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STUDIES ON THE ROLE OF SYNAPTIC ACTIVITY IN SYNAPSE DEVELOPMENT AND GENE EXPRESSION

Jildau Bouwman

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STUDIES ON THE ROLE OF SYNAPTIC ACTIVITY IN SYNAPSE DEVELOPMENT AND GENE EXPRESSION

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INTRODUCTION

Chapter 1

The mammalian brain controls many body functions and is essential for emotions and cognitive behavior, such as learning. Networks of nerve cells (neurons), which are the basic entities of the brain, process the information received from internal and external environments and coordinate physiological responses to these signals. The communication between neurons in the network occurs predominantly via the release of signaling molecules at specialized contact places, the synapses. Long extensions (axons and dendrites, together called neurites) make it possible to communicate between neurons. One of the major goals in neurobiology is to understand how neuronal networks develop and how these networks mature to form the adult brain.

During early stages of brain development the outgrowing neurites are already capable of releasing neurotransmitters. Several studies indicate that this developmental release has a role in network formation. After synapses have formed, these connections remain plastic; they can be strengthened or weakened as a result of previous experiences. This process is called synaptic plasticity. Synapse formation and synaptic plasticity may have several features in common. For instance, synaptic activity has a similar role in network formation and synaptic plasticity. As will be discussed below in greater detail, during synaptic plasticity and synapse formation gene expression is essential for sustained effects of synaptic activity. This activity-dependent gene expression may be essential for functional and morphological changes, both in development and in synaptic plasticity. The relation between synaptic activity and gene expression has been studied *in vitro* and *in vivo*.

This introduction will mainly focus on our current knowledge of the role of synaptic activity in neural development. Particularly, we will discuss the induction of gene expression by synaptic activity.

1 - Development of the nervous system

Three stages can be distinguished in the development of the nervous system: neurogenesis, synaptogenesis and maturation. During neurogenesis neurons proliferate and migrate to their correct positions and start to form dendrites and axons. Filopodial enlargements, called growth cones, at the end of outgrowing axons are guided by guidance cues from other cells. When the target is reached the formation of the synapse (synaptogenesis) starts. Synapses can then be either eliminated or stabilized and remain, this stage is called maturation.

For this thesis mainly the synapse formation and maturation is of importance, therefore these processes will be discussed in the next chapters in more detail.

1.1 Synaptogenesis

Communication between neurons is primarily restricted to the place where two cells contact each other (i.e., the synapse). The synapse consists of three elements: the presynaptic terminal, the postsynaptic target membrane and the synaptic cleft. In the presynaptic terminal, neurotransmitters are stored in vesicles. When an action potential reaches the terminal, neurotransmitter is released into the synaptic cleft, where it can diffuse to the postsynaptic target cell. In the postsynaptic cell membrane, neurotransmitter receptors are present to detect the released neurotransmitter. This triggers depolarization of the postsynaptic cell or activation of postsynaptic second messenger pathways.

Communication between growth cones and possible targets can initiate synapse formation. Some neurons form an overabundance of synapses, of which later in development several will be eliminated. Competition between neurons for target-derived factors is thought to be a central part in this elimination process (Vicario-Abejón, *et al.*, 1998). Pre- and post-synapse are considered to recognize each other by cell-specific surface proteins (like cell adhesion molecules) (see for review Bolz *et al.*, 1996). Their contact triggers intracellular signals, like calcium, that causes the pre-existing synaptic machinery to assemble (see for review Haydon and Drapeau, 1995; Marzban *et al.*, 2003). Incorporation of 80 nm dense-core vesicles in the presynapse delivers structural and functional components necessary for synaptic activity. The content of these vesicles influences the differentiation of the post-synapse (see for review Garner *et al.*, 2000). Time-lapse studies show that first functional presynaptic sites are formed (within 30 min after contact) and then postsynaptic receptor clusters are formed (after approximately 1 to 2 hours after initial contact; Friedman *et al.*, 2000). During synaptogenesis the pre- and postsynaptic terminal are thought to communicate with each other via the release of compounds or by interaction of molecules on the cell membrane. Synaptic release, for instance, can regulate filopodial motility and dendritic arbor growth and thus alter the number and stability of initial contacts (Cline, 2001; Lendvai *et al.*, 2000). In the TrkB and TrkC null mutant the number of synaptic contacts is lower, indicating that the release of neurotrophins is involved in the formation of synapses (Martinez *et al.*, 1998). Finally, the large diversity of cell adhesion molecules is most likely important for the formation of synapses (see for review Washbourne *et al.*, 2004). Especially the classical cell adhesion molecules, like cadherins and neuroligin-neurexin complexes are known to be involved in the initial synapse formation (see review Garner *et al.*, 2002).

1.2 Maturation of synapses

After initial synapse formation, synapses are modified both morphologically and functionally. We will define these adjustments of the synapse after initial synaptic

assembly as synaptic maturation (see also Zhang and Poo, 2001). For instance, myelination of axons is part of this maturation (see Itoh *et al.*, 1997). Myelination was used for a long time as criterion for maturation, as it is a clear morphological defined step in development. However, now it is known that most axons in the CNS are unmyelinated, and myelination is only one of the many steps in maturation (Pfrieger, 2002). During maturation of synapses the synaptic strength increases, which is probably caused by the recruitment of pre- and postsynaptic compounds. Other important aspects that can be distinguished at this point are the change in subunit composition of the neurotransmitter receptors, the increase in the number of receptors, and the recruitment of several scaffolding molecules, together strengthening the synapse. In addition, at this stage of development the postsynaptic side forms dendritic spines (Fiala *et al.*, 1998). Presynaptically, the number of synaptic vesicles increases during maturation (Mohrmann *et al.*, 2003) and the neurotransmitter that is released from the synapse can be changed (Nabekura *et al.*, 2004).

Communication between the pre- and postsynapse is important for synaptic maturation. For instance, the incorporation of certain subunits of NMDA and AMPA receptors in the post-synaptic membrane involves several activity-dependent pathways (Sheng and Kim, 2002; Crump *et al.*, 2001). In addition, in cultured rat neocortical neurons and in BDNF null mutant brain slices neurotrophins increase the protein levels of AMPA (Itami *et al.*, 2000; Narisawa-Saito, 1999). In addition, in the TrkB and TrkC null mutant the number of vesicles in the presynapse and the RNA levels of proteins involved in synaptic activity are lower. This indicates that presynaptic maturation is influenced by neurotrophins (Martinez *et al.*, 1998).

Moreover, cell adhesion molecules are important for the maturation of synapses. For instance, integrins are required for the functional maturation of synapses in the hippocampus (Chavis and Westbrook, 2001). Second, NCAM is necessary for the recruitment of the naturally occurring NMDA receptor switch in heterogenotypic cocultures of NCAM-deficient and wild-type neurons (Sytnyk *et al.*, 2002). Third, ephrin's and their receptors (EphB receptors) have been shown to promote the clustering of NMDA receptors (Dalva *et al.*, 2000). Finally, the expression of glycoproteins changes during the maturation of synapses and may play a role in cell adhesion (see for review Yamaguchi, 2002).

2 - Signaling in the nervous system

The preceding sections show that communication between the pre- and postsynaptic terminal is involved in several aspects of brain development. The next paragraphs will discuss the most important ways of communication between neurons in the nervous system.

2.1 Small clear cored vesicle release

Communication between neurons occurs in the adult brain mainly via fast chemical synapses. This method of communication is accomplished by release of neurotransmitter-filled synaptic vesicles (exocytosis) in response to calcium influx. Membrane fusion between vesicle and cell membrane during synaptic vesicle release involves many highly coordinated steps that result in a fast and spatially restricted process.

These efficient membrane fusion reactions require a conserved family of proteins called SNAREs (soluble N-ethylmaleimide-sensitive fusion protein (NSF)-attachment protein (SNAP) receptors). For fusion of membranes both a t-SNARE (at the target membrane) and a v-SNARE (on the vesicle) is needed. The synaptic vesicle protein Synaptobrevin2 (also known as VAMP2) and the plasma membrane-associated proteins Syntaxin-1 and SNAP-25 (Sollner and Rothman, 1996) are involved in synaptic release. These three proteins form a complex (the SNARE or core complex) (Sollner *et al.*, 1993), which is thought to facilitate the mixing of membrane lipids of the synaptic vesicle with the cell membrane (Chen *et al.*, 1999; McNew *et al.*, 2000). Other proteins are involved in the process of membrane fusion. For instance, members of the Sec1/Munc18 family (SM proteins) are essential for membrane fusion. Genetic deletion of the SM protein *munc18-1* results in a complete blockade of synaptic vesicle release (Verhage *et al.*, 2000). There has been no report of other protein deletions with such a drastic effect; even the deletion of some members of the SNARE complex does not result in a complete block of vesicle fusion (Schoch *et al.*, 2001; Washbourne *et al.*, 2002). In development, neurotransmitter release during outgrowth was thought to have two functions: first to add membrane to make elongation of axons possible and second to guide the outgrowth. In the *munc18-1* mutant, however, fibers pathways are found. Thus, synaptic vesicle release may have other functions in brain development, such as maturation of synaptic contacts.

2.2 Large dense cored vesicle release

Besides the release of classical neurotransmitters, synapses are capable of releasing other compounds like neuropeptides and neurotrophins. Neuropeptides and neurotrophins are stored in large dense core vesicles (LDCV) and co-released with neurotransmitters (Berg *et al.*, 2000; Wang *et al.*, 2002). The release of neurotrophins from axon terminals is increased by depolarization, calcium influx via N-type calcium channels and mobilization of calcium from intracellular stores (Wang *et al.*, 2002). Several of the proteins involved in the release of synaptic vesicles are shown to be involved in the release of LDCV as well. For instance, the calcium sensor synaptotagmin I is required for rapid, highly Ca^{2+}-sensitive release of both synaptic and LDCV vesicles. In addition, Munc18-1 is involved in both forms of release (Verhage *et al.*, 2000; Voets

et al., 2001). Tetanus toxin inhibits NT-3 release, suggesting that intact Synaptobrevin or Synaptobrevin-like molecules are required for LDCV release (Wang *et al.*, 2003). In PC12 cells Rabphilin and Noc2 were shown to be involved in LDCV release (Arribas *et al.*, 1997; Haynes *et al.*, 2001) and are probably recruited to dense-core vesicles through specific interaction with Rab27A (Fukuda *et al.*, 2004).

2.3 Retrograde signaling

Several studies indicate that, in addition to signals from pre- to postsynapse in so called anterograde signaling, the postsynapse can also signal to the presynapse. Retrograde messengers are hypothesized, because the postsynapse can affect presynaptic strength. These messengers may be soluble and released from vesicles in the postsynapse or may be membrane permeable and cross the post- and presynaptic membrane to exert its function. This signaling is thought to occur in response to activation of the presynapse.

For example, neurotrophins are hypothesized to act as retrograde messengers, as they can increase synaptic efficacy (see for review Schmidt, 2004). Several studies examined the activity-dependent release of neurotrophins from neurons. Most likely, the release of neurotrophins is induced by calcium that originates from internal calcium stores. For instance, NGF is released when hippocampal slices or cultured cells are activated by potassium-ions, carbachol (an acetylcholine receptor agonist) or veratridine (see for review Bonhoeffer, 1996). In addition, glutamate or depolarization with high concentrations of potassium activates the release of NGF and BDNF in hippocampal slices and primary hippocampal neuronal cultures. Neurotrophin secretion is blocked by the intracellular, high-affinity calcium chelator BAPTA and by blockade or depletion of intracellular calcium stores by dantrolene or thapsigargin (see Canossa *et al.*, 1997). The calcium stores are probably opened by activation of the inositol-triphophate pathway by neurotransmitter receptors in the membrane. Neurotrophins may originate from the postsynapse, since BDNF-GFP fusion protein is released upon high-frequency stimulation from postsynaptic structures of glutamatergic synapses in hippocampal neurons (Hartmann *et al.*, 2001), However, the neurons may also have axonal BDNF-GFP expression, because axons often grow along a dendrite and the axonal localization of BDNF-GFP may be undetected. Therefore, the effects of neurotrophins on the presynaptic cell may still be an effect of presynaptic release of these compounds.

Anterograde release of neurotrophins has been shown to be essential for presynaptic forms of long-term potentiation. For instance, restricted genetic deletion of BDNF in specific areas of the hippocampus showed that presynaptic derived BDNF is required for the induction of presynaptic potentiation (Zakharenko *et al.*, 2003).

In addition to neurotrophins, the membrane-permeable compounds nitric oxide,

endocannabinoids and arachidonic acid are thought to function as retrograde messengers as well. Nitric oxide (NO) can influence the presynaptic vesicle cycle and enhance neurotransmitter release (Micheva *et al.*, 2003; see for review Medina and Izquierdo, 1996). Nitric oxide synthase (NOS), the NO producing enzyme, has been shown to be located postsynaptically (Burette *et al.*, 2002). Furthermore, injecting NOS inhibitor in postsynaptic cells inhibits presynaptic LTP (see for review Medina and Izquierdo, 1996). Therefore, NO is a possible candidate to function as a retrograde messenger.

Arachidonic acid can influence neurotransmitter release, but the postsynaptic origin of this compound has not yet been shown (see for review Medina and Izquierdo, 1996). Therefore, arachidonic acid is not yet proven to function as a retrograde signal.

In addition, endocannabinoids may act as retrograde messenger. Endocannabinoid receptors are located presynaptically and agonists of endocannabinoids can induce presynaptic depression (Wilson and Nicoll, 2002; see for review Alger, 2002). Immunocytochemistry showed expression of FAAH, an enzyme, which produces an endocannabinoid in the cell bodies of Purkinje cells. Besides, CB1, a cannabinoid receptor, was expressed in the axons of granule cells and basket cells, neurons that are located presynaptically to Purkinje cells (Egertova *et al.*, 1998).

In conclusion, some compounds that influence presynaptic strength may originate from the postsynapse, however only for NO the function as retrograde messenger is proven. Retrograde signaling is most likely important for the regulation of synaptic strength, but which pathways are involved is still a matter of debate.

3 - Activity dependent adjustments of synapses

Synapses are plastic subcellular structures, of which both function and morphology can be influenced by activity. The effects of synaptic activity on synapse function and structures are mainly studied in adult neurons during synaptic plasticity. However, similar mechanisms have been shown to be important during the development of synapses as well. For two reasons it is difficult to study the effects of acvitity on the synase in great detail. First, functional and morphological changes can not always be separated, as functional changes can be accomplished by changes in synapse morphology and vise versa. Second, only the general effect of activity in the network on synapses can be studied, since action potential propagation leads to synaptic release. The synaptic release will in turn activate postsynaptic receptors. For that reason, affecting either one of these sequential events will also affect the activity of the others.

Several model systems, for instance the neuromuscular junction and the neocortex, have been exploited to analyze the activity-dependent changes in synapse function

and morphology. Most of them were either chosen for their accessibility or because their activity is easily blocked. Activity is often adjusted using pharmacological agents or by making lesions in certain brain areas.

3.1 Activity dependent functional changes

Synapses can be strengthened or weakened depending on the pattern of activity they experience. If the postsynaptic cell is activated just a few milliseconds after activation of the presynaptic cell, the synapse is often made stronger. Long-lasting enhancements of synaptic connections are called long-term potentiation (LTP). This process is important for the remodeling of neuronal circuits during development and for synaptic plasticity. Numerous studies show the relation between activity and synaptic strength during synaptic plasticity. However, less information is available about this relation during synapse development.

Some studies show an activity-dependent upregulation or change in the identity of receptors in the postsynaptic membrane during synapse development. For instance, some newly formed glutamatergic synapses only contain NMDA receptors. During maturation, AMPA receptors can be recruited to the synapse (Liao *et al.*, 2001). This insertion of AMPA receptors in the post-synaptic membrane probably depends on protein kinase A (Watson *et al.*, 2006). In addition, the subunit composition of the NMDA-receptor changes during synapse development depends on activity (Williams *et al.*, 1993).

In the presynapse, activity can change the neurotransmitter type that is released. For instance, in the rat, lateral superior olive neurons change from predominantly releasing GABA to predominantly releasing glycine (Nabekura *et al.*, 2004). Moreover, NMDA receptor activity modulates the maturation of GABAergic transmission in the colliculus by regulating the expression of the GABA synthesizing enzyme glutamate decarboxylase. During development potentiation of synapses is quickly reversed, which suggests long periods of activation are needed for effective induction of stable synapses (Zhou *et al.*, 2003). This indicates that synaptic adjustments can be made both in immature and mature synapses. However, the relation between synaptic activity and synaptic strength changes with time. For example, activation of Xenopus nerve-muscle cultures by brief bursts of action potentials only induced LTP in immature synapses with a low synaptic strength and not in mature synapses (Wan and Poo, 1999).

3.2 Activity dependent morphological changes

In addition to functional effects on the synapse, activity can also affect the shape of synapses, especially the postsynaptic spine and the number of terminals of the presynapse. For instance, during embryonic development each muscle fiber is

innervated by multiple motor axons and each motor axon innervates multiple muscle fibers. However during maturation of the neuromuscular junction the surplus of the synapses is eliminated and eventually only one synapse innervates one muscle fiber. For this process synaptic activity is essential (see for review Lichtman *et al.*, 2000). In the central nervous system synaptic activity is important for the regulation of innervation as well. Neurons in the visual cortex are organized in columns that respond mainly to one of the eyes (ocular dominance columns). These columns are formed before birth and only remain intact when synaptic activity is present (see for review Cohen-Cory, 2002). A similar rewiring of synapses is seen in the lateral geniculate nucleus (LGN). Blocking electrical activity by TTX or synaptic activity using glutamate receptor blockers in projections of the LGN prevents the segregation of eye-input (Shatz, 1996). In addition, the axons project primarily to the sub-plate of cortical areas, which is normally bypassed, rather than projecting to the visual cortex (Catalano and Shatz, 1998). Furthermore, synaptic transmission is involved in the formation of the barrel map in the primary somatosensory cortex (Lu *et al.*, 2006). Besides rewiring of synapses the presynaptic site of the synapse can also change morphology itself. Blocking activity by TTX changes the morphological development of retinogeniculate axon endings in the retinogeniculate pathway of the cat. The synaptic junctions in TTX treated animals were much larger than in the non-treated animals (Kalil *et al.*, 1986). Moreover, induction of activity using theta burst stimulation in hippocampal organotypic cultures increased the number of presynaptic protrusions (Nikonenko, 2003).

At the postsynaptic site the shape and the motility of the spine is influenced by the amount of synaptic activity as well. Deprivation of sensory input reduces the motility of protrusions of the developing barrel cortex (Lendvai *et al.*, 2000). However, activation of postsynaptic receptors in hippocampal cell cultures reduces the motility of spines (Fischer et al, 2000). From these contradictory data it is uncertain whether activity can increase or decrease spine motility. However, the morphology of spines is influenced by synaptic activity.

Moreover, active axons can influence their own myelination. Action potential firing of the axons can activate astrocytes, which in turn stimulate the oligodendrocytes to myelinate the axons (Ishibashi *et al.*, 2006).

In conclusion, activity can change the wiring of the brain and the morphology of synapses. How this activity dependent morphology is accomplished at the cellular and molecular level is yet unclear. For long-term morphological effects gene expression is most likely involved.

4 - Activity dependent gene expression

For consolidation of activity-dependent changes, induction of gene expression is needed (see for review Lynch, 2004). For instance, induction of LTP in several brain areas has been shown to induce expression of several early-response genes (especially transcription factors) and late response genes (like receptors; see for review: Lynch, 2004). This gene expression is probably important to produce proper connectivity and may be necessary to induce the levels of proteins involved in synapse function and structure. Moreover, this activity-dependent gene expression seems to be important for consolidation of the synapse (Bozon *et al.*, 2002; van Kesteren *et al.*, 2001). In this way activity-dependent gene expression may be essential for the formation of the adult brain. The transcription of some genes involved in synapse function and structure has been shown to be influenced by activity, especially the expression of NGF, BDNF and some neurotransmitter-receptors has been studied.

4.1 Neurotrophins

The relationship between activity and transcript levels is thoroughly studied for the neurotrophins. Their levels were found adjusted in several model systems using different ways to alter activity.

Neurotrophin levels in the visual system and the hippocampus increase upon stimulation, *in vivo* and *in vitro*. For instance, the RNA levels of NGF and BDNF are regulated by subtle light input (Castrén *et al.*, 1992). Maintaining rats in darkness reduced the BDNF levels in the cortex to about half of the control level, but NGF mRNA levels were unaffected (Lindholm *et al.*, 1994; Cellerino and Maffei, 1996). Intraocular injection of TTX, which blocks the electrical activity of retinal ganglion cells, also reduces BDNF transcript levels (Cellerino and Maffei, 1996). In the hippocampus of adult rats NGF and BDNF transcript levels increase a little when rats are kept in a stimulus-rich environment (Falkenberg *et al.*, 1992). Electrolytic lesions, that cause limbic seizures, increase NGF and BDNF mRNA levels in the hippocampal hilus of adult rats (Gall and Isackson, 1989). In cultured hippocampal neurons depolarization produced by an increase in potassium concentration or by veratridine (a sodium-channel inactivation inhibitor) increases NGF and BDNF mRNA levels (Zafra *et al.*, 1994). Blocking activity using kainate acid, an agonist of the non-NMDA-type glutamate receptors increases the transcript levels for NGF and BDNF in several brain areas *in vivo* (Zafra *et al.*, 1990). In addition, the activity-dependent increase during development and subcellular localization of BNDF transcripts have been shown to be isoform dependent (Pattabiraman *et al.*, 2005)

These studies show, that BDNF and NGF mRNA levels in the visual cortex and hippocampus are regulated by activity. The regulation of NGF and BDNF expression

is thought to be mediated through voltage-gated calcium channels, NMDA and non-NMDA receptors. This leads to an increase in intracellular calcium-concentration, which regulates gene expression via the calcium-response factor (CaRF), a transcription factor (Tao *et al.*, 2002). Moreover, calmodulin inhibitors block the activity-dependent increase in BDNF mRNA, which suggests that the activity-dependent effects are mediated by calcium-calmodulin-dependent protein kinases. However, in calcium-calmodulin-dependent protein kinase II alpha null mutants, activity-dependent induction of BDNF mRNA levels is not impaired. This indicates that other calmodulin-dependent mechanisms may be involved in regulating the BDNF mRNA levels (Lindholm *et al.*, 1994).

