V., Mukherjee, J., Maric, H.M., Schindelin, H., Sieghart, W., Moss, S.J., Gephyrin, the enigmatic organizer at GABAergic synapses. *Front Cell Neurosci*. 2012; 6:23.

Tretter, V., Moss, S.J., $GABA_A$ Receptor Dynamics and Constructing GABAergic Synapses. *Front Mol Neurosci.* 2008; 1:7.

Trujillo, K.A., Akil, H., Excitatory amino acids and drugs of abuse: a role for N-methyl-D-aspartate receptors in drug tolerance, sensitization and physical dependence. *Drug Alcohol Depend.* 1995; 38:139-54.

Tsujimoto,T. , Jeromin, A., Saitoh, N., Roder, J.C., Takahashi T., Neuronal calcium sensor 1 and activity-dependent facilitation of P/Q-type calcium currents at presynaptic nerve terminals. *Science* 2002; 295:2276–2279.

Tyagarajan, S.K., Ghosh, H., Yévenes, G.E., Nikonenko, I., Ebeling, C., Schwerdel, C., Sidler, C., Zeilhofer, H.U., Gerrits, B., Muller, D., Fritschy, J.M., Regulation of GABAergic synapse formation and plasticity by GSK3beta-dependent phosphorylation of gephyrin. *Proc Natl Acad Sci U S A.* 2011; 108:379-84.

Ulbrich, M.H., Isacoff, E.Y., Subunit counting in membrane-bound proteins. *Nat Methods.* 2007; 4:319-21.

Unwin, N., The structure of ion channels in membranes of excitable cells. *Neuron* 1989; 3:665-76.

Uusi-Oukari, M,, Korpi, E,R., Regulation of $GABA_A$ receptor subunit expression by pharmacological agents. *Pharmacol Rev.* 2010; 62:97-135.

Van der Kloot, W., Molgo, J., Facilitation and delayed release at about 0 degree C at the frog neuromuscular junction: effects of calcium chelators, calcium transport inhibitors, and okadaic acid *J. Neurophysiol* 1993; 63:717–729.

Varley, Z.K., Pizzarelli, R., Antonelli, R., Stancheva, S.H., Kneussel, M., Cherubini, E., Zacchi, P., Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. *J Biol Chem.* 2011; 286:20942-51.

Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Südhof, T.C., Brose, N., Neuroligins determine synapse maturation and function. *Neuron* 2006; 51:741-54.

Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Sudhof, T.C., Brose, N., Neuroligins determine synapse maturation and function. *Neuron* 2006; 51:741-754.

Varoqueaux, F., Jamain, S., Brose, N., Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol.* 2004; 83:449-56.

Vida, I., Halasy, K., Szinyei, C., Somogyi, P., Buhl, E.H., Unitary IPSPs evoked by interneurons at the stratum radiatum-stratum lacunosum-moleculare border in the CA1 area of the rat hippocampus in vitro. *J Physiol.* 1998;506: 755-73.

Wahl, L.M., Pouzat, C., Stratford, K.J. Monte Carlo simulation of fast excitatory synaptic transmission at a hippocampal synapse. *J Neurophysiol* 1996; 75:597-608.

Walker, M.C., Kullmann, D.M. Tonic $GABA_A$ Receptor-Mediated Signaling in Epilepsy. Jasper's Basic Mechanisms of the Epilepsies 4th edition. Bethesda; 2012.

Wang, X.J., Neurophysiological and computational principles of cortical rhythms in cognition. *Physiol Rev.* 2010; 90:1195-268.

Watkins, J.C., Jane, D.E., The glutamate story. *Br J Pharmacol.* 2006;147 Suppl 1:S100-8.

Whittington, M.A., Traub, R.D., Jefferys, J.G., Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation. *Nature* 1995; 373:612-5.

Wierengart, C.J., Becker, N., Bonhoeffer, T., GABAergic synapses are formed without the involvement of dendritic protrusions. *Nat Neurosci.* 2008; 11:1044-52.