Only a few studies have examined the relation between synaptic activity and the expression of the neurotrophin receptors. One study shows that activity is not involved in the expression of Trk receptors; TrkB receptor levels increase during development, but dark-reared animals show the same protein level and pattern of TrkB RNA level induction compared to age-matched, normally reared controls (Tropea and Domenici, 2001).

4.2 Neurotransmitter-receptors

Not only the expression of neurotrophins is affected by activity. Several studies describe an effect of activity on the expression of neurotransmitter-receptors, especially for NMDA, acetylcholine (AChR), and GABA receptor subunits.

For instance, the levels of the NR1 and NR2B transcript (which code for NMDA-receptor subunits) in the rat superior colliculus change dramatically during the first postnatal weeks. When AP-5, a blocker of the NMDA receptor, is chronically applied to the colliculi, this increase in NR1 transcript levels fails to occur. This suggests that activity dependent upregulation of NR1 normally takes place in this system (see for review Scheetz and Constantine-Paton, 1994). NR2A mRNA levels in neocortical cultures are increased by activity, as blocking the postsynaptic glutamate receptors in these cultures resulted in a lower of NR2A transcript levels. In contrast, NR2B RNA levels were not influenced by this treatment. Moreover, as shown pharmacologically, the regulation of NR2A mRNA levels was mediated by postsynaptic Ca^{2+} influx through both NMDA receptors and L-type Ca^{2+} channels (Hoffmann *et al.*, 2000). Activity is also important for the synaptic targeting of NMDA, via the activity-dependent increase in Drebrin A (Takahashi at al., 2006). In summary, these studies demonstrate that activity is a key factor in the regulation of NMDA receptor subunit composition during neocortical development.

During development, the levels of AChR protein and transcript increase in avian neurons. This increase coincides with the time when pre- and post-synaptic connections are formed. The normal 10- to 15-fold increase in the β2-subunit mRNA

levels does not occur in chick optic neurons deprived of innervation. It was shown that mRNA levels of the α3, α4 and α5-subunits are reduced to 60 % of the control situation in absence of innervation or by input-deprivation (see for review Schwartz *et al.*, 1995). Also the levels of extrasynaptic α7-nAChR-subunit are regulated by activity (Brumwell *et al.*, 2002). This suggests a role for activity in the regulation of AChR subunit-composition and levels. For the induction of nAChR transcript levels calcium/calmodulin-activated protein kinase II is probably necessary (Macpherson *et al.*, 2002).
Besides upregulation of excitatory neurotransmitter-receptors, also the GABA receptors are upregulated by synaptic activity. Evoked seizures increases $GABA_B$-mediated responses and $GABA_B$ receptor binding in thalamus of the adult rat. Blocking the input from one eye, using TTX, reduced the RNA levels of $GABA_B$ receptors in the dorsal lateral geniculate nucleus (LGN). A similar decrease was shown for the α1, α2, α4, α5, β1, γ2 subunits of the $GABA_A$ receptor in response to monocular deprivation (Muñoz *et al.*, 1998). These results indicate that activity induces both the inhibitory and the excitatory system.
In conclusion, these investigations imply that activity regulates the amount of mRNA of receptors in the postsynaptic cell. Influx of calcium is probably the main second messenger that regulates the expression of receptors. However, whether all receptors are regulated by the same magnitude of influx of calcium is unknown.

4.3 Proteins of the nerve terminal

In the presynapse, activity influences the expression of neuropeptides, and neurotransmitter producing enzymes. This effect can be caused by three processes, by retrograde signaling from the postsynaptic cell, by depolarization of the cell itself or via a feedback in the network. For example, making lesions in the cortex decreases the RNA levels of VGF, a secretory-peptide precursor, in the ipsilateral cortex (Snyder, *et al.*, 1998) and increases the RNA levels of glutamate decarboxylase (GAD), cholecystokinin, neuropeptide Y, and somatostatin in the homolateral cortex (Jacobs, *et al.*, 1994). Neuronal stimulation with the depolarizing agent veratridine increases the RNA and protein levels of somatostatin in cortical cultures (Tolon, *et al.*, 1996). Furthermore, blocking activity in the dorsal lateral geniculate nucleus by intraocular tetrodotoxin injection rapidly decreases the transcript levels of VGF (Snyder, *et al.*, 1998). Blocking GABA-ergic inhibition in hippocampal slice cell cultures of 7-day old rats increases the number of Somatostatin and Neuropeptide Y immunoreactive cells, whereas blocking the glutamatergic excitatory activity decreases the amount of immunoreactive cells (Marty and Onteniente, 1997). When activity is blocked in the primary visual cortex of a monkey by monocular deprivation the RNA levels of GABA and glutamic acid decarboxylase (GAD) are profoundly decreased. This decrease in immunoreactivity is restricted to the ocular dominance columns that had previously

received input from the removed eye. Similar effects were observed after monocular injection of TTX (Benson *et al.*, 1994). When the striatum is denervated the mRNA levels of preproenkephalin are increased by at least 55 % (Campbell and Bjorklund, 1994). During development, regulation of neuropeptide levels by activity has also been shown (see chapter 4).

In addition to genes coding for secreted compounds, also genes coding for proteins that are involved in vesicle release are regulated by activity. Blocking synapses of the chick ciliary ganglion by preventing reuptake of choline, increased the mRNA levels of the synaptic vesicle protein synaptophysin II, but not of synaptotagmin I (Plunkett *et al.*, 1998). Increasing the calcium influx by expression of the P/Q-type calcium channel or adding an ionophore to HEK293 cells increases the transcript levels of syntaxin-1A (Sutton *et al.*, 1999). When activity is induced by electrical stimulation (0.6 Hz) in the hippocampal dentate gyrus the mRNA levels of the synaptic vesicle proteins, syntaxin 1B and synapsin I, are upregulated (Hicks *et al.*, 1997).

Thus, activity modulates the mRNA levels of synaptic vesicle proteins, neuropeptides, and neurotransmitter producing enzymes. This upregulation of presynaptic compounds may increase synaptic transmission during development, and have a role in induction of LTP.

4.4 Other proteins

The expression of several other proteins has been shown to be regulated by activity, including cytoskeleton-associated proteins, and cell adhesion molecules, which will be described in the next section. Influencing the expression of these structural proteins may be responsible for the morphological effects of synaptic activity.

During development the mRNA level of the cell adhesion molecule N-cadherin in myotubules is decreased. This normal decrease in N-cadherin can be prevented by blocking activity with d-tubocurare in cell culture (see Itoh *et al.*, 1997). Several other cell adhesion molecules are regulated by activity, however their induction is cell type and frequency dependent. For instance, the transcript levels of L1 are downregulated by specific frequencies of electrical impulses in mouse DRG neurons *in vitro*. Stimulation of dorsal root ganglia with 0.1 Hz, but not with 0.3 or 1 Hz gave a 13-fold downregulation of the L1 mRNA. The levels of NCAM mRNA were not significantly altered by any of the frequencies, whereas all frequencies of stimulation decreased N-cadherin mRNA levels (Itoh *et al.*, 1997). Thus, different cell-adhesion molecules are targets for activity-dependent regulation, but their regulation depends on cell type and stimulus. Activity may regulate target selection in this way.

An unanswered question remains: How does the cell make a distinction between different stimulation frequencies in its gene expression? It is possible that a critical calcium concentration is needed for the activation of gene expression. This critical

calcium concentration may be different for various proteins, for instance by activating distinct calcium-dependent pathways that have different calcium sensitivities (see for review Bito, 1998).

Several other structural proteins are upregulated by activity. For instance, the mRNA levels of neuritin, a GPI-anchored protein, which is expressed on the surface of neurons and promotes neurite outgrowth, are induced by activation of kainate glutamate-receptors. When hippocampal and cortical cultures were treated with NMDA, AMPA or depolarizing concentrations of KCl the mRNA of neuritin was increased 5-fold. In the visual cortex physiological light-induced neuronal activity increases neuritin mRNA levels (Naeve *et al.*, 1997).

The transcript levels of the cytoskeleton-associated protein Arc are increased in the hippocampus of high frequency stimulated rats. The same raise in Arc mRNA levels was found in cultured PC12 cells (Lyford *et al.*, 1995). AMPA receptors have recently been shown to be involved in the regulation of Arc transcription (Rao *et al.*, 2006). In addition, Arc can be induced by cAMP and calcium (Waltereit *et al.*, 2001), this induction requires the activity of protein kinase A and mitogen-activated protein kinase (Waltereit *et al.*, 2001). Because lowering the expression of structural proteins will influence synaptic strength, morphological changes induced by synaptic activity and induction of LTP may be intermingled.

Expression of alivin, a protein that promotes survival of neurons, is regulated by KCl and/or NMDA concentrations in cultured cerebellar granule neurons. Alivin mRNA levels are regulated by the Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels. Levels of alivin mRNA in cultured cortical neurons were lower when their electrical activity was blocked by tetrodotoxin (Ono *et al.*, 2003). How alivin regulates survival of neurons cannot be deduced from these data, but it shows that the survival of neurons depends on synaptic activity.

A few studies examined activity-dependent changes in gene expression of large groups of genes. For instance, using microarray hybridization a specific increase in the RNA levels of proteins involved in intracellular structure and vesicular trafficking by activation of NMDA-receptors was shown (Hong *et al.*, 2004). Changes in RNA levels induced by seizures in area CA1 were studied using cDNA microarrays containing 9000 genes. Fourteen transcripts were more then 1.8 times regulated. One of them was coding for the orphan nuclear receptor, Nur77, which had 12 times higher trancript levels in the dentate gyrus after seizures as was shown by in situ hybridization (French *et al.*, 2001). Kainate, an agonist of non-NMDA-glutamate receptors, increases the mRNA levels of 52 genes in the hippocampal dentate gyrus. Several of these genes encoded transcription factors, or vesicle- and synapse related proteins (Nedivi *et al.*, 1993). In Drosophila seizure mutants, the increased activity leads to a changed mRNA level of 250 trancripts, including transcripts coding for cell adhesion molecules, membrane excitability and cellular signaling proteins (Guan *et al.*, 2005)

Summarizing, several molecules have been identified that are regulated by activity, including: cytoskeleton-associated proteins, cell adhesion molecules, neuropeptides, neurotrophins, transcription factors, and receptors. These effects may result in physiology and morphology changes of the synapse. These changes may be responsible for the activity-dependent modulation of synaptic efficacy in neural circuit development and experience-dependent plasticity. The structural and physiological effects are most likely not separate effects, since both effects may change the strength of a synapse.

Several second messenger pathways are involved in the activity-dependent regulation of gene expression. Especially, the influx of calcium-ions through NMDA, non-NMDA and voltage gated calcium channels is thought to have a role in this regulation. In addition, the CAMKII-pathway and the MAPK-pathway are involved in activity-dependent gene expression (Brosenitsch *et al.*, 2001; Wu *et al.*, 2001).

5 - Short-term versus long-term effects of activity

Some of the effects of activity on the synaptic strength are mediated within minutes and are probably a consequence of posttranslational modifications, because gene expression takes hours to have an effect. For instance, the activity-induced cell surface expression of PSA (a regulator of cell surface interaction that is bound to NCAM) and NCAM is restored within 1 hour when the pre-existing PSA is enzymatically removed. This restoration is too quick to be explained by induction of gene expression (Kiss *et al.*, 1994).

In addition, BDNF application to microcultures of postnatal rat hippocampal neurons, enhanced the synaptic transmission by 143 %. This increase in synaptic transmission is an acute effect, because BDNF was applied during the recordings (Leßmann and Heumann, 1998). The enhancement of the synaptic transmission is accompanied by a 3-fold increase in the NMDA receptor open probability, which was abolished by the Trk-receptor blocker k252A indicating that Trk-receptors are involved in this process (Levine *et al.*, 1998).

NGF has been shown to modulate calcium current in PC12 cells within minutes, which indicates that NGF may have local effects (Jia *et al.*, 1999). Local effects of NGF may be exerted by NO, because NGF can reduce the NOS activity to 61% of the baseline levels (Lam, *et al.*, 1998).

In conclusion, some of the structural and physiological changes of synapses during development are rapid and transient, these effects will be induced in other ways than gene expression. Possibly, posttranslational modifications are involved in these short-term effects.

6 - Retrograde regulation of presynaptic gene expression

The activity-dependent modulation of neurons could either require the activation of the presynaptic neurons themselves, or activation of their target cells (Marty *et al.*, 1997).

Especially neurotrophins are thought to act as retrograde messengers and they have been shown to regulate the expression of several presynaptic genes. Neurotrophins can influence the neurotransmitter phenotype of neurons in the central nervous system by modulating the expression of neuropeptides and neurotransmitter synthesizing enzymes (see Cellerino and Maffei, 1996). In particular, BDNF can regulate the expression of several neuropeptides in distinct neurons. For example, BDNF regulates the neuropeptide expression in cortical and hippocampal interneurons *in vivo*. At postnatal day 15, BDNF deficient mice show reduced RNA levels of NPY in the neocortex and hippocampal formation, while the number of cells remain normal (see for review Marty *et al.*, 1997). When cultures of immature hippocampal neurons are stimulated by BDNF, the NPY transcript levels in the GABA-containing neurons are increased (Marty *et al.*, 1997). In addition, an increase in GAD and proenkephalin mRNA levels and a decrease in preprotachykinin caused by denervation of the rat striatum is counteracted by BDNF and NT-4/5 (Sauer *et al.*, 1995).

Besides BDNF, NGF and NT-3 are both also able to change gene expression. For instance, NGF can signal retrogradely to induce RNA levels of both tyrosine hydroxylase and p75 neurotrophin receptor (Toma *et al.*, 1997). In addition, in hippocampal neurons NT-3 stimulates the increase in neuropeptides transcripts in GABA-containing neurons. All these effects can be blocked by k252A, a tyrosine-kinase inhibitor, with a preference for Trks (see for review Thoenen, 1995).

Not only the expression of neuropeptides, neurotransmitter synthesizing enzymes, and receptors are regulated by neurotrophins. Also the mRNA levels of other proteins, which are involved in synapse morphology and transmission, are affected by neurotrophins, like neuritin and calbindin. Neuritin message can be induced by an intraventricular injection of BDNF into neonatal rat pups in the hippocampus and cortex. Inhibition of TrkB with k252A blocked the induction of neuritin mRNA by BDNF (Naeve *et al.*, 1997). Calbindin (which binds Ca^{2+}) protein levels are induced by NT-3 in hippocampal neurons *in vitro* (see for review Marty *et al.*, 1997).

Another way of investigating the relation between neurotrophins and gene expression is to compare the transcript levels in neurotrophin receptor (Trk) mutant mice with normal mice. In TrkB null mutant mice the synaptic vesicle proteins levels were examined by immunocytochemistry. Especially the synaptic proteins: Synapsin I (38-71 %), Synaptophysin (38-71 %), Synaptotagmin I (31-56 %), Syntaxin I (60-68 %), SNAP-25 (40-56 %), and Rab3a (53-63 %) were reduced in the TrkB null mutant (Martínez *et al.*, 1998).

These studies show that neurotrophin-signaling is responsible for some of the activity-dependent effects on the expression of presynapse proteins. However, because the postsynaptic origin of neurotrophins has not been proven, the effect may also be a feedback mechanism of the presynapse. The effects of neurotrophins on the presynaptic cell may be mediated by the tyrosine kinase receptors (Trk) or the p75-receptor. Activated receptors are retrogradely transported from the nerve terminal to the cell body (see Lam, *et al.*, 1998), where they may change expression.

7 - Aim of this thesis

The aim of this thesis is to analyze the relation between the usage and strength of synapses and the expression of synaptic genes. We hypothesize that especially genes involved in synapse function and structure are regulated by activity. This gene expression may be important for the maturation of synapses, because an increase in synapse structure and function genes may increase the synaptic strength.

To study the role of synaptic activity in development we used the *munc18-1* null mutant. This mutant lacks both evoked and spontaneous secretion and the secretion of large dense core vesicles that contain neuromodulatory substances, is severely impaired. To our knowledge, the *munc18-1* mutant is the first model defective in all these aspects of presynaptic signaling. *Munc18-1* null mutant embryos are completely paralyzed. The mutants are unable to breathe and therefore die shortly after birth. As a consequence, for all measurements embryonic day 18 animals (E18) were used, just before birth (Verhage *et al.*, 2000). Electrophysiological recordings of GABA-ergic neocortical and cholinergic neuromuscular junction synapses at E18 revealed an absolute lack of neurotransmitter release. However, the cells responded normally to exogenously applied neurotransmitter, indicating that post-synaptic receptors were functional. Besides neurotransmitter release, measurements in chromaffin cells and the pituitary showed also a severely reduced release from large dense core vesicles (Voets *et al.*, 2001; Korteweg N. *et al.*, 2005). Electron microscopic analysis of the marginal zone of the cortex of E18 *munc18-1* null indicated that the formation of morphologically defined synapses does not require synaptic activity. Initially all brain structures developed normally but degenerated in order of appearance (i.e., brain structures that developed first also showed degeneration first). However, the cortex was still intact and did not show any signs of degeneration just before birth. Therefore, in this study we analyzed the neocortex of this mutant.

In several other studies lesions or pharmacological agents were used to study the effects of activity on the brain. We now use a novel approach by studying a mutant that lacks activity. Because the environment of neurons in the *munc18-1* null mutant is not affected except for synaptic activity, this mutant is a powerful model to study

effects of synaptic activity on synapse development. In addition, many model systems do not block spontaneous secretion, which is the main form of synaptic activity during synapse development.
Therefore, the analysis of these mice allowed us to answer two important questions concerning the role of synaptic activity in brain development *in vivo*. First, how synaptic activity influences initial brain development and synapse formation. And second, which genes are regulated by synaptic activity during synapse development.

8 - Outline of this thesis

The studies described in this thesis aim to address the role of synaptic activity in synapse formation and developmental gene expression. The *munc18-1* mouse model, which lacks synaptic release, is mainly used to answer these questions.
Chapter 2 aims to unravel the role of activity in synapse development *in vivo*. In this chapter we studied the morphology and number of synapses and localization of synaptic proteins in the *munc18-1* null mutant brain. The ultra-structure of mutant synapses was compared to control animals.
Synaptic usage may be coupled to the expression of synaptic proteins. To examine this, we analyzed transcript levels in the *munc18-1* null mutant and compared it to wild type littermates. In **chapter 3** we exploited microarray technology and quantitative PCR to study RNA levels in general. We hypothesized a feedback mechanism of synaptic activity on the expression of genes involved in this process. For instance, an increase in synaptic activity may induce the expression of synaptic genes. Chapter 4 studies the expression of genes that are important for synaptic signaling, like neuropeptides, neurotransmitter producing enzymes and their receptors specifically. Munc18-1 is binding to several molecules in the presynaptic terminal. Protein levels of these binding partners were shown earlier to be reduced in mutant whole brains. This reduction may be caused by a reduction of gene expression. **Chapter 5** addresses the question whether all differential gene expression in the *munc18-1* null mutant depends on activity, or whether Munc18-1 itself is able to regulate gene expression directly. This chapter describes the relation between the RNA levels of the SNARE complex member, Syntaxin1 and the proteins levels of Munc18-1.
Finally, **chapter 6** summarizes the main findings, attempts to generate a model for the role of synaptic activity in the development of synapses and suggests directions for future experiments.

QUANTIFICATION OF SYNAPSE FORMATION AND -MAINTENANCE *IN VIVO* IN THE ABSENCE OF SYNAPTIC RELEASE

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Chapter 2

ABSTRACT

Outgrowing axons in the developing nervous system secrete neurotransmitters and neuromodulatory substances, which is considered to stimulate synaptogenesis. However, some synapses develop independent of presynaptic secretion. To investigate the role of secretion in synapse formation and –maintenance *in vivo*, we quantified synapses and their morphology in the neocortical marginal zone of *munc18-1* deficient mice which lack both evoked and spontaneous secretion (Verhage *et al.*, 2000). Histochemical analyses at embryonic day 18 (E18) showed that the overall organization of the neocortex and the number of cells were similar in mutants and controls. Western blot analysis revealed equal concentrations of pre- and post-synaptic marker proteins in mutants and controls and immunocytochemical analyses indicated that these markers were targeted to the neuropil of the synaptic layer in the mutant neocortex. Electron microscopy revealed that at E16 immature synapses had formed both in mutants and controls. These synapses had a similar synapse diameter, active zone length and contained similar amounts of synaptic vesicles, which were immuno-positive for two synaptic vesicle markers. However, these synapses were 3 times less abundant in the mutant. Two days later, E18, synapses in the controls had more total and docked vesicles, but not in the mutant. Furthermore, synapses were now 5 times less abundant in the mutant. In both mutant and controls, synapse-like structures were observed with irregular shaped vesicles on both sides of the synaptic cleft. These 'multivesicular structures', were immuno-positive for synaptic vesicle markers and were 4 times more abundant in the mutant.

We conclude that in the absence of presynaptic secretion immature synapses with a normal morphology form, but fewer in number. These secretion-deficient synapses may fail to mature and instead give rise to multivesicular structures. These two observations suggest that secretion of neurotransmitters and neuromodulatory substances is required for synapse maintenance, not for synaptogenesis. Multivesicular structures may develop out of unstable synapses.

INTRODUCTION

In the developing nervous system, outgrowing axons secrete neurotransmitters, such as acetylcholine and GABA, from their growth cones in a quantal manner (Xie and Poo 1986; Sun and Poo, 1987; Gao and Van Den Pol, 2000). Blocking or promoting this quantal secretion was reported to inhibit axon outgrowth and promote synaptogenesis, respectively (Han *et al.*, 1991; Osen-Sand *et al.*, 1993; Kabayama *et al.*, 1999). On the other hand, a variety of studies *in vitro* and *in vivo* suggested that presynaptic neurotransmitter release is not necessary for the formation of normal

synaptic networks. For instance, blocking receptors or the propagation of action potentials does not block the formation of normal synaptic networks in cultured neurons (see for instance Van Huizen *et al.*, 1985, Verderio *et al.*, 1994, but note Nakagami *et al.*, 1997), and eliminating impulse activity in amphibians does not disturb retinotectal projection development (Harris, 1980). Also, neuromuscular junctions form correctly in the absence of postsynaptic neurotransmitter receptors in fish (Westerfield *et al.*, 1990) and develop without synaptic transmission in the fly (Featherstone *et al.*, 2002, Saitoe *et al.*, 2001). In *C. elegans* synaptic connectivity is established, and receptors cluster normally in the absence of one of its most abundant neurotransmitters; GABA (Jin *et al.*, 1999; Gally and Bessereau, 2003), Finally, we recently showed that the mouse brain is assembled correctly, including the formation of synapses, in the absence of quantal secretion (Verhage *et al.*, 2000; see also Varoqueaux, 2002).

Nevertheless, synaptic secretion plays a role in establishing mature neuronal networks. When transmission is blocked, *in vitro* networks mature slower (Van Huizen *et al.*, 1985), massive cell death has been observed *in vivo* (Fawcett *et al.*, 1984; Ikonomidou *et al.*, 1999, Verhage *et al.*, 2000) and in the absence of acetylcholine the mouse neuromuscular junction develops aberrant nerve branching and receptor clustering (Brandon *et al.*, 2003). Furthermore, synaptic activity is important for reshaping of multiple innervation in the neuromuscular junction and the visual system (see Lichtman and Colman, 2000: Chapman *et al.*, 1986; Goodman and Shatz, 1993). Hence, while the initial steps in synapse formation may not always require synaptic activity, the subsequent phases of synapse maturation probably do.

In addition to (evoked) synaptic transmission, nerve terminals continuously secrete transmitters by spontaneous fusion of vesicles with a low incidence. The significance of this spontaneous release for synapse development remains elusive. In *Drosophila* neuromuscular junction spontaneous release was suggested to be necessary for receptor clustering (Saitoe *et al.* 2001). However, another study using similar mutant flies did observe clustering in the absence of spontaneous release (Featherstone *et al.*, 2002). Thus, the role of spontaneous activity remains unclear.

In addition to neurotransmitters, other presynaptically delivered factors, like neuropeptides, neurotrophins and other neuromodulatory substances, are known to be important for synapse formation. In *C. elegans* the presence of the presynaptic cell, but not its main transmitter, GABA, is essential for correct receptor clustering (Gally and Bessereau, 2003) and presynaptic genes like agrin and NARP are essential for synaptogenesis (O'Brien, 2002; see for review Bezakova and Ruegg, 2003).

The *munc18-1* null mutant mouse is a vertebrate model in which both evoked and spontaneous secretion of synaptic vesicles is completely blocked (Verhage et al, 2000), and also the secretion of large dense core vesicles that contain neuromodulatory

substances is severely impaired (Voets *et al.*, 2001; Korteweg *et al.*, 2005). To our knowledge, the *munc18-1* mutant is the first model defective in all these aspects of presynaptic signaling.

To study the role of presynaptic signaling on synapse formation and -maintenance in the developing brain *in vivo*, the *munc18-1* mutant was analyzed in two ways. First, we analyzed the protein levels of pre- and post-synaptic marker proteins by Western blot and their location by immuno-histochemistry. Second, we quantified the number of synapses and analyzed their morphology using electron microscopy at different time points in development.

EXPERIMENTAL PROCEDURES

Animals

Generation of germ line-transmitting *munc18-1*-deficient mice and PCR genotyping was described previously (Verhage *et al.*, 2000). Exons 2 to 5 of the *munc18-1* gene were replaced with a neomycin-resistance gene using homologous recombination. The mouse colony was maintained by breeding of heterozygous animals. Heterozygous males were mated with synchronized heterozygous females for one night. If the following morning a vaginal plug was detected, this was considered embryonic day (E) zero. Embryos were harvested from mothers rapidly sacrificed after timed matings at E16 and E18. Embryo heads were used for electron microscopy and a posterior paw was sampled for genotype analysis. Experimental procedures were carried out with local regulatory approval for animal experimentation, according to the Dutch law for animal welfare. All efforts were made to minimize the number of animals used and their suffering.

Immuno blotting

Mouse brains were obtained at E18 by caesarean section from heterozygous matings. Brains were solubilized with a tissue homogenizer and were then sonicated on ice (3 x 10 sec) in 10 mM Hepes with proteinase inhibitors. Then NaCl and Triton-X100 were added (final concentration: 150 mM and 1%, respectively) and the samples were rotated at 4°C for 1 h. Homogenates were centrifuged for 5 min at 16,100g. Solubilized proteins in the supernatant were quantified using a BCA protein assay (Pierce, Rockford, Illinois, USA) and equal amounts (approximately 50 µg) were loaded on a gradient SDS-PAGE (6 to 15%). Three mutant and three control homogenates were loaded on one gel. Proteins were transferred to a PVDF membrane (Biorad, Hercules, California, USA). Blots were preblocked using 5% blocking agent (Amersham, Buckinghamshire, UK) and immunostained with various antisera. Data were corrected

using Tau (Microtubulin associated protein) as internal standard, which is an abundant protein present in most neurons and not in glial cells and is expressed from early development onwards.
The following monoclonal antibodies were used: munc18-1 (BD Biosciences, Palo Alto, California, USA), syntaxin1 (Sigma, Saint Louis, Missouri, USA, HPC-1) SynaptobrevinII (Synaptic systems, Göttingen, Germany, Cl69.1), SNAP25 (BD Biosciences, Palo Alto, California, USA, SMI81), Tau (Chemicon, Hofheim, Germany, MAB361). The following polyclonals antisera were used: PSD95 (L667), Synapsin I (P610), GDI (D633), Synaptotagmin (W855), Rabphilin3A (I734), Doc2B (13.2).

Immunohistochemistry

E18 mouse were decapitated and the heads were rapidly frozen in liquid nitrogen vapor. Coronal sections of 14 µm were cut with a cryostat (Leica, Jung CM3000) and stored at –20˚ C. Sections were thawed for 30-45 seconds at room temperature, immersed in a solution of 0.1 % paraformaldehyde in 0.1 M PBS (pH 7.4) and immediately irradiated in a microwave oven (30 seconds at 700 W). Then, they were then transferred into 0.01 M PBS (pH 7.4) and processed for immunofluorescence staining.
Briefly, sections were blocked with 10 % NGS/0.05 % Triton X-100 in PBS for 30 min, and incubated overnight at room temperature with primary antibodies diluted in 3% NGS/0.05% Triton X-100 in PBS. We used a mouse monoclonal antibody against synaptobrevin/VAMP-2 (Synaptic System, Göttingen, Germany, clone 69.1; 1:1000) and a rabbit polyclonal antibody against AMPA (GluR2/3; Chemicon, Temecula, California, USA; 1:100). Secondary antibodies were goat-anti-mouse IgG-Alexa Fluor 488 (Molecular Probes, Eugene, Oregon, USA) and goat anti–rabbit-Cy3 conjugate (Jackson ImmunoResearch).
Fluorescent images were captured with a Zeiss (Heidelberg, Germany) AxioCam Hrc camera (using AxioVision 3.0 software) mounted on Nikon (Tokyo, Japan) eclipse E800 microscope with a Nikon Plan Fluor lenses. Images were prepared for printing using Adobe Photoshop. Controls omitting the first antibodies were all negative.

Electron microscopy preparation

E16 and E18 mouse embryos were decapitated. In order to give fast access to the brain for the fixative a few drops of Karnovsky's fixative (Karnovsky, 1965) (4% paraformaldehyde, 5% glutaraldehyde, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 M Na-cacodylate pH 7.4) or tri-aldehyde fixative (2.5% DMSO, 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein in 0.1 M Na-cacodylate pH 7.4) were poured onto the heads, and the skin and skull were peeled off. Brains were severed sagittally between the two hemispheres. The heads were then put in tri-aldehyde fixative for 1h at room temperature, and then

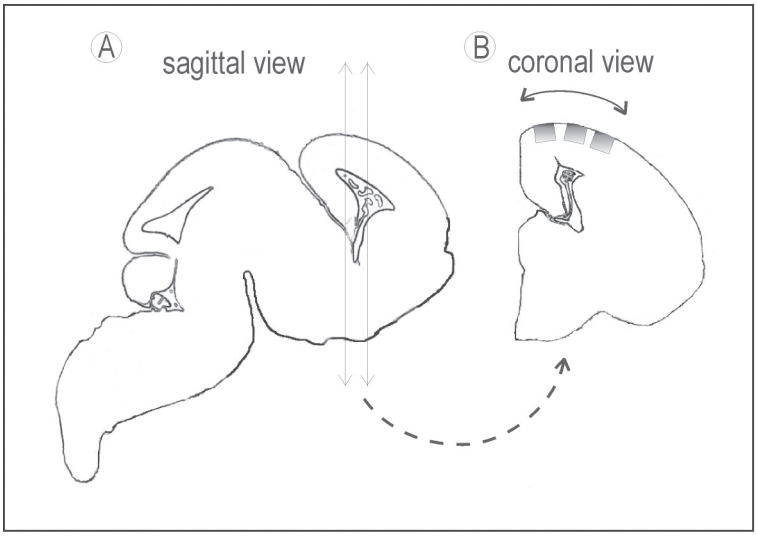

Fig. 1: Scheme showing the dissection procedure used for a consistent sampling of the neocortical marginal zone (layer I) (A) Sagittal view of the brain. Double arrows indicate the planes of sectioning resulting in the thick slice visible in B. **(B)** Coronal view of the brain slice obtained. Double arrows indicate the area where quantification in ultrathin sections occurred. Dark squares indicate the small areas taken for immunogold labeling (see Experimental Procedures).

overnight at 4° C. The next day, further dissection was carried out, as indicated in Fig. 1. Afterwards, we followed a protocol described elsewhere (Gorgels, 1991). Briefly, the area of interest was post-fixed for 2h at room temperature in 2% OsO_4 and 4% $KRuCN_6$ in Na-cacodylate buffer (0.4 M, pH 7.4). After dehydration, the area was embedded in EPON. For a consistent sampling of the same brain area, semi-thin sections were examined after toluidine blue staining. Ultrathin sections were mounted on non-coated grids and contrasted with uranyl acetate/lead citrate. Four animals per group at each sample point were used and at least three non-consecutive sections per animal were examined, representing more than 5000 μm^2 in total.

Immunogold labeling

E18 mouse embryos were decapitated. We followed the same procedure as described above except that the fixative consisted of 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer (Slot *et al.*, 1991). Dissected brains were incubated in the fixative for 2h at room temperature and then overnight at 4° C. The area of interest was dissected into small blocks and washed in PBS with 0.02 M glycine. Blocks were embedded in 12% gelatin in PBS and solidified on ice. After infiltration with 2.3 M sucrose at 4˚ C, blocks were mounted on aluminum pins and frozen in liquid nitrogen. Ultrathin sections were picked up in a 1:1 mixture of 2% methylcellulose and 2.3 M sucrose for improved ultra-structural preservation (Liou *et al.*, 1996). Immuno-labeling

according to the protein A gold method was carried out as previously described (Slot *et al.*, 1991)
Mouse monoclonal antibodies against VAMP/synaptobrevin (Cl69.1; (Edelmann *et al.*, 1995) and Rab3 (Cl42.1, Matteoli *et al.*, 1991) were obtained from Synaptic Systems (Göttingen, Germany). As a bridging antibody for protein A conjugated 10 nm gold we used rabbit-anti-mouse IgG (DAKO, Denmark).

Data collection and statistical analysis

The synaptic parameters chosen (see Results) were quantified directly from the microscope's calibrated scale. The total areas scanned during these quantifications were also measured directly from the electron microscope. Low magnification micrographs (x1,200) of the cortical marginal zone were taken and used for the extracellular space and cell number measurements. A cell soma was counted only when the cell nucleus was visible. Extracellular space measurement was done following the stereology method (Steer, 1981). Raw data were log-transformed and tested using the 2-sided Student's t-test (α=5 %). Values are given as mean ± standard error of the mean (SEM).

RESULTS

The synaptic layer of the mutant neocortex has a normal overall morphology

To investigate synaptogenesis, we choose the marginal zone of the neocortex. Some brain areas in the *munc18-1* mutant mouse showed signs of degeneration, especially in the rhombencephalon. However, the entire cortex of the mutant and control were alike until birth, although the total size of the brain and the cortex were slightly lower in the mutant, but there was no conspicuous cell degeneration, and the number of cells and the layering were similar in mutant and wild type (Verhage *et al.*, 2000). Furthermore, the synapses in the marginal zone originate largely from cortical plate neurons, with little extra-cortical input (some fibers from midbrain catecholamine systems and thalamic input are present at this developmental stage; see Marin-Padilla, 1998; Miyashita-Lin *et al.*, 1999). Thus, marginal zone synapses are largely formed by two cortical neurons and the quantification of synapse formation will not be disturbed by the fact that some lower brain areas start to degenerate in the mutant.
First, we quantified the protein levels and their distribution in the whole neocortex, to test whether pre- and post-synaptic proteins are present and at the correct location to form functional synapses. We compared, with Western blotting, the protein levels of pre- and post-synaptic markers in control and mutant littermates (Fig. 2). As an

internal standard we used the ubiquitous protein Tau. The protein levels of the synaptic vesicle protein Synaptotagmin1 were unchanged, as were the levels of the post-synaptic anchoring protein, PSD-95. The levels of valosin-containing protein, a protein involved in membrane trafficking, were similar in mutant and wild type. Also, the expression of the SNARE-complex members that do not interact with Munc18-

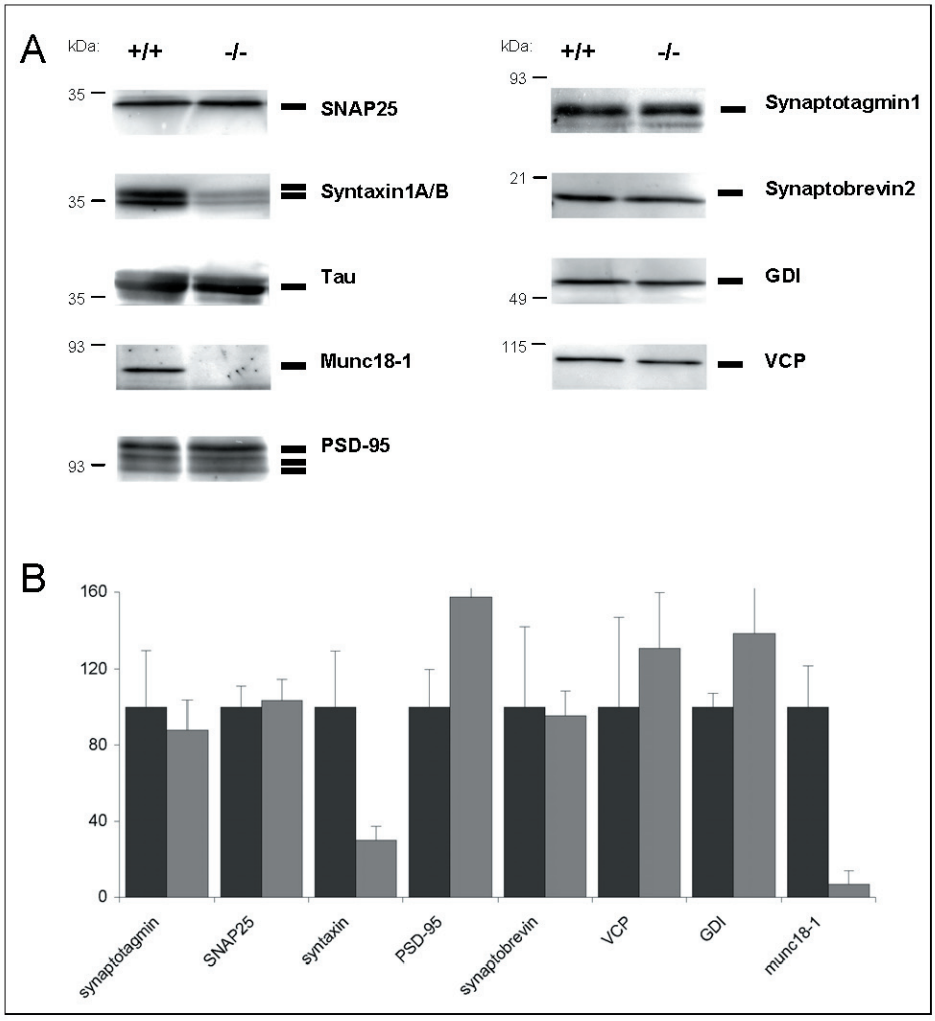

Fig. 2: Quantification of protein levels in the cortex of the munc18-1 mutant and wild type. A: Immunoblots were loaded with 50µg total protein from littermates of wild type and mutant mice. B: Quantification of the western data. Indicated is the average of 3 animals standardized to the staining of tau on the same blot (black bars: wild type animals and grey bars: mutant animals).

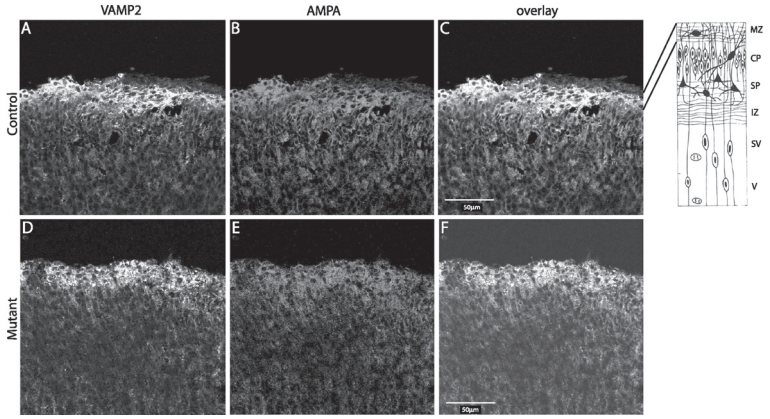

Fig. 3: Localization of pre- and postsynaptic proteins in neurons. Immunostaining for AMPA-receptor GluR2/3 shows receptors are targeted similar in wild type (B) and mutant (E). Staining for VAMP2 indicates that the localization of presynaptic proteins is unaffected in mutant (D) compared to wild type(A). The overlay of these two stainings (C,F) shows adjacent staining for the pre- and postsynaptic marker. The scheme to the side (Uylings *et al.*, 1990) showed what part of the cortex is represented in the pictures. Abbreviations: V, ventricular zone; SV, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; MZ, marginal zone.

1, namely SNAP25 and VAMP, were unaffected. The presynaptic Munc18-1 binding partners Syntaxin1 and DOC2 were present but at lower levels in the mutant. The protein levels of the two isoforms of Syntaxin1, Syntaxin1A and Syntaxin1B, which are expressed in neurons, were both 70% lower in the mutant, and the Munc18-1 binding partners DOC2A and DOC2B were 40% lower. We previously showed that the staining patterns for B50/GAP43, Synapsin1 and VAMP/Synaptobrevin are similar in whole brain at a low magnification (Verhage *et al.*, 2000). Here, we studied the localization of a post-synaptic AMPA receptor (GluR2/3) and a presynaptic protein (VAMP2) in wild type and mutant slices of the cortex at a higher magnification; the staining patterns of VAMP2 and AMPA were equal in wild type and mutant (Fig. 3, color figure see pag. 92). Punctate immuno-fluorescence was observed in all cortical layers and in particular in the marginal zone. Bright clusters of postsynaptic staining were present (Fig. 3B, E, color figure see pag. 92). Because the marginal zone contains very few cell bodies and because the staining pattern was punctuate, the data suggest that the antigens were present in the neuropil and, therefore, the antigens were targeted from their site of synthesis (soma) to their correct location (axons/dendrites). However, we cannot exclude that at the synaptic level, the distribution of synaptic markers may be different.

We used light and electron microscopy to study to the cortex structure in the mutant. No difference in the layering of the cortex in the mutant compared to wild type was observed using light microscopy, indicating that the migration of cells is normal in the mutant (Fig. 4A,B). In line with this observation, we observed no differences in the

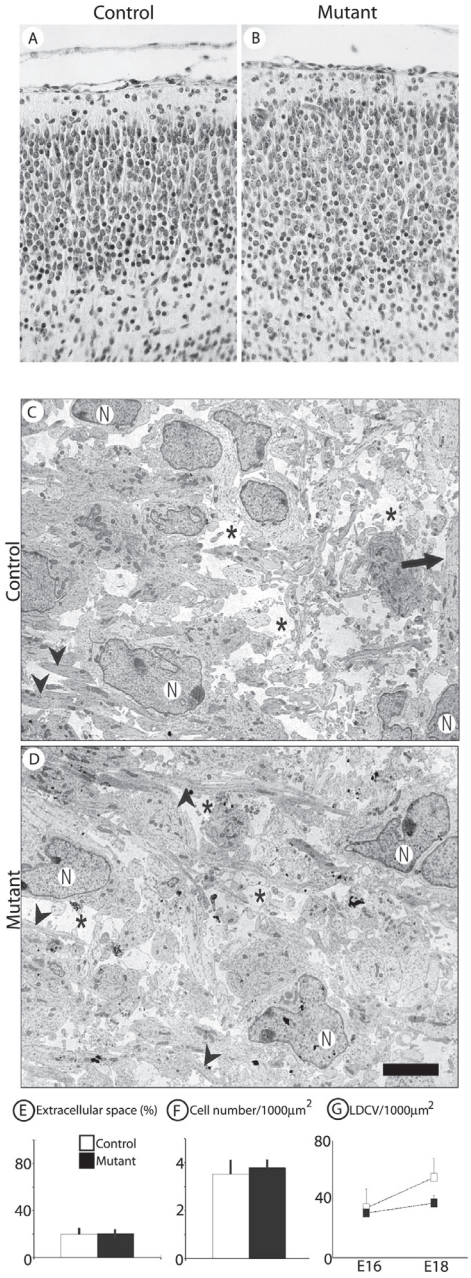

Fig. 4: Overview of neocortex E18 and quantification of general tissue parameters. (A) control and **(B)** *munc18-1* null-mutant neocortex. Layering and number of cells is similar in mutant and wild type (see also Verhage *et al.* 2000). **(C)** control **(D)** *munc18-1* null-mutant marginal zone (layer I). Cortical plate neurons are to the left and pial to the right. Controls **(C)** and mutants **(D)** had few somata compared to axons (arrow heads) and dendrites (arrow). Immature neurons (N) were found scattered in the marginal zone. **(E)** The percentages of extracellular space (asterisks in A/B) were similar in both groups. **(F)** The numbers of cells per 1000µm² of neocortical marginal zone were also similar. Bar: 6µm. **(G)** The number of LDCVs per 1000µm².

ultra-structure of the mutant marginal zone at a low magnification in the electron microscope. The marginal zone is composed of few somata and many axons and dendrites (Fig. 4C,D). At E18, the marginal zone contained a similar number of cells in both the mutant and control brains (Cajal-Retzius cells and migrating or uncommitted neurons): control 3.5 ± 0.6 per 1000 μm^2; mutant 3.8 ± 0.3 per 1000 μm^2 (Fig. 4E; Table 1). The percentage of extracellular space in the marginal zone was also similar (control 19.6 ± 5.3 %; mutant 20.2 ± 3.5 %; n=4; Fig. 4F; Table 1). Thus, the overall organization of the mutant marginal zone was similar to control.