Wittner, L., Huberfeld, G., Clémenceau, S., Eross, L., Dezamis, E., Entz, L., Ulbert, I., Baulac, M., Freund, T.F., Maglóczky, Z., Miles, R., The epileptic human

hippocampal cornu ammonis 2 region generates spontaneous interictal-like activity in vitro. *Brain*. 2009; 132:3032-46.

Wu, L.G., Borst, J.G., The reduced release probability of releasable vesicles during recovery from short-term synaptic depression . *Neuron* 1999; 23:821–832.

Xu, J., Wu, L.G., The decrease in the presynaptic calcium current is a major cause of short-term depression at a calyx-type synapse. *Neuron* 2005; 46: 633–645.

Yang, J., Thio, L.L., Clifford, D.B., Zorumski, C.F., Electrophysiological properties of identified postnatal rat hippocampal pyramidal neurons in primary culture. *Brain Res. Dev. Brain Res.* 1993; 71:19–26.

Yu, W., Jiang, M., Miralles, C.P., Li, R.W., Chen, G., de Blas, A.L., Gephyrin clustering is required for the stability of GABAergic synapses. *Mol Cell Neurosci.* 2007; 36:484-500.

Yu, Y., Maureira, C., Liu, X., McCormick, D., P/Q and N channels control baseline and spike-triggered calcium levels in neocortical axons and synaptic boutons*. J Neurosci* 2010; 30: 11858–11869.

Yue, C., Yaari, Y., Axo-somatic and apical dendritic Kv7/M channels differentially regulate the intrinsic excitability of adult rat CA1 pyramidal cells. *J Neurophysiol* 2006; 95: 3480–3495.

Zacchi, P., Dreosti, E., Visintin, M., Moretto-Zita, M., Marchionni, I., Cannistraci, I., Kasap, Z., Betz, H., Cattaneo, A., Cherubini, E., Gephyrin selective intrabodies as a new strategy for studying inhibitory receptor clustering. *J Mol Neurosci.* 2008 ; 34:141-8

Zhang, C., Atasoy, D., Araç, D., Yang, X., Fucillo, M.V., Robison, A.J., Ko, J., Brunger, A.T., Südhof, T.C., Neurexins physically and functionally interact with GABA(A) receptors. *Neuron* 2010; 66:403-16.

Zhang, X., Poo, M.M., Localized synaptic potentiation by BDNF requires local protein synthesis in the developing axon. *Neuron* 2002; 36: 675–688.

Zita, M.M., Marchionni, I., Bottos, E., Righi, M., Del Sal, G., Cherubini, E., Zacchi, P. Post-phosphorylation prolyl isomerisation of gephyrin represents a mechanism to modulate glycine receptors function. *EMBO J* 2007; 26:1761-71.

Zucker, R.S., Regehr, W.G., Short-term synaptic plasticity. *Annu. Rev. Physiol.* 2002; 64: 355–405.

Zweier, C., de Jong, E.K., Zweier, M., Orrico, A., Ousager, L.B., Collins, A.L., Bijlsma, E.K., Oortveld, M.A., Ekici, A.B., Reis, A., Schenck, A., Rauch, A., CNTNAP2 and NRXN1 are mutated in autosomal-recessive Pitt-Hopkins-like mental retardation and determine the level of a common synaptic protein in Drosophila. *Am J Hum Genet.* 2009; 85:655-66.

ACKNOWLEDGMENTS

I am deeply grateful to my supervisor Prof. Cherubini for his continuous support, encouragement and especially for creating a very pleasant lab atmosphere. I will treasure most of his teaching.
A special thank is for Laura and Marilena. Your friendship has been very important through my PhD (but remember that I still remember that Monday ;-).
I am also very grateful to Claudia, Assunta, Roberta, Michele, Sudhir and Zeynep.
Thanks to Angelo, Angela, Giada and Elettra for sharing lunch break and endless smile ;-).
A big thank is for Paola, I am sure I will miss funny moments we shared :-).
Last but not least I would like to thanks all the friends known at SISSA.