It was previously shown that in the absence of Munc18-1 protein and during overexpression of *syntaxin-1A*, the Golgi-complex and rough endoplasmic reticulum underwent deformation, and that expression of *munc18-1* prevented such deformation. This suggests that the Syntaxin-1A protein in the absence of *munc18-1* may produce cellular abnormalities (Rowe *et al.*, 1999). However, these abnormalities observed by Rowe *et al.* were not observed in the cortical plate cells of the *munc18-1* mutant (Fig. 4).

Table 1: Quantification of tissue and synaptic parameters in the marginal zone of the *munc18-1* null-mutant and control littermates at E16 and E18. Data represent means +/- SEMs, n=4 animals in each group. Statistical significant differences between controls and mutants are indicated in bold + italic. N.D. = not determined.

		E16		E18		
		control	mutant	control	mutant	
tissue characteristics	cells	N.D.	N.D.	3.5 ± 0.6	3.8 ± 0.3	per 1000 μm^2
	extracellular space	N.D.	N.D.	20 ± 5	20 ± 4	%
	LDCVs	34 ± 8	31 ± 5	55 ± 13	38 ± 5	per 1000 μm^2
	synapses	***8.5 ± 2.1***	***3.1 ± 0.5***	***15.5 ± 2.3***	***3.6 ± 1.4***	per 1000 μm^2
	with docked vesicle	***5.1 ± 2.0***	***1.6 ± 0.5***	***8.6 ± 1.4***	***1.7 ± 0.9***	per 1000 μm^2
	'empty synapse'	***35 ± 5.1***	***13 ± 1.7***	***24.1 ± 2.6***	***8.1 ± 0.7***	per 1000 μm^2
	multivesicular struct.	7.0 ± 1.9	6.6 ± 2.5	***11.5 ± 3.5***	***22.2 ± 2.6***	per 1000 μm^2
synapse characteristics	diameter	0.68 ± 0.04	0.73 ± 0.09	0.58 ± 0.04	0.65 ± 0.02	μm
	active zone length	0.26 ± 0.01	0.27 ± 0.02	0.29 ± 0.01	0.28 ± 0.01	μm
	synaptic vesicles	4.5 ± 0.9	5.4 ± 1.6	***9.8 ± 0.2***	***5.0 ± 0.8***	per synapse
	docked vesicles	1.2 ± 0.5	1.2 ± 0.4	***2.1 ± 0.1***	***0.9 ± 0.5***	per synapse

We quantified the number of LDCV in the marginal zone. Both groups had similar numbers of LDCV at E16 (control 34 ± 8 per 1000 μm^2; mutant 31 ± 5 per 1000 μm^2; p=0.805) and at E18 (control 55 ± 13 per 1000 μm^2; mutant 38 ± 5 per 1000 μm^2; Fig. 4G and Table 1).

At E16, synapse morphology is similar in controls and mutants

Scanning the marginal zone at high electron microscope magnification revealed that both control and mutant brains contained developing synapses (Fig. 5A-H, see also Verhage *et al.*, 2000). We characterized synapse morphology by quantifying the presynapse profile diameter, the active zone length and the numbers of morphologically apparently docked vesicles (<30 nm from the membrane). Control and mutant had similar presynapse profile diameters (control 0.68 ± 0.04 μm; mutant 0.73 ± 0.09 μm), active zone length (control 0.26 ± 0.01 μm, mutant 0.27 ± 0.02 μm) and total number of synaptic vesicles (control 4.5 ± 0.9; mutant 5.4 ± 1.6) and of docked synaptic vesicles (control 1.2 ± 0.5; mutant 1.2 ± 0.4) per synapse (Fig. 5K-M; Table 1). Thus, at E16 the synapse morphology of controls and mutants was similar.

At E18, synapse morphology differs between controls and mutants

At E18, presynapse profile diameters (control 0.58 ± 0.04 μm, mutant 0.65 ± 0.02 μm) and active zone lengths (control 0.29 ± 0.01 μm, mutant 0.28 ± 0.01 μm) were similar in both groups. However, control animals showed two times more synaptic vesicles per synapse, while mutants had similar numbers as 2 days earlier (control: 9.8 ± 0.2 vesicles per synapse; mutant 5.0 ± 0.8; $p < 0.005$). The amount of docked vesicles was also 2 times higher in control synapses, but the mutant contained similar numbers as at E16 (control 2.1 ± 0.1; mutant 0.9 ± 0.5, Fig. 5K-M and Table 1). Thus, whereas in the controls the numbers of docked and total vesicles per synapse doubled in two days, the synapse morphology in the mutant was still similar to that of two days before. The staining for the synaptic proteins VAMP2 and Rab3 colocalized often with the small vesicles found in the synapses. This observation makes it even more likely that these vesicles are synaptic vesicles (Fig. 5D,E,I,J).

Synapses are less abundant in the mutant

In the marginal zone of the neocortex, the first morphologically identifiable synapses were reported at E15 (König et al, 1975). At E16, we observed few synapses in the control (8.5 ± 2.1 per 1000 μm^2). The mutant showed even fewer synapses (3.1 ± 0.5 per 1000 μm^2; $p < 0.014$), which is almost 3 times less than in controls (Fig. 5N; Table 1). Two days later, controls showed almost twice as many synapses, while mutants had similar values as 2 days before (control 15.5 ± 2.3; mutant 3.6 ± 1.4; $p < 0.005$; Fig. 5N;

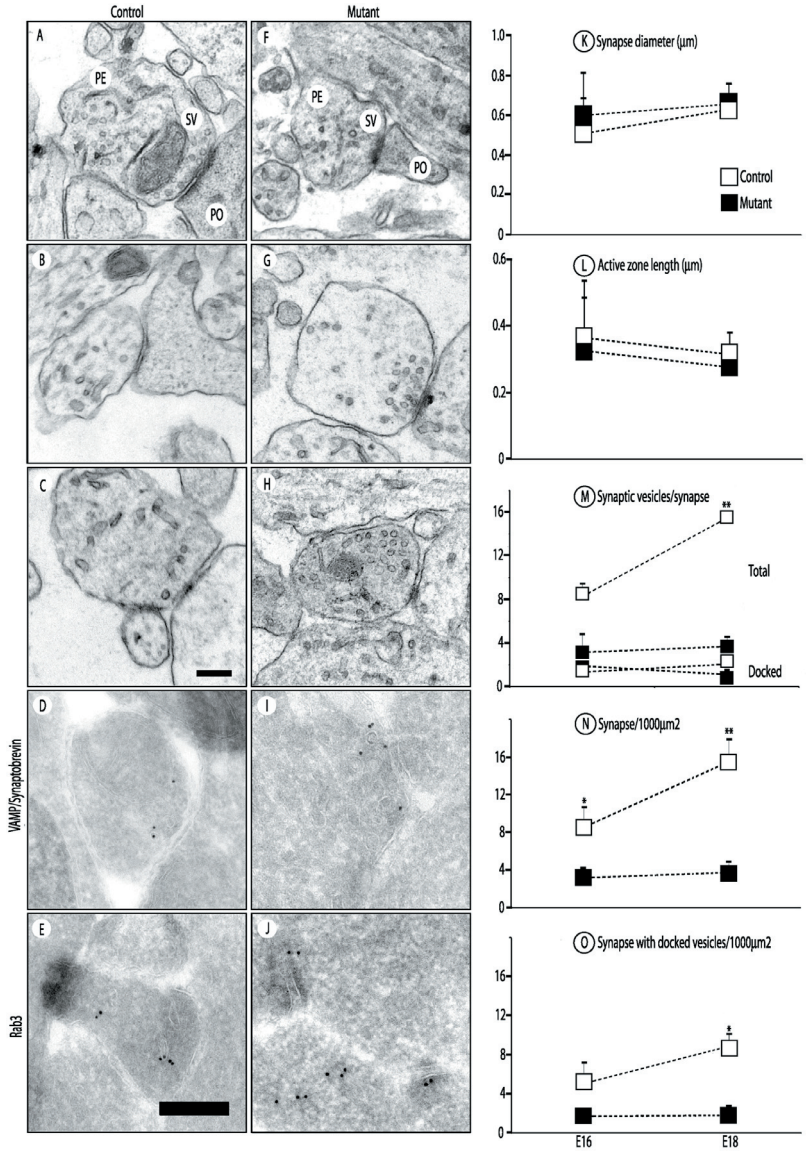

Fig. 5: Electron micrographs of marginal zone synapses in the *munc18-1* null-mutant (F-H) and control littermates (A-C) at E18 and quantification of synaptic parameters. Controls and mutants had similar developing synapses composed of a presynaptic element (pe) containing electron-lucent round and/or electron-lucent pleomorphic synaptic vesicles (sv), and a postsynaptic element (po). Immunogold labeling of VAMP/synaptobrevin (D,I) and Rab3 (E,J). Quantification of synaptic parameters and (K-O) the number of synapses in the marginal zone. * = P<0.05, ** = P< 0.01. Bars: 200 nm.

Table 1). Thus, at E18, controls had almost 4 times more synapses than mutants. At E16, 43 synapses were counted and analyzed in the mutant, and 116 in controls; at E18, 50 synapses in the mutant and 222 in control animals were analyzed.
At both time points, approximately half of the synapses showed docked synaptic vesicles. Similar to the quantification of all synapses (see above), we found that the number of synapses with docked vesicles almost doubled between E16 and E18 in controls, while the number of these synapses did not increase in the mutant. Consequently, synapses with docked vesicles were 3 times more abundant in controls at E16 (control 5.1 ± 2.0; mutant 1.6 ± 0.5; $p < 0.060$) and 5-fold at E18 (control 8.6 ± 1.4; mutant 1.7 ± 0.9; $p < 0.022$; Fig. 5O; Table 1).
Thus at E16, controls and mutants had similar synapse morphology, but controls had many more synapses. Between E16 and E18, control synapses matured and increased in number, while the morphology and number of synapses in the mutant remained the same.

The mutant marginal zone contains fewer "empty synapses"

We observed that the membranes of certain axons and/or dendrites were connected by electron-dense material, denser than the rest of the membrane, and resembling the membrane specializations in synapses, but with no vesicles in the vicinity (Fig. 6). High osmolarity Karnovsky fixative, which tends to shrinken embryonic tissue probably, caused a protein smear between the membranes (Fig. 6C). This suggests that specific proteins are present in this space. They may be involved in cell recognition or synapse formation. Such structures were also reported, for instance, in the rat embryo marginal zone and visual cortex and pontine nuclei of newborn rats, as well as in the chick embryo optic tectum (König *et al.*, 1975; Panzica and Viglietti-Panzica, 1980; Blue and Parnavelas, 1983; Mihailoff and Bourell, 1986). They may represent either a synapse early in its development or a section through a part of the synapse that is devoid of vesicles, but they may also represent (in part) other forms of transitory intercellular junctions. Here, these structures are referred to as "empty synapses". The lengths of their electron-dense membranes were similar between controls and mutants (E16: control 0.230 ± 0.004 µm; mutant 0.239 ± 0.008 µm; E18: control 0.245 ± 0.012 µm; mutant 0.226 ± 0.014 µm). However, the empty synapse incidence was significantly different between the two groups. At E16, control animals had almost 3 times more empty synapses than mutants (control 35 ± 5.1; mutant 13 ± 1.7; $p=0.002$). The number of these empty synapses decreased between E16 and E18 in both groups, but remained significantly different, controls having 3 times more, empty synapses than mutants (control 24.1 ± 2.6; mutant 8.1 ± 0.7; $p=0.0004$; Fig 6D-E; Table 1). Thus, the empty synapses, are a normal feature during development, are more regularly found at E16 than at E18 and less frequently observed in the mutants.

The mutant contains more 'multivesicular structures' at E18

We observed that the membranes of two axons and/or dendrites were connected by electron dense material, and both sides contained round and/or pleomorphic vesicles (Fig. 7A,B,D). Such structures have been described in the developing brain, for instance, in the marginal zone (König *et al.*, 1975), in the olfactory lobe (Pinching and Powell, 1971) and in the pontine nuclei (Mihailoff and Bourell, 1986) and may reflect the elimination of a synaptic contact or the degeneration of the entire axon and/or dendrite, starting a the synapse. We refer to these structures as 'multivesicular structures', because they have vesicles at both sides of the electron dense membrane.

The length of the electron-dense region of the membrane of the multivesicular structures was similar in controls and mutants (at E16: control 0.261 ± 0.006 µm; mutant 0.264 ± 0.027 µm; at E18: control 0.313 ± 0.025 µm; mutant 0.276 ± 0.017 µm).

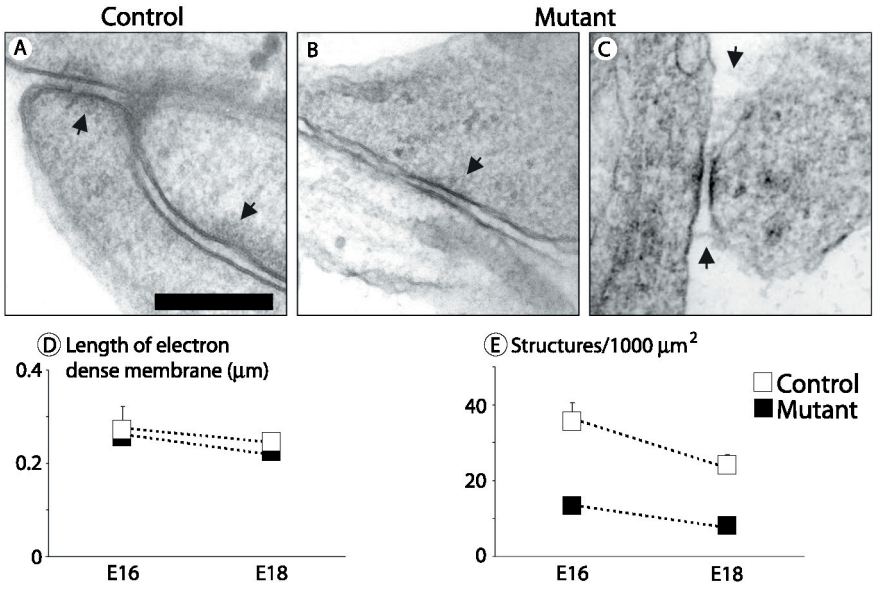

Fig. 6: Electron micrographs of electron dense membranes, 'empty synapses', in the marginal zone of the *munc18-1* null-mutant (B,C) and control littermates (A) at E18 and quantification. (A,B) Electron dense membrane (arrow) between axons and/or dendrites in tri-aldehyde fixative and (C) the same structure in Karnovsky fixative (see Experimental Procedures and Results sections). Notice in C the smear of proteins (arrows) between the two cell processes. Bar: 200 nm. (D,E) Quantification. ** = $p < 0.01$, *** = $p < 0.001$.

At E16, the incidence of multivesicular structures was similar in both groups (control 7.0 ± 1.9 per 1000 µm^2; mutant 6.6 ± 2.5 per 1000µm^2), but at E18 it was 2 times more abundant in mutants (control 11.5 ± 3.5 per 1000 µm^2; mutant 22.2 ± 2.6 per 1000µm^2; $p < 0.05$; Fig. 7F,G; Table 1).

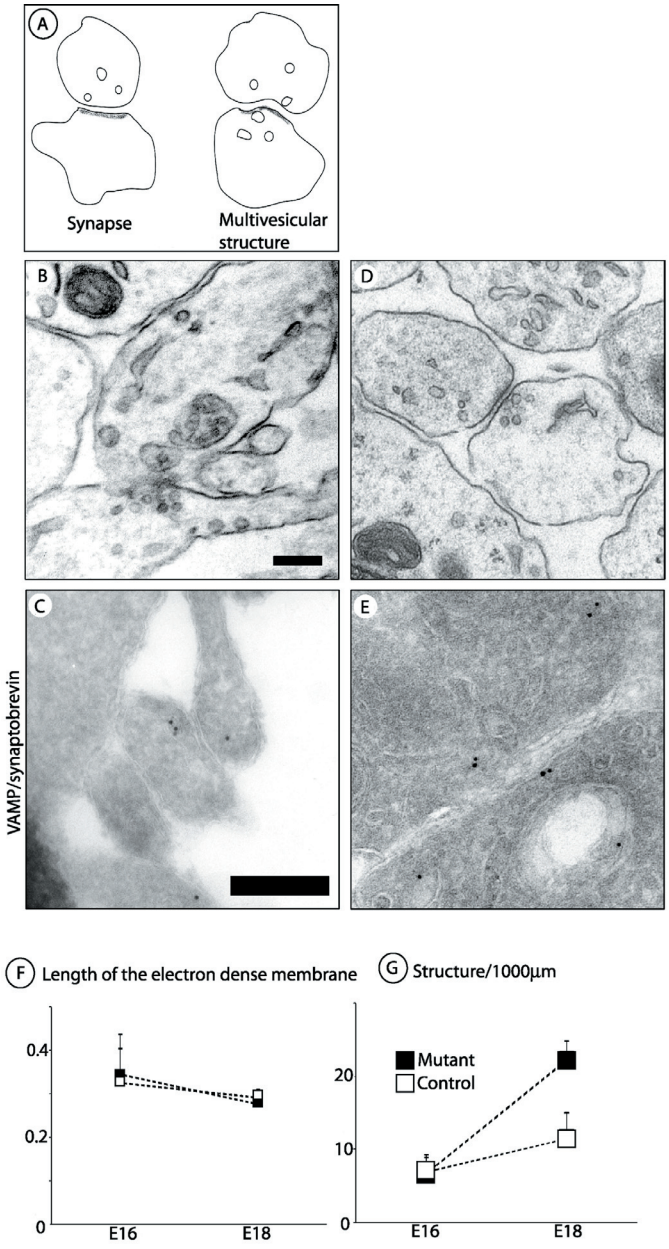

Fig. 7 : Electron micrographs of multivesicular structures in the marginal zone of the *munc18-1* null-mutant (D,E) and control littermates (B,C) at E18 and quantification. (A) Cartoon of a synapse and a multivesicular structure, showing that the only difference is that vesicles (round or pleomorphic) are present at both sites of the membrane. (B,D) Multivesicular structures, i.e. two cell processes containing round and/or pleomorphic vesicles (v) joined by an electron dense material. (B,D) Immunogold labeling of VAMP/Synaptobrevin. Multivesicular structures were labeled in both groups. Bar: 200 nm. (E,F) Quantification of multivesicular structures.

To assess the nature of the round and pleomorphic vesicles in the synapses and in the multivesicular structures, we performed immunogold labeling for two synaptic proteins on brain sections from mutants and controls at E18. Although the loose and immature tissue was difficult to prepare and immunoreactivity for markers of the (mature) synapse was low, we observed significant VAMP/Synaptobrevin (Fig. 5D,I) and Rab3A immunoreactivity (Fig. 5E,J) at the vesicle membranes. In both groups, multivesicular structures round and pleomorphic electron-lucent vesicles were labeled with gold particles. Immunogold labeling also revealed that the multivesicular structures were immuno-positive for VAMP/Synaptobrevin in both groups (Fig. 7C,E).

DISCUSSION

In the present study we characterized and quantified synapse-formation and -maintenance in the absence of presynaptic signaling, using the *munc18-1* null-mutant as a model. We found that the overall organization of the neocortical marginal zone was normal. Proteins needed for synaptic function were expressed and targeted to the correct location. In the mutant, immature synapses had formed. Initially, the morphology of these synapses was similar to synapses in control littermates at the same age (E16), but controls had 3 times more synapses. Between E16 and E18, the number of control synapses increased and the number of vesicles in the synapses also increased, while the morphology and number of secretion-deficient synapses remained the same. Instead, the number of multivesicular structures increased in the mutants. Immunogold labeling indicated that both synapses and multivesicular structures of the mutants contained synaptic vesicle markers.

The lower amount of synapses is not the result of degeneration in other brain areas

In the *munc18-1* null-mutant, massive degeneration takes place in lower brain regions between E16 and E18 (Verhage *et al.*, 2000). However, this is unlikely to explain the lower density of synapses in the neocortex of the mutants. Most synapses found in the marginal zone are formed between two cortical neurons (see Marin-Padilla, 1998; Miyashita-Lin *et al.*, 1999). These neurons do not show signs of degeneration because they revealed neither apoptotic bodies (through standard histology) nor TUNEL staining (Verhage *et al.*, 2000 and this manuscript). In addition, the levels of most synaptic proteins were not lower in the mutant relative to the control proteins, which would be expected if synapses of lower brain areas were an important input to the cortex. Furthermore, the distribution of the AMPA receptor was similar in mutant and wild type. Therefore, we conclude that the lower density of synapses is not a consequence of neuronal degeneration in other brain areas. The lower density

and failure to mature may rather be the primary consequence of deleting *munc18-1* expression and/or the resulting loss of presynaptic signaling.

Synaptic vesicles do not accumulate in the mutant

Despite the blockade of secretion in the *munc18-1* null mutant we observed similar amounts of vesicles at E16 and at E18 in the secretion-deficient synapses and fewer vesicles per synapse than in controls. Hence, vesicles do not accumulate after secretion was blocked. This suggests that the normal increase in the number of vesicles during synapse maturation depends primarily on local activity in the synapse than on supply from the cell body (independent of local activity). Alternatively, the *munc18-1* gene may be required for targeting a normal number of precursor vesicles to the terminal. In any case, the lack of vesicle accumulation contrasts with the observation that mutation of a *S. cerevisiae* munc18 ortholog leads to vesicle accumulation at the target membrane (Novick and Schekman 1979).

Secretion-deficient synapses may not mature and may transform to multivesicular structures instead

Between E16 and E18 both the number of synapses and the number of vesicles per synapse did not increase in the absence of presynaptic signaling, while these parameters approximately doubled over the same period in controls. This may be explained by assuming that after E16 no further synapses were formed in the mutant and that synapses formed earlier remained static for two days. However, since synapses are highly dynamic structures, we favor the explanation that synaptogenesis in itself is unimpaired, but that synapses do not mature in the mutant and disassemble instead after some time. Together with ongoing assembly of new immature synapses this disassembly can explain why we observed fewer and only immature synapses both at E16 and E18. Furthermore, the multivesicular structures that were frequently observed in the mutant at E18 may be the result of the disassembly of immature synapses observed at E16. The fact that these multivesicular structures were labeled with antisera for a synaptic vesicle marker strengthens the suggestion that they have a synaptic origin. In the *munc18-1* mutant the multivesicular structures may be the first step in a cascade of events that ultimately leads to general degeneration of the whole neuronal network as observed in lower brain areas (Verhage *et al.*, 2000). The marginal zone contains a number of different classes of synapses. We cannot exclude that the difference in synapse density between controls and mutants is caused by a selective or disproportional loss of certain classes of synapses.

Interestingly, elimination of neuromuscular synapses depends on the activity of strong synapses, producing 'punishment signals' to weaker synapses (see for a review Lichtman and Colman, 2000). Since the neocortex in the *munc18-1* null

mutant completely lacks neurotransmission, even spontaneous synaptic events, a different mechanism must cause this synaptic disassembly. Alternatively, the synaptic disassembly in the *munc18-1* null-mutant and the appearance of many multivesicular structures maybe is initiated from the somata, which are devoid of neural inputs. It was previously shown that after silencing neuronal networks in culture, the first signs of cell death are observed at the synapses (Williamson and Neale, 1998). In addition to the lack of synaptic input or output, the reduced Syntaxin levels may also contribute to the loss of synapses and the accumulation of multivesicular structures. However, albeit reduced, there is still considerable Syntaxin staining, also in the synaptic layers and in terminals of cultured null mutant neurons (Toonen et al, 2005). Furthermore, at E16 the organization of immature synapses is still normal in the mutant, while syntaxin levels are already reduced.

Synapse maturation may require the concerted effects of different presynaptic signals

In addition to the *munc18-1* null mutant, a second mouse model was recently generated, the *munc13-1/munc13-2* double null mutant (dko), that is also completely devoid of both spontaneous and evoked neurotransmitter release at least in cultured neurons of the hippocampus (Rosenmund *et al.*, 2002; Varoqueaux *et al.*, 2002). In contrast to the *munc18-1* null mutant, this mutant was found to produce normal, mature synapses in culture, and no conspicuous degeneration in the brain (Varoqueaux *et al.*, 2002). It is not certain if as many neurons are silent in the *munc13* dko as in the munc18-1 mutant, has the same amount of synapses *in vivo* as in control animals and if multivesicular structures are also present in the former model. Still, these data suggest that the lack of evoked and spontaneous secretion of (classical) neurotransmitters does not prevent formation of normal, mature synapses. In line with this suggestion we observed that *munc18-1* null mutant neurons cannot be rescued by co-culturing them with wildtype (transmitting) neurons or supplying exogenous neurotransmitters (Heeroma *et al.*, 2004), indicating that additional defects in the *munc18-1* null mutant are involved in the proposed instability of synapses and the eventual disassembly of neuronal networks. One candidate for such an additional defect is the defective secretion of neuromodulatory substances from large dense core vesicles (LDCVs) observed in *munc18-1* null mutant chromaffin cells and pituitary (Voets *et al.*, 2001, Korteweg *et al.*, 2005). By contrast, in the *munc13* dko chromaffin cells do not show impaired secretion of LDCVs (Ashery, Brose, personal communication). In conclusion, while presynaptic signaling is dispensible for the formation of initial immature synapses, the effects of classical neurotransmitters or neuromodulatory substances are probably required to form normal, mature synaptic networks. Alternatively, the proposed disassembly of synapses and the

neurodegeneration in the *munc18-1* mutant may result from an intrinsic defect caused by the absence of the protein rather than from blocking presynaptic signaling. However, not all neurons degenerate in the *munc18-1* mutant. For instance, primary sensory neurons normally express *munc18-1* but do not degenerate in the null mutant. This argues against a cell-intrinsic defect as a primary cause for the observed effects reported in this study.

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DIFFERENTIAL GENE EXPRESSION IN THE DEVELOPING NEOCORTEX OF A MOUSE MODEL LACKING SYNAPTIC ACTIVITY

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Chapter 3

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ABSTRACT

Neuronal activity has an important role in neuroplasticity, as activity can affect synaptic strength and morphology. Some of these effects are long-lasting and involve gene expression. We assessed the role of activity-dependent gene expression in the development of synapses. Thereto, a developmental transcript level profile was generated using an oligonucleotide-microarray (7k) analysis, in which neocortical expression of *munc18-1* null mutant mice, which are deprived of evoked and spontaneous neurotransmitter release, was compared to control animals. We hypothesized that genes induced by synaptic activity will have differences in transcript levels in mutants compared to controls. The RNA levels of most genes (>90%) were similar, including gene groups involved in cellular metabolism and apoptosis. At embryonic day 18, the mutant had a pronounced reduction in the RNA levels of a number of genes involved in neuronal transmission. However, these genes were unaffected earlier in embryonic life, when synaptic activity is not yet present. These data were validated using real-time quantitative PCR. Thus, during development synaptic activity increases the expression of several neuronal transmission compounds.

INTRODUCTION

Synapses are dynamic structures and probably often exist transiently. Both their function and structure can be altered in response to various signals in developing and mature networks. The synergy of functional and morphological changes affects the strength of the synapse. Functional changes include changes in synaptic probability and the number of receptors in the postsynaptic membrane (Sheng and Kim, 2002). In addition, the shape of the synapse can be regulated, especially of the postsynaptic spine (Fischer *et al.*, 1998; Majewska et al, 2003). These two types of changes can lead to short- or long-lasting strengthening or weakening of synaptic transmission. For some of the long-lasting effects, an increase of compounds involved in synaptic transmission is needed. This increase may involve regulation of transcription. For instance, adjusting the expression of genes involved in synapse function can be important for the maintenance phases of synaptic potentiation, and changes in the expression of structural genes may underlie morphological rearrangements.

Activity regulates the expression of several synaptic genes, like cell adhesion molecules and synaptic release molecules. For instance, the expression of cell adhesion molecules is influenced by activity in some cases in a frequency dependent way. The RNA levels of cell adhesion molecule L1 are decreased by 0.1 Hz stimulation in mouse DRG neurons *in vitro*, while higher frequencies did not change the levels. In contrast the RNA levels of N-cadherin were lowered at both low and high frequencies (Itoh *et al.*, 1997). In addition, the RNA levels of the cytoskeleton-associated protein, Arc are increased in

high frequency stimulated rat hippocampi and in cultured PC12 cells (Lyford *et al.*, 1995). Synaptic activity can also increase the expression synaptic vesicle release genes (Sutton *et al.*, 1999), neuropeptides (MacArthur and Eiden, 1996) and receptors (Hoffmann *et al.*, 2000).

Effects of activity on the transcript levels of large numbers of genes were studied using microarray technology. The effect of NMDA-receptor activation on RNA levels, was measured using microarray hybridization. Especially the RNA levels of genes involved in intracellular structure and vesicular trafficking were increased (Hong *et al.*, 2004). In addition, application of kainate, an agonist of non-NMDA-glutamate receptors, to the hippocampal dentate gyrus increases the mRNA levels of genes encoding transcription factors and vesicle- and synapse related proteins (Nedivi *et al.*, 1993). These data show that the expression of particular synaptic proteins is regulated by activity. The increase in synaptic proteins most likely underlies the increase in synaptic strength that occurs during development.

Until now, effects of synaptic activity on gene expression are mainly studied by massive over-stimulation of neurons. We now took a novel approach by exploiting activity-dependent changes in transcript levels in the *munc18-1* null mutant, which is devoid of activity. In the *munc18-1* null mutant all studied synapses are deprived of evoked and spontaneous release of classical neurotransmitters, and have a reduced secretion from large dense core vesicles (Verhage *et al.*, 2000; Voets *et al.*, 2001). We used this mouse model to perform an open screen using microarray technology, and compared RNA levels with wild type littermates in the neocortex during late embryonic development. In addition to synaptic activity also *munc18-1* itself may be responsible for differential expression of genes in this mutant. Therefore, we examined the transcript levels before (E14), during (E16) and after (E18) the first synapses had formed to distinguish effects induced by activity and by expression of *munc18-1*. For instance, at E14 synapses have not yet formed in the neocortex, but *munc18-1* is already expressed in wild type animals. Therefore, at this stage only genes that are regulated by Munc18-1 will be differentially expressed. While at E18, genes will be regulated both by synaptic release and by expression of Munc18-1. Strikingly, especially the transcrip levels of genes involved in neuronal transmission were lower in the mutant at E18 and not earlier in development.

EXPERIMENTAL PROCEDURES

Laboratory animals

Munc18-1 deficient mice were generated as described before (Verhage, *et al.*, 2000). Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous matings. The day a vaginal plug was observed was considered to be

embryonic day 0 (E0). Embryos were harvested from mothers rapidly sacrificed after timed matings at E14 E16 and E18. Experimental procedures were carried out with local regulatory approval for animal experimentation, according to the Dutch law for animal welfare.

RNA isolation

RNA was isolated from mutant and wild type animals (for qPCR and microarray RNA from different animals was used) by using the Trizol method (Invitrogen, San Diego, USA). The amount of RNA was measured with a spectrophotometer and a Bioanalyzer (Agilent technologies, Palo Alto, USA), and similar amounts of RNA was used in the cDNA reactions or loaded on a Northern gel. Genomic DNA was removed by a DNAse I treatment (1 U, Invitrogen), which was apparently sufficient since genomic primers developed to detect an HPRT intron did not result in a PCR product.

Microarrays

Custom slides

Microarray expression analysis was carried out as described by Buermans *et al.* (2005), with minor changes. Microarrays were produced by spotting mouse oligonucleotides (65 base-pairs C6-modified; 7524 set Sigma/Compugen, Jamesburg, USA). cDNA synthesis was performed on total RNA (60-75 µg per sample) with aminoallyl-dUTP (Ambion, Austin, Texas, USA). RNA was removed by hydrolysis (0.03 M NaOH) and non-incorporated nucleotides were removed using a Microcon YM-30 filter (Millipore, Billerica, MA, USA). Each mutant sample was coupled to either Cy3 or Cy5, whereas control RNA was pooled and separated into two before dye coupling, one tube for Cy3 and one for Cy5. Uncoupled dye was removed using a PCR purification column (Qiagen, Hilden, Germany). For each slide labeled cDNA (mutant and pooled control) was mixed with Cot-1 DNA (30 µg), tRNA (75 µg) and poly-A (15 µg) and ethanol-precipitated. Labeled DNA was dissolved in hybridization mix (460 µl) to achieve a final composition of 50% formamide, 10% dextran sulphate, 2 X SSC, 2% SDS, 8% glycerol and 10 µg of tRNA. The arrays were analyzed with Imagene 5.0 standard settings (10% lower and 5% upper cut-off in signal and background, threshold value 0.55, empty spots setting 2.4). At E14 and E18 spots were also manually flagged, if dust was on the spot area or if circles were placed outside the printed area. Local or general background subtraction was performed (no difference in results) and the values were ^{2}log-transformed and duplicate spots were averaged. Normalization was performed to the average signal of non-flagged spots in the channel (Cy3 and Cy5 separately). The data were analyzed with a Student's *t*-test, α=5% and also in SAM (Significance Analysis of Microarrays; 23). Genes were counted as measurable if they were non-flagged in at least 75% of the

arrays. A transcript was defined as regulated if the levels were significantly different (Student's *t*-test; α= 5%) in the mutant compared to control animals. The genes were grouped according to Gene Ontology assignments (Van der Wiel *et al.*, 2005). We have adhered to this classification throughout the manuscript. Genes were clustered using the programs Cluster and Treeview http://rana.lbl.gov/EisenSoftware.htm).

Microarray setup

For the E14 micro-array experiment 6 mutant and 6 control animals were used and hybridized to 6 microarray slides (n=6), for E16 n=4, and for E18 n=11 (Fig. 1). For each developmental stage, the control material was used as common reference. Therefore,

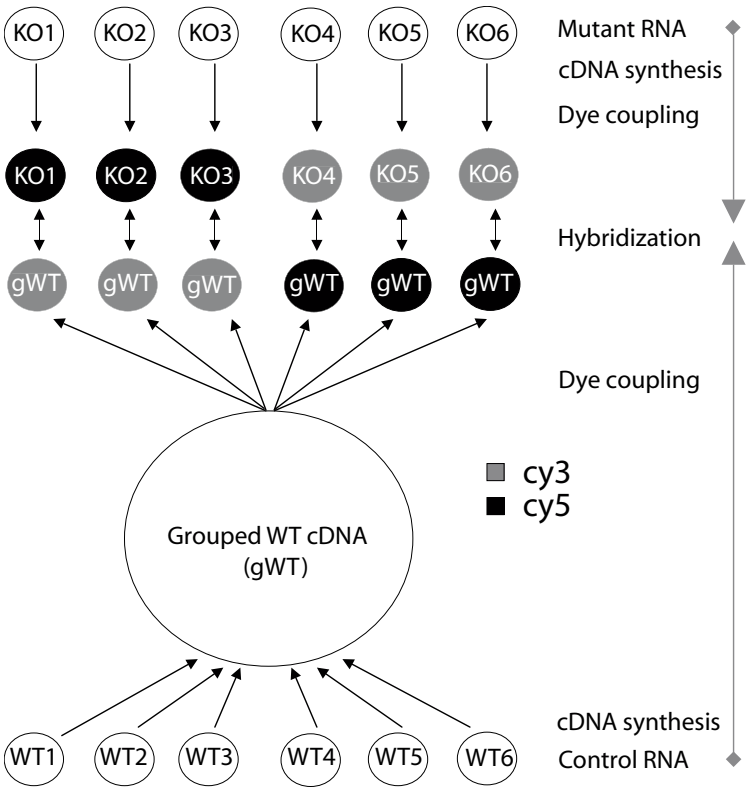


Figure 1: Scheme showing the procedure used for the microarray performed on E14, E16 and E18 mutant and control animals. In this example of the experimental set-up 6 mutant (KO) and 6 control (gWT) animals are indicated. RNA of control animals (n=6 for E14, n=4 for E16 and n=11 for E18) was pooled. Half of the control material was labeled with Cy3 and half of it with Cy5. The mutants were kept separately and half of there numbers were labeled with Cy3 and the other half with Cy5. Then the hybridization with 1 mutant and a part of the control material was performed.

it was pooled not only after the RNA isolation but also after the cDNA reaction. A dye-swap was performed by labeling half of the RNA of the mutant animals with Cy3

and half with Cy5, and of the control material the other way around. We performed a control experiment using *munc18-1* heterozygote material (n=2). We hybridized two aliquots of one heterozygote cortex, one labeled with Cy3 and one with Cy5, to the same array. The data showed a normal distribution and no relation between the abundance of the transcript and the Cy3/Cy5 ratio (Fig. 2), indicating the absence of dye-bias and reproducibility of the procedure for high and low abundant transcripts. The normal distribution had a standard deviation of 0.1 and 72% of the data are within this standard deviation. Original data files for all arrays were uploaded in MIAME format for expression arrays at GEO, http://www.ncbi.nlm.nih.gov/geo/ accession numbers GSE3637, GSE3538 and GSE3639.

qPCR

Oligonucleotide primers were designed to amplify a 80-120 bp amplicon. HPRT was chosen as an internal standard. Primers were designed using Primer Express software 1.0 (PE Applied Biosystems, Foster City, CA, USA). PCR reactions (20 µl) were set up and run as described by the manufacturer, shortly the reactions contained 10 µl SYBR Green PCR Core Kit (PE Applied Biosystems, Foster City, CA, USA), 20 pmol of each primer (Sigma or Eurogentec, Seraing, Belgium); and 3 µl of cDNA template (equivalent to 1 ng RNA). Amplification, data acquisition, and data analysis were carried out in the ABI 7700 Prism Sequence Detector (once at 2 min, 50° C; 10 min, 95° C; and 40 cycles at 95° C, 15 s; 59° C, 1 min). The calculated cycle threshold values (Ct) were exported to Microsoft Excel for analysis using the ΔΔCt method (Spijker *et al.*, 2004a). Dissociation curves (Dissociation Curves 1.0 f. software, PE Applied Biosystems, Foster City, CA, USA) of PCR products were run to verify amplification of the correct product. As negative control primers were chosen in the region of *munc18-1* that is removed in the mutant. In another study we showed that qPCR has the selectivity to detect transcripts over a dynamic range of 5 orders of magnitude, including the very low-abundant transcripts. In addition, we showed that it has the resolution to discriminate subtle changes in RNA levels significantly if they are larger then 1.4 fold (Spijker *et al.*, 2004a).

RESULTS

To address the relation between synaptic activity and gene expression during synapse development, we studied the RNA levels in the *munc18-1* null mutant at embryonic day 14, 16 and 18 relative to the levels in control littermates, using DNA array technology and quantitative real-time PCR.

We performed two control experiments using *munc18-1* heterozygote material. We hybridized two aliquots of one heterozygote cortex, one labeled with Cy3 and one with Cy5, to the same array (Fig. 2A). The data showed a normal distribution (Fig. 2B) and no

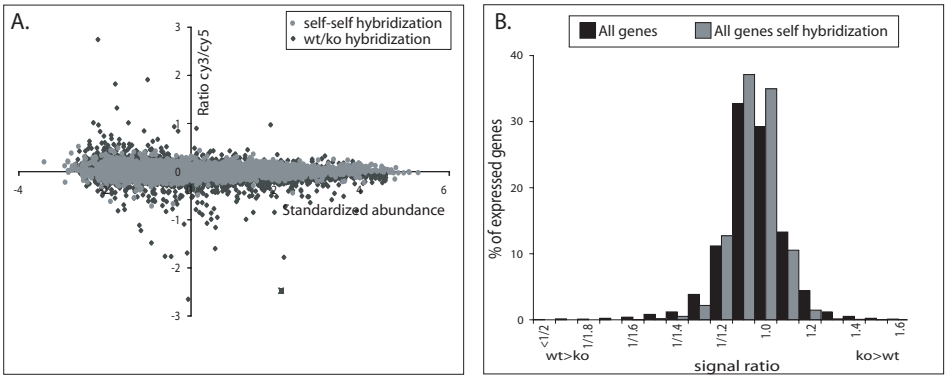


Figure 2: Relation between abundance of a transcript and its regulation. A: The standardized intensity of the spot is indicated on the x-axis and the Cy3/Cy5 ratio on the y-axis. All expressed genes measured in two control hybridizations with heterozygote material are shown. One aliquot of the heterozygote RNA was labeled with Cy3 and a second aliquot of the same material with Cy5 (in grey and in black a E18 experiment with a mutant and wild type sample). B: For each group, all expressed genes were classified into signal intensity difference intervals (0.1 bins) according to their Cy3/Cy5 signal ratio. Transcripts in the "1" bins had identical Cy5 versus Cy3 signal intensity ratio and were thus not regulated. Positive values indicate that the levels are higher in mutants (ko), while negative values indicate higher levels in control animals (wt). The y-axis shows the percentage of genes in a certain bin. The black bars indicate the distribution of all expressed genes across eleven comparisons of mutant and control animals. Grey bars denote the percentage of expressed genes in a certain bin across two control hybridizations with heterozygote material. Per array, one aliquot of heterozygote material was labeled with Cy3 and a second aliquot of the same heterozygote mRNA was labeled with Cy5 and hybridized to an array.

relation between the abundance of the transcript and the Cy3/Cy5 ratio, indicating that the procedure is reproducible for high and low abundant genes and transcripts are not binding to one of the colors specifically. The normal distribution had a standard deviation of 0.1 and 72% of the data lay within this standard deviation.

The expression of a few genes is altered in the mutant animal

To identify activity-dependent gene expression, we compared the RNA levels in cortices from control and *munc18-1* deficient mice at E14 (n=6), E16 (n=4) and E18 (n=11). Cortices of E18 null mutant animals were somewhat smaller than the cortices of wild type littermates, while no difference was observed at E14 and E16. At E18, the RNA yield of null mutants was also lower; about 70% of control levels. The microarray data were standardized to the average background on the total array. The normalized raw data of the mutant of the six separate E14, four E16 and eleven E18 experiments were hierarchically clustered. The mutant data of the three embryonic days clustered in separate groups (Fig. 3). This indicates that the gene profiles of experiments within a certain embryonic age were more similar than between ages. The E14 mutant data were the most separate relative to the E16 and E18 data. Of 7524 printed transcripts, our assay detected expression of 66.5% (5010 transcripts) in the neocortex at E14, 77.4% at

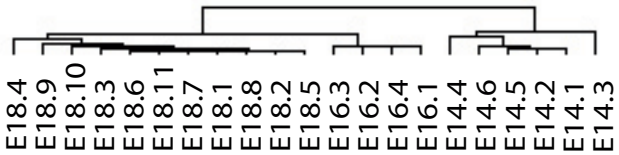


Figure 3: Clustering of separate arrays. Standardized mutant intensity levels of the changed genes were clustered. The arrays with samples of the same embryonic age cluster together. The distance between the E14 cluster and the E16 and E18 cluster is larger then the distance between the E16 and E18 cluster.

E16 (5831) and 74.2% at E18 (5586).

At the early embryonic ages there were virtually no differences in RNA levels between the mutant and wild type, at E14 30 genes (0.6%) and at E16 129 genes (2.2%, $p<0.05$) had different RNA levels. However, many more transcripts levels were different at E18 comparing the mutant and control animals (505 transcripts (9.0%), $p<0.05$) (Table1).

Table 1: Quantification of genes that are measurable or changed on the array of mutant and control mice. Table 1 shows the quantification of genes measurable in numbers and percentage relative to the total amount of genes printed (7524) on the array at E14, E16 and E18. The table also shows the number (#) of changed genes on the array at the different time points.

	Number measurable	Percentage measurable	Number changed	Percentage changed	# higher in control	# higher in mutant
E14	5010	66.5	30	0.6	8	22
E16	5831	77.4	129	2.2	377	69
E18	5586	74.2	504	9.0	271	233

Analysis of the data with SAM (Significance Analysis of Microarrays; Tusher *et al.*, 2001), which is a Student's *t*-test extended with a multiple testing correction, gave a more stringent view: only 2 genes were significantly regulated at E14 (FDR < 5 %) and E16 (FDR < 5 %) and at E18 175 genes were significantly regulated (FDR < 5 %).

The most upregulated transcripts in the mutant at E18 were glial fibrillary acidic protein, lysosomal-associated protein transmembrane 5, and secreted phosphoprotein 1 (Fig 4). Tachykinin 1, and somatostatin were within the most downregulated transcripts in the mutant. The difference in their transcript levels was in the range of munc18-1, the deleted gene. Residual hybridization for munc18-1 in the mutant can be explained by the truncated RNA for this gene (Verhage *et al.*, 2000) or by binding of munc18-2 or munc18-3. In conclusion, only at E18 a significant amount of transcripts had different levels, consistent with the idea that synaptic activity influences gene expression.

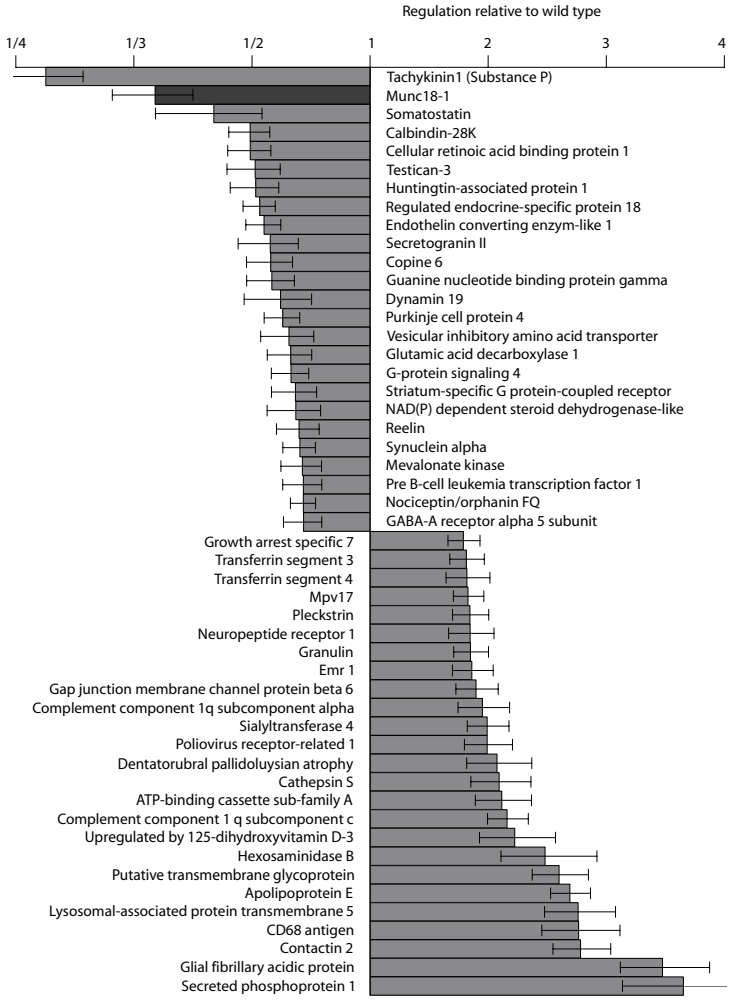


Figure 4: Most extreme regulated genes. Expression levels of the 5% highest and lowest regulated genes at E18 are shown relative to control animals (average ± SEM). Half of the genes are high expressed in the mutant, half of them high in control animals. Some of the genes that have a relative low expression in mutant animals are involved in synapse function. Some genes high expressed in the mutant are involved in immune response.

Genes that were found to have differences in transcript levels (according to the Student's *t*-test) with a similar function were grouped according to Gene Ontology (GO) assignments. The three organizing classes of GO are biological process (Fig. 5), molecular function and cellular component (Fig. 6). Within these classes, groups of genes are defined based on their common properties. This organization is not specifically developed for the nervous system and has redundancy in class-assignments. An overrepresentation-analysis, which indicates that the function or process in which the group is involved can be viewed as affected by our experiments, was performed using the biological process class as it was the

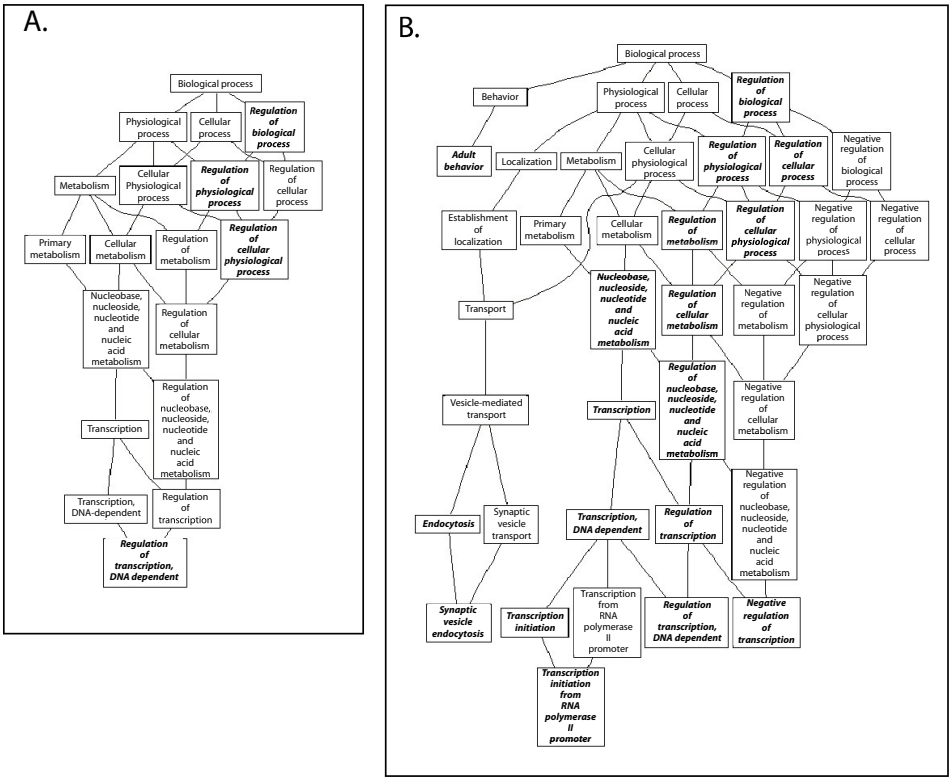
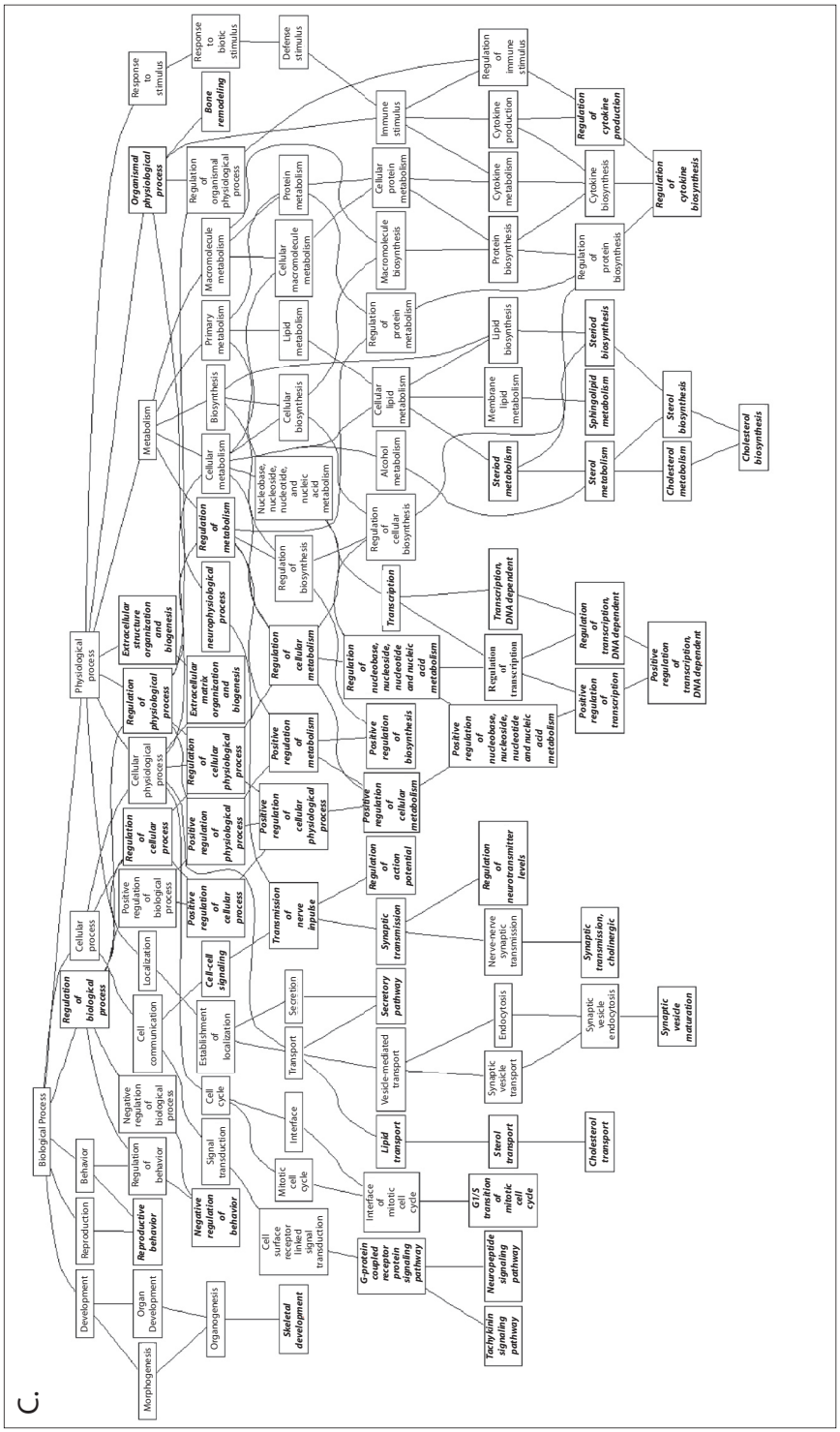


Figure 5: DAG (directed asymmetric diagram) view E14, E16, E18 for Biological process. Overview over the regulated GO groups in Biological process (bold-italic) and their relation. A: E14, B: E16 and C: E18 (see page 60).

most suitable for neurons. Within this class, 4 groups were relatively enriched at E14, 18 at E16 and 52 groups were enriched at E18 (Fig. 5 and Table 2). For all ages, the RNA levels of genes involved in basal cell function were hardly affected. For instance, cellular metabolism was not enriched at any of the embryonic ages. This suggests that the expression of only specific gene groups is affected in the *munc18-1* mutant.


C.
Biological Process
Regulation of biological process
Cellular process
Positive regulation of biological process
Cellular physiological process
Regulation of cellular process
Positive regulation of physiological process
Positive regulation of cellular process
Regulation of physiological process
Regulation of cellular physiological process
Positive regulation cellular physiological process
Extracellular structure organization and biogenesis
Extracellular matrix organization and biogenesis
Physiological process
neurophysiological process
Regulation of metabolism
Regulation of cellular metabolism
Positive regulation of metabolism
Positive regulation of biosynthesis
Positive regulation of cellular metabolism
Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Transcription
Regulation of transcription, DNA dependent
Transcription, DNA dependent
Positive regulation of transcription, DNA dependent
Regulation of transcription
Positive regulation of transcription
Regulation of biosynthesis
Regulation of cellular biosynthesis
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism
Alcohol metabolism
Cellular lipid metabolism
Regulation of protein metabolism
Cellular protein metabolism
Protein metabolism
Regulation of immune stimulus
Immune stimulus
Cytokine production
Regulation of cytokine production
Cytokine metabolism
Cytokine biosynthesis
Regulation of cytokine biosynthesis
Regulation of protein biosynthesis
Protein biosynthesis
Macromolecule biosynthesis
Cellular macromolecule metabolism
Macromolecule metabolism
Regulation of organismal physiological process
Organismal physiological process
Bone remodeling
Response to biotic stimulus
Defense stimulus
Response to stimulus
Metabolism
Cellular metabolism
Biosynthesis
Cellular biosynthesis
Primary metabolism
Lipid metabolism
Lipid biosynthesis
Membrane lipid metabolism
Steriod biosynthesis
Sphingolipid metabolism
Steriod metabolism
Sterol metabolism
Cholesterol metabolism
Sterol biosynthesis
Cholesterol biosynthesis
Transmission of nerve impulse
Regulation of action potential
Synaptic transmission
Regulation of neurotransmitter levels
Nerve-nerve synaptic transmission
Synaptic transmission, cholinergic
Synaptic vesicle endocytosis
Synaptic vesicle maturation
Endocytosis
Synaptic vesicle transport
Vesicle-mediated transport
Secretory pathway
Secretion
Transport
Establishment of localization
Localization
Cell communication
Cell-cell signaling
Regulation of behavior
Behavior
Reproduction
Reproductive behavior
Negative regulation of behavior
Negative regulation of biological process
Cell cycle
Signal transduction
Interface
Mitotic cell cycle
Interface mitotic cell cycle
G1/S transition of mitotic cell cycle
Cell surface receptor linked signal transduction
G-protein coupled receptor protein signaling pathway
Neuropeptide signaling pathway
Tachykinin signaling pathway
Lipid transport
Sterol transport
Cholesterol transport
Morphogenesis
Development
Organ Development
Organogenesis
Skeletal development

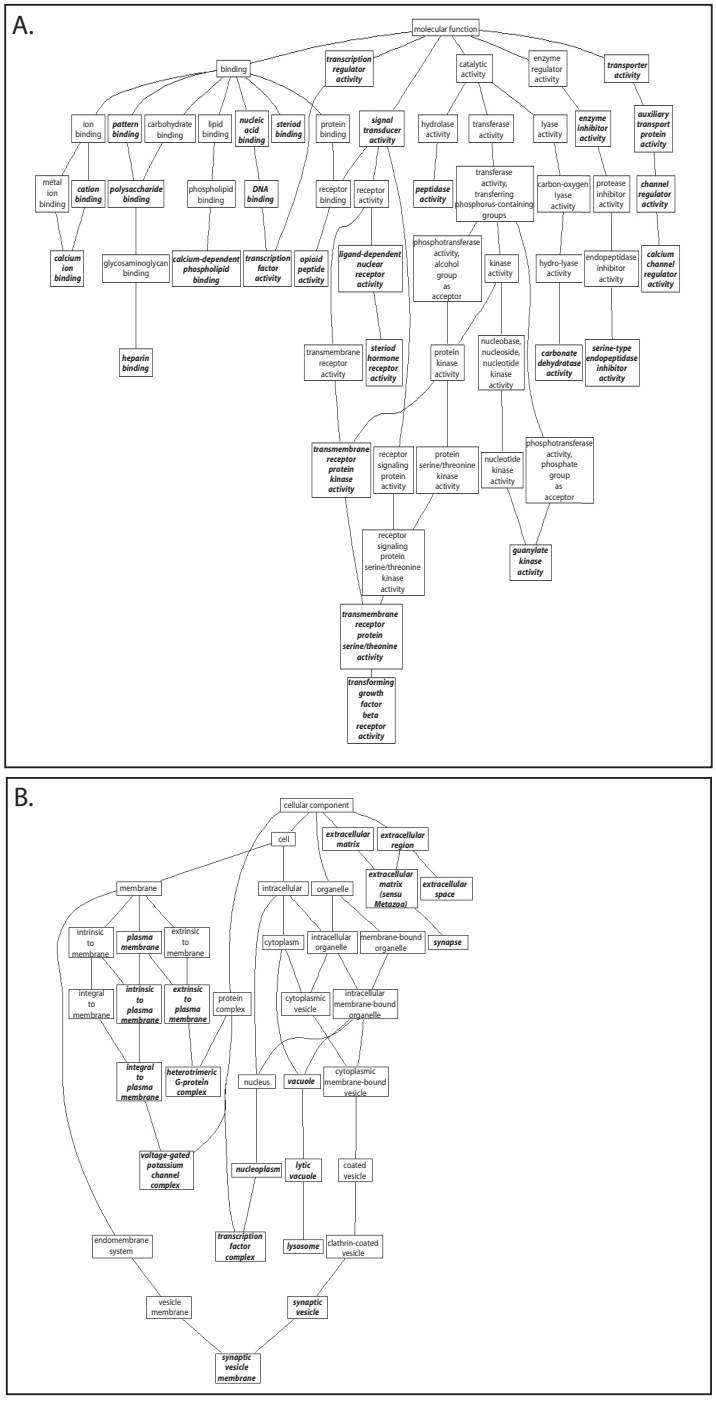


Figure 6: DAG view E18 Molecular function (A) and cellular component (B). Overview over the regulated GO groups (bold-italic) and their relation at E18.

Gene groups involved in neuronal transmission have high numbers of differentially expressed genes

More detailed analysis of gene groups indicated by the overrepresentation analysis showed that most gene groups were not enriched in the mutant. Of the groups containing more than 10 differentially expressed genes those involved in (regulation) of transcription were the most enriched groups at E14 and E16. At E18, groups involved in regulation of action potential, tachykinin signaling pathway, sterol transport and cholesterol transport were the only groups that were more than 6 times enriched (Table 2). However these groups only contained a low number of genes (2-3 genes).

Table 2. Enriched GO groups at E14, E16 and E18. The changed genes (according the Student's *t*-test) are grouped by the biological process, molecular function and cellular component. Indicated are the numbers of genes that are regulated in those groups (Observed) and the numbers that was expected in that group (Expected). The enrichment of the group is indicated (R) and the significance of this enrichment (P).

In biological process		Observed	Expected	R	P
E14	regulation of cell migration	2	0.15	13.33	0.0094
	regulation of cell motility	2	0.15	13.33	0.0094
	regulation of transcription\. DNA-dependent	11	5.25	2.1	0.0087
	transcription\. DNA-dependent	11	5.32	2.07	0.0096
	regulation of cellular physiological process	14	7.55	1.85	0.0072
	regulation of cellular process	14	7.73	1.81	0.0090
	regulation of physiological process	14	7.68	1.82	0.0084
	regulation of biological process	15	8.17	1.84	0.0053
E16	adult behavior	2	0.16	12.5	0.0097
	nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism	41	26.33	1.56	0.0009
	regulation of nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism	34	18.42	1.85	0.0001
	positive regulation of transcription\, DNA-dependent	5	1.08	4.63	0.0041
	positive regulation of transcription from RNA polymerase II promoter	4	0.85	4.71	0.0099
	regulation of transcription	34	18.26	1.86	0.0001
	regulation of transcription\, DNA-dependent	33	17.93	1.84	0.0002
	transcription	34	18.74	1.81	0.0002

	transcription\, DNA-dependent	33	18.13	1.82	0.0002
	transcription from RNA polymerase II promoter	8	2.81	2.85	0.0066
	transcription initiation from RNA polymerase II promoter	2	0.09	22.22	0.0029
	transcription initiation	2	0.16	12.5	0.0097
	regulation of cellular metabolism	34	19.43	1.75	0.0004
	regulation of cellular physiological process	41	26.06	1.57	0.0007
	endocytosis	7	1.53	4.58	0.0007
	synaptic vesicle endocytosis	2	0.09	22.22	0.0029
	regulation of cellular process	41	26.89	1.52	0.0013
	regulation of metabolism	34	19.86	1.71	0.0005
	regulation of physiological process	43	26.84	1.6	0.0003
	regulation of biological process	44	28.73	1.53	0.0007
E18	cell-cell signaling	24	9.58	2.51	0.0000
	transmission of nerve impulse	18	6.71	2.68	0.0000
	regulation of action potential	3	0.32	9.38	0.0017
	synaptic transmission	15	6.23	2.41	0.0005
	G-protein coupled receptor protein signaling pathway	32	21.08	1.52	0.0075
	neuropeptide signaling pathway	10	3.19	3.13	0.0004
	tachykinin signaling pathway	2	0	NA	0.0000
	sterol metabolism	12	3.19	3.76	0.0000
	cholesterol metabolism	11	3.03	3.63	0.0000
	cholesterol biosynthesis	7	1.28	5.47	0.0000
	sterol biosynthesis	8	1.44	5.56	0.0000
	regulation of cytokine biosynthesis	4	0.8	5	0.0028
	steroid biosynthesis	9	2.56	3.52	0.0002
	steroid metabolism	15	4.47	3.36	0.0000
	regulation of nucleic acid metabolism	63	33.86	1.86	0.0000
	positive regulation of nucleic acid metabolism	9	2.4	3.75	0.0001
	positive regulation of transcription	9	2.4	3.75	0.0001

	positive regulation of transcription\, DNA-dependent	6	1.76	3.41	0.0036
	regulation of transcription	63	33.06	1.91	0.0000
	regulation of transcription\, DNA-dependent	62	31.79	1.95	0.0000
	transcription	65	34.02	1.91	0.0000
	transcription\, DNA-dependent	63	32.42	1.94	0.0000
	regulation of cellular metabolism	70	37.38	1.87	0.0000
	positive regulation of cellular metabolism	12	3.35	3.58	0.0000
	regulation of cellular physiological process	95	73.47	1.29	0.0019
	positive regulation of cellular physiological process	22	11.98	1.84	0.0023
	potassium ion transport	8	3.19	2.51	0.0084
	sterol transport	2	0	NA	0.0000
	cholesterol transport	2	0	NA	0.0000
	secretory pathway	10	4.31	2.32	0.0063
	regulation of cellular process	97	76.99	1.26	0.0041
	positive regulation of cellular process	23	13.26	1.73	0.0040
	biomineral formation	6	1.92	3.13	0.0063
	ossification	6	1.92	3.13	0.0063
	skeletal development	8	3.19	2.51	0.0084
	determination of symmetry	4	0.96	4.17	0.0074
	determination of bilateral symmetry	4	0.96	4.17	0.0074
	determination of left/right symmetry	4	0.96	4.17	0.0074
	positive regulation of biosynthesis	4	0.8	5	0.0028
	regulation of metabolism	73	40.09	1.82	0.0000
	positive regulation of metabolism	13	3.83	3.39	0.0000
	organismal physiological process	62	42.65	1.45	0.0007
	bone remodeling	7	2.08	3.37	0.0018
	regulation of cytokine production	4	0.8	5	0.0028
	neurophysiological process	28	14.85	1.89	0.0004
	regulation of physiological process	100	76.83	1.3	0.0011

	positive regulation of physiological process	22	12.46	1.77	0.0039
	regulation of biological process	108	85.93	1.26	0.0025
In molecular function					
E14					
E16	nucleic acid binding	40	26.42	1.51	0.0022
	DNA binding	35	21.02	1.67	0.0009
	enzyme activator activity	5	1.24	4.03	0.0076
	transcription regulator activity	22	12.92	1.7	0.0078
E18	polysaccharide binding	11	5.15	2.14	0.0085
	heparin binding	9	3.7	2.43	0.0065
	cation binding	67	48.72	1.38	0.0021
	calcium ion binding	39	26.69	1.46	0.0066
	phospholipid binding	7	2.25	3.11	0.0032
	calcium-dependent phospholipid binding	5	1.45	3.45	0.0075
	DNA binding	69	46.95	1.47	0.0002
	transcription factor activity	30	12.22	2.45	0.0000
	opioid peptide activity	3	0.32	9.38	0.0017
	steroid binding	4	0.64	6.25	0.0007
	carbonate dehydratase activity	2	0	NA	0.0000
	guanylate kinase activity	2	0	NA	0.0000
	signal transducer activity	91	72.52	1.25	0.0065
	ligand-dependent nuclear receptor activity	5	0.16	31.25	0.0000
	steroid hormone receptor activity	5	0.16	31.25	0.0000
	transcription regulator activity	39	17.04	2.29	0.0000
	transporter activity	68	47.27	1.44	0.0005
	auxiliary transport protein activity	3	0.32	9.38	0.0017
	channel regulator activity	3	0.32	9.38	0.0017
	calcium channel regulator activity	3	0.32	9.38	0.0017
	potassium channel activity	6	1.77	3.39	0.0037

	voltage-gated potassium channel activity	4	0.96	4.17	0.0075
In cellular component					
E14					
E16	chromosome	9	2.67	3.37	0.0013
	chromatin	5	1.32	3.79	0.0099
	nucleoplasm	15	6.86	2.19	0.0030
	transcription factor complex	13	5.77	2.25	0.0045
E18	synaptic vesicle	8	1.47	5.44	0.0000
	synaptic vesicle membrane	3	0.49	6.12	0.0046
	vacuole	14	5.4	2.59	0.0003
	lytic vacuole	14	4.91	2.85	0.0001
	lysosome	14	4.91	2.85	0.0001
	nucleoplasm	21	8.84	2.38	0.0001
	transcription factor complex	17	5.4	3.15	0.0000
	heterotrimeric G-protein complex	5	1.31	3.82	0.0042
	integral to plasma membrane	37	24.57	1.51	0.0047
	voltage-gated potassium channel complex	3	0.33	9.09	0.0018
	intrinsic to plasma membrane	37	24.57	1.51	0.0047
	plasma membrane	78	59.95	1.3	0.0045
	extracellular region	142	115.8	1.23	0.0012
	extracellular space	128	105.64	1.21	0.0040

Of the gene groups containing 15 or more genes with different RNA levels the most enriched (more than 2 times) groups were synaptic transmission (Fig. 5), steroid metabolism, transmission of nerve impulse, and cell-cell signaling (Table 2). The groups synaptic transmission and transmission of nerve impulse are subgroups of the group cell-cell signaling (Fig. 1c). None of these groups were enriched at E14 or E16. Most of the genes involved in synaptic transmission and the neuropeptide genes had lower transcript levels in the mutant (Fig. 7)

The groups cell-cell signaling, secretory pathway and neurotransmitter transport contain synapse specific genes. In the cell-cell signaling group many genes had similar transcript levels, including 33 neurotransmitter receptors. The enriched group secretory pathway

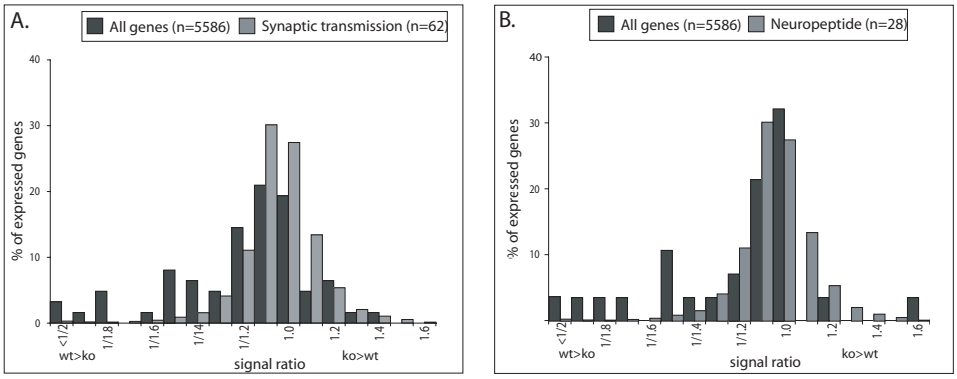


Figure 7: Distribution of gene expression differences for all genes and in certain gene groups. A: For each group, all expressed genes were classified into signal intensity difference intervals (0.1 bins) according to their Cy3/Cy5 signal ratio. Transcripts in the "1" bins had identical Cy5 versus Cy3 signal intensities and were thus not regulated. Positive values indicate that the levels are higher in mutants (ko), while negative values indicate higher levels in control animals (wt). The y-axis shows the percentage of genes in a certain bin. In all the panels, the black bars indicate the distribution of all expressed genes across eleven comparisons of mutant and control animals. Grey bars denote distribution of expressed genes in the synaptic transmission group, B: neuropeptide group.

contained several genes involved in synaptic vesicle release, like synaptotagmin1 and SNAP25, which had similar transcript levels. In this group also DOC2A (a binding partner of Munc18-1) was present, for which we found that the RNA levels were lower in the mutant. This group included several molecules of the CASK/Lin2-Veli/Lin7-Mint1/Lin10 complex, some of these were affected in the mutant, like veli, neurexin II and neurexin III. The group neurotransmitter transport, including the munc18-1 binding partner syntaxin1A (which was significantly changed), was not significantly enriched. In this group 9 neurotransmitters carriers were present, of which only the transcript of the GABA carriers were different. All these genes were significantly different as well if tested using SAM.

In the GO-enriched gene group cell-cell signaling, several genes were coding for neuropeptides. RNA levels of all neuropeptide genes (6) on the array were higher in controls than in the mutants (significant both in Student's *t*-test and in SAM) and those genes were among the most severally differentially expressed genes (Fig. 7). The RNA levels of the somatostatin and substance P genes showed the largest differences (see chapter 4 of this thesis). Transcript levels of the dynorphin and enkephalin gene were 1.5 fold higher in the controls and cholecystokinin levels were also significantly higher (1.2 fold). The transcript levels of other secreted substances were lower in the mutant as well, for instance the neurotrophic gene BDNF (1.5 fold), FGF1 (1.5 fold) and Reelin (1.8 fold).

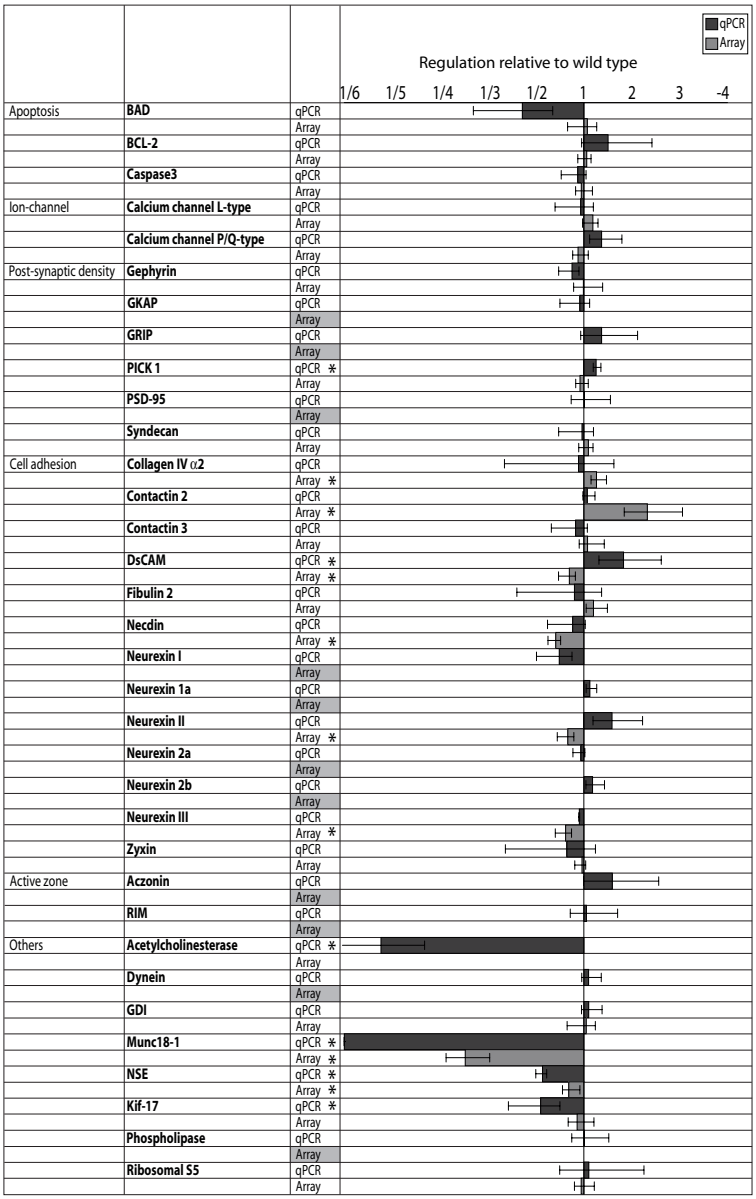
Immune or glial genes are higher expressed in the mutant at E18

Although gene groups with high numbers of genes with different RNA levels contained mainly genes with lower RNA levels in the mutant, in general as many genes had higher as had lower transcript levels in the mutant (Fig. 4). Genes that had a more than 2 times higher levels in the mutant were; secreted phosphoprotein 1 (ossification), macrophage-specific gene (steroid metabolism), P lysozyme structural (signal transduction), glial fibrillary acidic protein (cytoskeleton organization and biogenesis), contactin 2 (cell adhesion), CD68 antigen (cell adhesion), and lysosomal-associated protein transmembrane 5 (vacuolar transport). Most of the genes that had higher RNA levels in the mutant have a function in glial cells, immune cells or lysosomes. The group regulation of cytokine production was enriched (Fig. 5). This may indicate that an immune response occurred in the mutant. This may explain not only the increase in immune response genes, but also the increase in glial and lysosomal genes.

Quantitative PCR validates differential expression

To validate the differences in transcript levels, we studied other RNA samples with qPCR. We studied to most affected genes in the array experiment and several post-synaptic genes and cell-adhesion molecules that were not printed on the array, because the literature shows that these genes may be affected by synaptic activity. Several synaptic structure and function genes, like ion-channels, proteins of the post-synaptic density, cell adhesion molecules, and proteins of the active zone were studied in the *munc18-1* null mutant at embryonic day 18.

The RNA levels of two ion-channels, six post-synaptic density proteins, thirteen cell adhesion molecules, two active zone proteins, and some other proteins were measured using both qPCR (n=3) and array analysis (n=11). The levels were measured relative to hypoxanthine phosphoribosyl transferase (HPRT). Many of the tested genes had similar transcript levels in the mutant compared to control animals (Student's *t*-test α=5%), which was shown both by qPCR and microarray hybridization (Fig. 8). The RNA levels of the genes coding for acetylcholinesterase, NSE and Kif-17 were lower in the mutant. The levels of NSE were also lower in the mutant if using microarray analysis. The levels of acetylcholinesterase were too low to detect using microarray hybridization. A few synaptic structure transcripts had different RNA levels, but only if measured by microarray analysis; the RNA levels contactin 2, and contactin3 were higher in the mutant, whereas the levels of DsCAM, Necdin, NeurexinII and NeurexinIII were lower. However, the differences in transcript levels were very small. In general the measured transcript levels using array technology or qPCR were similar, only for a few transcripts the two methods gave different results.



Apoptosis	**BAD**	qPCR	
		Array	
	BCL-2	qPCR	
		Array	
	Caspase3	qPCR	
		Array	
Ion-channel	**Calcium channel L-type**	qPCR	
		Array	
	Calcium channel P/Q-type	qPCR	
		Array	
Post-synaptic density	**Gephyrin**	qPCR	
		Array	
	GKAP	qPCR	
		Array	
	GRIP	qPCR	
		Array	
	PICK 1	qPCR *	
		Array	
	PSD-95	qPCR	
		Array	
	Syndecan	qPCR	
		Array	
Cell adhesion	**Collagen IV α2**	qPCR	
		Array *	
	Contactin 2	qPCR	
		Array *	
	Contactin 3	qPCR	
		Array	
	DsCAM	qPCR *	
		Array *	
	Fibulin 2	qPCR	
		Array	
	Necdin	qPCR	
		Array *	
	Neurexin I	qPCR	
		Array	
	Neurexin 1a	qPCR	
		Array	
	Neurexin II	qPCR	
		Array *	
	Neurexin 2a	qPCR	
		Array	
	Neurexin 2b	qPCR	
		Array	
	Neurexin III	qPCR	
		Array *	
	Zyxin	qPCR	
		Array	
Active zone	**Aczonin**	qPCR	
		Array	
	RIM	qPCR	
		Array	
Others	**Acetylcholinesterase**	qPCR *	
		Array	
	Dynein	qPCR	
		Array	
	GDI	qPCR	
		Array	
	Munc18-1	qPCR *	
		Array *	
	NSE	qPCR *	
		Array *	
	Kif-17	qPCR *	
		Array	
	Phospholipase	qPCR	
		Array	
	Ribosomal S5	qPCR	
		Array	

Figure 8: Quantification of RNA levels of in control and *munc18-1* mutant mice at E18 by qPCR and array. RNA levels of E18 embryos of both control and mutant mice were measured using both qPCR and array for transcripts that have a function in apoptosis, ion-channels, post-synaptic density, cell adhesion, active zone and some others. First column: Group. Second column: Name of the transcript. Third column: Measured using qPCR or microarray analysis. Asterisks indicate a significant change (Student's *t*-test; α=5%). Fourth column: Amount of transcript present in the mutant relative to control as measured with qPCR (black) and array (gray). For each transcript the amount was standardized to HPRT (qPCR) or to the total signal (microarray). Data represent mean ± SEM of 3 animals (qPCR) or 11 animals (microarray) per transcript. The SEM are asymmetric for the qPCR data, because the log scale qPCR data are transposed to the linear fold change scale. For proprotein convertases and secretory protein 6 animals per sample (qPCR) were used.

The expression of apoptotic genes is not different in the mutant

To test whether apoptosis was induced in the cortex E18 mutants, we analyzed the transcript levels of genes involved in apoptosis.
The transcript levels of three apoptotic genes were measured using both qPCR (n=3) and array analysis (n=11). In the qPCR, the RNA levels were measured relative to HPRT (Fig. 8). Of the 88 printed apoptotic transcripts were 63 detectable in the array. The highest abundant transcript was caspase3, whereas BAD had the lowest RNA levels. The levels for the tree apoptotic genes that were measured using qPCR were equal in the mutant compared to control mice (Student's *t*-test α=5%) in the qPCR and microarray analysis. Of the other apoptotic genes present on the array only 10% was changed at E18 and none of these were changed at E14. Because the apoptotic gene group is affected in the mutant to a low extend, no additional apoptosis occurs in the mutant neocortex relative to control animals.

DISCUSSION

In this paper we studied the effect of synaptic activity on gene expression in the developing brain. Therefore we analyzed transcript levels in the *munc18-1* null mutant, which is deprived of evoked and spontaneous release of classical neurotransmitters and has a reduced secretion from large dense core vesicles, relative to control littermates using microarray analysis and quantitative PCR. The transcript levels of many genes and gene groups, especially of genes that have a basal function, were not different in the mutant. In addition, we found minor changes in the levels of a few apoptotic genes. Several genes that have a function in glial cells, immune cells or lysosomes had higher transcript levels in the mutant. Gene groups containing high numbers of genes with affected RNA levels were synaptic transmission, steroid metabolism, transmission of nerve impulse, and cell-cell signaling. At E14 and E16, when synapses are not yet functional in the cortex, these gene groups were not enriched. These results were confirmed using qPCR.

Model and methods are well controlled

Measuring the RNA levels using qPCR and microarray analysis showed comparable results. Both methods showed differences in transcript levels for several genes and many genes were shown to have similar levels in the mutant compared to control animals using both methods. However, for several cell adhesion molecules a small difference in the transcript levels were detectable using microarray analysis, but qPCR did not confirm these differences. In addition, the differences were often larger when measured by qPCR than when measured by microarray analysis, as was observed by

others (see for instance: Musatov *et al.*, 2004 and McMullen *et al.*, 2004). This may be a result of the overlapping spectra of Cy3 and Cy5 or by auto-fluorescence of the spotted probes. The transcript levels of acetylcholinesterase were measurable using qPCR, but was too low to be detected by microarray analysis. In conclusion, both methods gave very similar results, however qPCR is a more sensitive method.

A thorough previous analysis at the cellular level suggests that at E14 mutants have no detectable phenotype in the brain (Verhage, *et al.*, 2000). The changes in RNA levels at this stage of development may be considered for that reason as non-specific effects or may be caused by transcription regulation of Munc18-1 and will not be caused by synaptic activity. For this reason we used this E14 animals as control for effects other than caused by activity. The amount of genes with differences in RNA levels at E14 is much smaller compared to the other embryonic days and no synaptic function genes had different RNA levels at this time point. Therefore, comparing the transcript levels in mutant and control animals at E14 is a good control for effects that are not caused by activity in the mutant.

The lower expression level for synapse function proteins is not the result of apoptosis

In the *munc18-1* null-mutant, massive degeneration was observed in lower (non-cortical) brain areas between E16 and E18 (Verhage *et al.*, 2000). However, it is unlikely that this degeneration affects the analyses in the neocortex described here. First, the array data showed no significant change in the RNA levels of gene groups involved in apoptosis and the qPCR measurements showed not significant change in BAD, BCL-2 or caspase 3 (Fig. 8) in the neocortex. Second, no morphological signs of degeneration were observed in a detailed analysis of the neocortex (chapter 2). Third, if cell death would be involved, RNA levels of other synaptic and non-synaptic genes is expected to be affected equally, which was not observed. Finally, lower innervation, and thus synaptic input, of the neocortex from degenerating brain areas is unlikely to affect the observed differences because the embryonic neocortex is poorly innervated by neurons in other areas and most synapses are corto-cortical connections. Again, also in this scenario the transcript levels of all genes should be affected equally. However although several synaptic genes were affected in the mutant, several synaptic proteins were not had similar transcript levels in the mutant, like SNAP25, VAMP2 and synaptotagmin 1. Therefore the regulation of some synaptic transcripts is not caused by apoptosis of a specific part or a changed innervation of the cortex. In conclusion, apoptosis in the mutant brain cannot explain the differences in transcript levels in the mutant cortex.

Genes expressed in immune and glial cells and genes with a lysosomal function had relatively high RNA levels in the mutant at E18. Some of these transcripts are similar in neurons (Holness *et al.*, 1993; Schafer *et al.*, 2000). These transcripts may have higher

levels in the mutant as a result of infiltration of the brain by immune cells to remove the apoptotic cells in the lower brain areas. Therefore, the increase in some immune response transcripts may be an indirect effect of the synaptic defects.

Expression of synapse function genes is regulated by synaptic release

Groups of genes involved in synapse function contained the highest amount of regulated genes and most pronounced affected genes. Most of these transcripts were lower in the mutant at E18, but their levels were similar at E14 and E16, when synapses are not yet functional in the cortex (Konig, *et al.*, 1975). For instance, the levels for Doc2, syntaxin 1a (both Munc18-1 binding proteins), syntaxin 3A and synaptogyrin 3 were lower in the mutant at E18 (see chapter 5). A plausible explanation for these observations is that normally synaptic release increases the RNA levels of genes involved in synapse function, but is not increased in the *munc18-1* mutant that lacks synaptic release. Other studies have shown a regulation by activity of these synaptic genes (Sutton *et al.*, 1999; Davis *et al.*,, 1998 ; Belizaire *et al.*, 2004). However, in this study the expression of these genes may also be reduced as a direct effect of the low levels of their binding partners. In addition, dark-rearing postnatal mice leads to changes in the expression of synaptic proteins (Tropea *et al.*, 2006). However, this study especially showed an increase in the expression of receptor subunits, this in in contrast to our results. In the group secretory pathway also veli, neurexin II and neurexin III had different transcript levels in the mutant. Munc18-1 is connected to the CASK/Veli/Mint complex via binding to Mint1, this complex is hypothesized to have a role in synapse formation (Scheiffele, 2003). However, these differences in RNA levels could not be confirmed using qPCR and therefore might be a false positive of the array (see chapter 5).

The gene group cell mobility is another group containing a high number of regulated genes. The increase in the levels of cell mobility genes, like cytoskeleton genes, may be necessary in control animals for the morphological changes that occur during synapse maturation. Some cytoskeletal genes have been shown to be regulated by synaptic release (Lyford *et al.* 1995) and some have been shown to be essential for spine mobility (Ackermann and Matus, 2003; Meng *et al.*, 2003). However, in the *munc18-1* mutant the affected cell mobility genes were mainly involved in synapse function, like calbindin-28K, and calretinin. This observation provides more proof that especially synapse function expression is affected in the mutant.

In this study genes involved in synaptic function had mainly lower RNA levels in the mutant, whereas genes involved in cell adhesion had both higher and lower levels. This difference may occur because cell adhesion molecules have a very diverse role in the brain. Cell adhesion molecules are not only important in neurons, but also in glial cells and immune cells, whereas synapse function molecules have a more specific role.

Synapse instability is not influenced on gene expression level

At E16 and E18 less synapses are present in the cortex of the mutant (chapter 2), possibly because synapses cannot be preserved without synaptic activity. In control animals, activity may increase the expression of several genes. This increase may be essential for the maturation of synapses. Without activity, such as in the mutant, the expression of these genes will not be increased and synapses may be eliminated. Candidate genes, involved in this phenomenon, may have a function in synapse structure, such as cell adhesion molecules or PSD-molecules. However, the transcript levels of these genes are not severely reduced in the mutant. Thus, the instability of synapses in the mutant is probably caused by an intrinsic effect of the deletion of *munc18-1* (see also Heeroma *et al.*, 2004). On the other hand, the reduction in the RNA levels of several genes involved in cell-cell signaling is most likely regulated by synaptic release, because the expression of these genes was similar in mutant and control animals at E14 and E16. In control animals this synaptic activity-dependent increase in neuronal secretion genes may be essential for the formation of mature synapses.

REDUCED EXPRESSION OF NEUROPEPTIDE GENES IN A MOUSE MODEL LACKING SYNAPTIC ACTIVITY

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4

Chapter 4

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ABSTRACT

Activity-dependent changes in synapses rely on functional changes in resident proteins and on gene expression. We addressed the relation between synapse activity and the expression of synaptic genes by comparing RNA levels in the neocortex of normal mice versus synaptically silent *munc18-1* null mutants, using microarray analysis, quantitative PCR and Northern blotting. We hypothesized that RNA levels of synaptically released compounds will be reduced in mutants, relatively to controls. We found that all neuropeptide genes detectable on the microarray had different transcript levels, being 3-20 fold higher in control cortex. Transcript levels for genes encoding for their receptors and most other synaptic components were similar in the mutant. Differences in the transcript levels of neuropeptide genes were confirmed by qPCR analysis. *In situ* hybridization indicated that the difference in neuropeptide expression was uniform and not due to the loss of specific cells in the mutant. In primary sensory neurons, which do not depend on synaptic activity for their input, the differences in neuropeptide transcript levels were not observed. These data reveal no simple relation between activity of synapses and expression of their resident proteins, but identified a unique feature of neuropeptide gene expression, which appears to depend on synaptic activity.

INTRODUCTION

The strength of synaptic coupling between neurons is regulated by a number of different mechanisms acting on different time scales both during development and in mature networks. Most of the short-term changes probably depend on local, activity dependent changes in resident proteins, e.g. translocation, phosphorylation etc. Long-term changes probably involve changes in protein composition of the synapses involved. Indeed, gene expression is essential for some forms of synaptic plasticity (see for instance (Nguyen *et al*., 2002). Several lines of evidence suggest a link between synapse use and the supply of synaptic components via activity dependent gene expression. Firstly, interference with sensory input decreased the expression of synaptic genes (Munoz *et al*., 1998). Secondly, pharmacological interference with action potential propagation or synaptic activity decreased the expression of synaptic genes (Missias *et al*., 1996; Corriveau *et al*., 1998; Hoffmann *et al*., 2000; Loeb *et al*., 2002). Conversely, agents that induce synaptic activity also induced the expression of several synaptic components (Nedivi, 1999; Lou and Bixby, 1995; Plunkett *et al*., 1998).

In addition to genes encoding resident synaptic proteins, synaptic activity may also influence the expression of synaptic 'consumables', like secreted neuropeptides, neurotrophins and their co-factors. For instance, BDNF RNA levels are regulated by LTP

and injury (Hughes *et al*., 1999) and BDNF immunoreactivity was reduced after visual deprivation in retinal ganglion cells (Seki *et al*., 2003). Lesions in the cortex decreased VGF RNA levels in the ipsilateral cortex (Snyder *et al*., 1998) but increased the RNA levels of cholecystokinin, neuropeptide Y, and somatostatin (Jacobs *et al*., 1994). Furthermore, intraocular tetrodotoxin injection rapidly decreased the levels of VGF in the geniculate nucleus (Snyder *et al*., 1998) and the depolarizing agent veratridine increased the RNA and protein levels of somatostatin in cortical cultures (Tolon *et al*., 1996).

Together these lines of evidence identify a relationship between neuronal activity or synapse use and the expression of certain synaptic components and secreted factors. Such a coupling may be a general principle that contributes to the proper establishment of functional synapses during brain development and to various long-term changes in synaptic coupling in the adult brain. However, it is currently unclear how such principles may operate, whether expression of all synaptic components is regulated in a concerted manner, when synapses have just formed and in the adult brain, and whether expression is primarily coupled to the activity of the whole neuron or directly to the activity of its synapses.

To get a better insight into these issues, we examined the effect of synaptic activity on the RNA levels *in vivo* in a mouse model devoid of synaptic activity. The *munc18-1* null mutant mice are characterized by the complete loss of both evoked and spontaneous release events from both inhibitory and excitatory neurons in the brain, but do show spontaneous action potentials in individual cells and morphologically normal synapses (Verhage *et al*., 2000; Bouwman *et al*., 2004; Heeroma *et al*., 2003). The release from large dense cored vesicles is also severely impaired (Voets *et al*., 2001, Korteweg *et al*., 2005). Consequently, these mutants will also fail to induce expression of genes that are normally induced by synaptic activity in normal animals. By comparing RNA levels between wildtypes and mutants at the time when synapses become active, such activity dependent genes can be identified. For this purpose we performed an open microarray screen and found that few genes were differentially expressed between mutants and wildtypes. However, striking differences in the expression of neuropeptide genes were observed, but not of their receptors, neurotransmitter producing enzymes and neurotransmitter-receptors. We investigated this further by real-time quantitative PCR and Northern blot analysis and used in situ hybridization to exclude that the loss of specific cells accounted for the differences in neuropeptide mRNAs between mutants and controls.

EXPERIMENTAL PROCEDURES

Laboratory animals

Munc18-1 deficient mice were generated as described before (Verhage *et al*., 2000). Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous matings. The day a vaginal plug was observed was considered to be embryonic day 0 (E0). Embryos were harvested from mothers rapidly sacrificed after timed matings at E14, E16 and E18. Experimental procedures were carried out with local regulatory approval for animal experimentation, according to the Dutch law for animal welfare.

RNA isolation

See chapter 3.

Microarrays

See chapter 3.

Northern

Total RNA was isolated from brains of E18 embryos by using Trizol (Invitrogen, San Diego, USA). The RNA (10 µg/lane) was subjected to electrophoresis (1.0% agarose, 0.04 M 3-[*N*-morpholino]propanesulfonic acid (pH 7.0), and 1.75% formaldehyde), transferred to a charged nylon membrane (Amersham, Uppsala, Sweden) and UV cross-linked (3 min). Sequential hybridizations were performed to cDNA probes for enkephalin and GAPDH, respectively. After prehybridization (65° C, 30 min, 0.5 M $NaHPO_4$ pH 7.0, 1% BSA, 1 mM EDTA, 7% SDS) hybridizations were performed overnight at 65°C in the same buffer now containing 0.2×10^6 cpm/ml probe, labeled with α-^{32}P dCTP to a specific activity of 2×10^9 cpm/µg, using the Random Primers DNA Labeling System (Roche, Basel, Switzerland). After hybridization, the membrane was washed twice in hybriwash 5 (65° C, 5 min, 40 mM $NaHPO_4$ pH 7.2, 1 mM EDTA, 5% SDS) and twice in hybriwash 1 (65° C, 15 min, 40 mM $NaHPO_4$ pH 7.2, 1 mM EDTA, 1% SDS) and then exposed up to 48 hr in a PhosphoImager screen (Fuji, Tokyo, Japan). Signals were analyzed with Aida 3.0 software.

qPCR

See chapter 3.

In situ hybridization

In situ hybridization was carried out as described by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993), with minor changes. Cryostat sections of the whole brain, cut at 16 µm thickness, were thaw-mounted onto poly-L-lysine coated slides, dried and fixed for 10 minutes in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS sections were acetylated for 10 minutes in a solution containing 245 ml H_2O, 0.013% triethanolamine, 438 µl HCl (37%) and 625 µl acetic anhydride. Sections were then washed with PBS and prehybridized for 2 hours at room temperature in a hybridization solution (50% deionidized formamide, 5X SSC (20X SSC: 3 M sodium chloride, 0.3 M sodium citrate), 0.1% Ficoll 400, 01% polyvinylpyrrolidone, 0.1% bovine serum albumine, 250 µg/ml baker's yeast RNA and 500 µg/ml herring sperm DNA). Hybridization was performed overnight at 72° C with 300-1300 ng/ml digoxygenin-labeled RNA probe in 150 µl hybridization buffer. Sections were then washed shortly with 2X SSC and washed for 2 hours in 0.2X SSC at 72° C. Preincubation with 1 ml buffer 1 (100 mM Tris-HCl pH 7.4; 150 mM NaCl) with 10% heat inactivated fetal calf serum (hiFCS) was performed for 1 hour at room temperature in a humidified chamber. The sections were then incubated overnight at 4° C with alkaline phosphatase-conjugated mouse anti-digoxygenin Fab fragment (Roche, Basel, Switzerland), 1:5000 diluted in buffer 1 with 1% hiFCS. Following washing with buffer 1 and equilibration with buffer 2 (100 mM Tris-HCl, pH 9.5; 50 mM $MgCl_2$; 100 mM NaCl), the color reaction was performed in the dark for 24 hours at room temperature with 200 µl NBT/BCIP solution (Roche, Basel, Switzerland) and 2.4 mg levamisole in buffer 2.

Extraction of Substance P from brain

Brains or cortices were solubilized with a tissue homogenizer and were then sonicated on ice (3 x 10 sec) in 10 mM Hepes (pH 7.4) with proteinase inhibitors (Roche, Basel, Switzerland). Then NaCl and Triton-X100 were added (final concentration: 150 mM and 1%, respectively) and the samples were rotated at 4°C for 1 h. Homogenates were centrifugated for 5 min at 16,100g.

Reverse-phase columns (1.0 ml C18) were activated with methanol, followed by rinsing with TFA (trifluoroacetic acid; 7.5 mM). Samples (supernatant of homogenate) were loaded on columns and the columns were washed two times with TFA. Substance P was eluted from the columns with 1 ml of 60% acetonitrile in 7.5 mM TFA. The samples were dried in a speed-vac until 10 µl was left and 100 µl EIA buffer was added (Cayman Chemical Co., Ann Arbor, MI, USA). Solubilized peptides in the sample were quantified using a BCA protein assay (Pierce, Rockford, Illinois, USA).

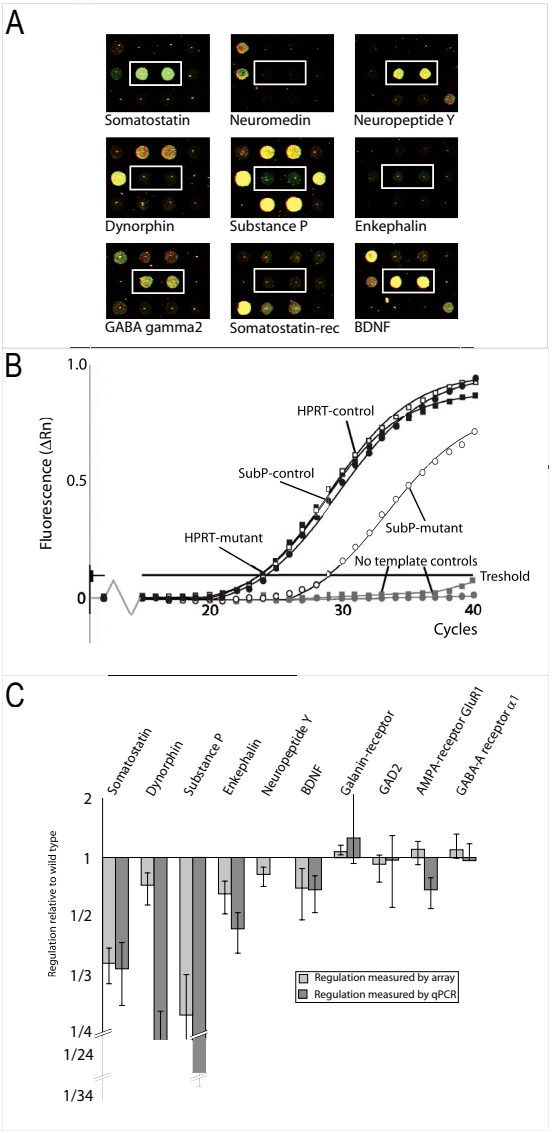

Figure 1: Quantification of expression levels in control and mutant animals by array, qPCR and Northern blot. A: Quantification of RNA levels using microarray technology. Control RNA was labeled with Cy3 (green) and mutant with Cy5 (red). Levels were standardized to the total level in the channel (green or red). Each picture represents a certain gene in duplicate with the surrounding spots on the array. Green: higher in control. Red: higher in mutant. Yellow: equal expression. The intensity of the spot indicates the abundance of the transcript. For results see figure 2. B: Raw data of a qPCR experiment on wild type and mutant for substance P and HPRT. X-axis: PCR cycles numbers, Y-axis: fluorescence. If the curve passes the threshold the number of cycles that the PCR has performed at that point is the read-out for the abundance of the transcript. C: Comparison of qPCR data and array data for neuropeptide genes, indicating that the results from the qPCR and array experiments are similar. An important observation is that the array experiment gives less pronounced differences than the qPCR. Dark gray bars represent qPCR regulation data and lighter gray bars represent array data. Error bars indicate the SEM.

Quantification of substance P; enzyme immunoassay

The quantification of Substance P in brain samples was accomplished by an antigen competition enzyme immunoassay (EIA) (Cayman Chemical Co., Ann Arbor, MI, USA). In the assay substance P coupled to acetylcholinesterase and substance P in the lysed brain sample compete. The acetylcholinesterase hydrolyzes acetylthiocholine to thiocholine and this in turn reacts with 5,5'-dithio-bis-2-nitrobenzoic acid, producing a 5-thio-2-nitrobenzoic acid absorbing at 412 nm. To the wells, which are pre-coated with monoclonal anti-rabbit antibody, 50 µl of the lysed brain sample was added. Then 50 µl of the acetylcholinesterase-conjucated substance P and 50 µl of rabbit anti-substance P was added, except to the non-specific binding wells, in which buffer was added. The plates were then incubated at 4° C overnight and washed for 5 times with wash buffer. Then, 50 µl of the enzyme substrate was added and incubated for 1 hour at room temperature, in the dark with rotation. The absorbance was measured at 405 nm in a microplate reader (Biorad, Hercules, CA, USA). We calculated the net optical density (OD) dividing the sample values by the non-specific binding values. The OD values were divided by the amount of peptide in the sample (that was measured with the BCA) and compared between mutant and control. The results were standardized to synaptobrevin and GDI levels, which were quantified by Western blot.

RESULTS

Microarray analysis identifies neuropeptide genes as the main differentially expressed gene group.

To address the relation between synaptic activity and neuropeptide expression, we compared the RNA levels between *munc18-1* null mutant and control neocortex at embryonic day (E) 14, 16 and 18. We analyzed the neocortex because some other brain areas suffer from degeneration at E18 in the null mutant, but the neocortex shows no signs of impaired viability or aberrant organization (chapter 2). Comparisons were made at this late embryonic stage because the number of synapses in the neocortex was previously found to increase 3 fold between E16 and E18 (chapter 2) and synaptic secretion events were readily observed in control neocortical slices at this stage (Verhage *et al*., 2000). Hence, there is a suitable time window of massive synaptogenesis during prenatal development to study induction of synaptic gene expression in lethal mutants.

We analyzed the transcript levels using microarray analysis of a 7k oligonucleotide library (Fig. 1A). More than 90% of the detected mRNAs showed no differences between the mutant and controls. Genes which were affected in the mutant with a similar function were grouped according to Gene Ontology (GO) assignments

	Literature: Cortex	Literature: Development	Fold regulation measured by array	Fold regulation measured by qPCR	Abundance (ΔCt relative to HPRT)
Neuropeptides					
Cholecystokinin (8&58)	?	E15[16]	-1.20*	1.11	medium (0.9)
Dynorphin	+	E4-E11[1]	-1.46*	-4.13*	medium (0.9)
Enkephalin	+	E11[2]	-1.61*	-2.20*	high (-1.5)
Neurotensin	?	?	N.A.	1.48	high (-1.1)
Somatostatin	+	E14[3]	-2.78*	-2.94*	very high (-4.7)
Substance P	+	E15[4]	-3.65*	-24.3*	medium (0.4)
Neuropeptide Y	?	?	-1.28*	N.A.	
Neuropeptide receptors					
Arg-vasopressin-receptor1A	?	?	N.D.	1.03	low (2.2)
Galanin-receptor-1	?	?	-1.09	1.33	low (3.2)
Neuromedin-K receptor	?	?	N.A.	1.09	low (2.8)
Neurotensin-receptor-I	+	E18[6]	1.11	1.47	high (-1.3)
Neuropeptide-Y-receptor-1	?	?	1.60*	1.47	medium (-0.4)
Somatostatin-receptor-1	+	E18[5]	1.25	1.13	medium (-0.5)
Substance-P receptor	?	?	N.D.	-1.29	very low (4.5)
VIP-receptor-1	?	?	N.A.	1.53	low (3.7)
Neurotransmitter-enzymes					
Glutamic acid decarboxylase 2	?	?	1.11	-1.04	high (-2.1)
Nitric oxide synthase	?	?	-1.09	1.31	medium (-1.0)
Tyrosine hydroxylase	?	?	N.D.	-3.23*	low (3.6)
Neurotransmitter-receptors					
AMPA-receptor subunit GluR1	+(rat)	low E18[13]	-1.06	-1.54	high (-1.5)
AMPA-receptor subunit GluR3	+(rat)	?	-1.07	1.15	medium (-0.4)
D1-receptor	?	?	-1.21	-1.53	low (2.9)
GABA-$_A$-receptor subunit α1	++	E18[7]	1.12	-1.05	medium (0.3)
GABA-$_A$-receptor subunit α2	+	E17[9]	-1.12	1.46	low (1.1)
GABA-$_A$-receptor subunit α3	+	E17[9]	1.05	N.A.	N.A.
GABA-$_A$-receptor subunit α4	++	+[10]	-1.17	1.01	low (3.0)
GABA-$_A$-receptor subunit α5	++	E17[9]	-1.79*	-1.44	medium (-1.0)
GABA-$_A$-receptor subunit α6	?	?	-1.03	N.A.	N.A.
GABA-$_B$-receptor subunit 1	?	?	1.00	-1.00	high (-2.2)
Kainate-receptor subunit GluR6	+/-(rat)	?	1.01	1.12	high (-1.5)
NMDA-receptor subunit NR1	+++	E18[11]	1.01	1.47	medium (0.3)
NMDA-receptor subunnit NR2A	+	low E18[12]	1.23	-1.33	medium (0.5)
Serotonin-receptor 1B	?	?	N.D.	-2.89*	very low (4.4)
Proprotein convertase associated protein					
7B2	+	E11[14]	-1.07	1.05	high (-2.5)
Proprotein convertases					
PC1	+	?	N.A.	-1.59	low (3.9)
PC2	?	?	N.A.	-1.21	low (3.9)
Neurotrophin					
BDNF	+	?	-1.51*	-1.56*	very low (4.4)
Controls					
Munc18-1	+	E13[15]	-3.48*	-91.5*	high (-3.6)
Dynein	+	+	1.06	1.10	high (-3.5)
GAPDH	+	+	1.01	-1.21	high (-6.0)

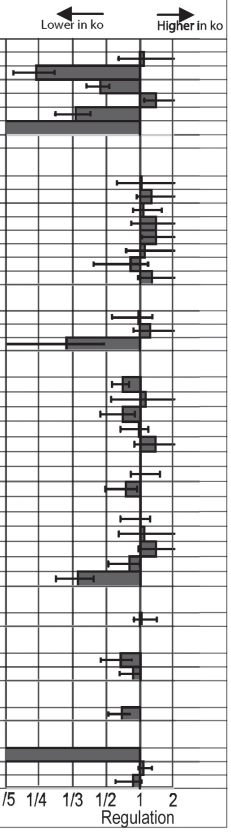

Figure 2: Quantification of RNA levels in control and *munc18-1* mutant mice at E18 by qPCR and array. A: RNA levels of E18 embryos of both control and mutant mice were measured using both qPCR and microarrays. First column: Name of the transcript and group to which the transcript belongs. Second column: Expression data from literature, whether or not the transcript is expressed in the mouse cortex (? = not found in literature, +/- = medium expression levels, + = high expressed, ++ = very high expressed, +++ = extremely high expression levels). Third column: First detected time point in embryonic development of expression of the transcript in literature (? = not found in literature). Fourth column: RNA levels of several transcript were measured by microarray. Indicated is the relative difference (fold regulation) in expression in mutant as compared to control animals. Data are averages, n = 11 (N.A. is not analyzed, N.D. is not detectable). Only the neuropeptides cholecystokinin, dynorphin, enkephalin, neuropeptide Y, somatostatin and substance P and BDNF, neuropeptide-Y receptor 1 and the GABA-A receptor subunit α5 were significantly different in the mutant relative to control mice (*, α = 5%). Fifth column: RNA levels were measured by qPCR and standardized to hypoxanthine phosphoribosyl transferase (HPRT). Indicated is how many times the transcript is more expressed in mutant relative to control (n = 3 except for proprotein convertases and secretory protein: n = 6: N.A. is not present in oligoset). Between brackets the standard error of the mean (SEM). Only Dynorphin, Enkephalin, Somatostatin, Substance P, BDNF, Tyrosine hydroxylase and the Serotonin receptor 1B differed significantly between mutant and control (*, α = 5%). Sixth column: A estimate of the abundance of a certain transcript is obtained by the difference between the cycle threshold of HPRT and the specific transcript (ΔCt value) of the qPCR: very low: ΔCt > 4, low:

1<ΔCt<4, medium: -1 < ΔCt < 1, high: -4 < ΔCt < -1, very high: ΔCt <-4. Graph: Amount of transcript present in the mutant relative to control as measured with qPCR. For each transcript the amount was standardized to HPRT. Data represent mean ± SEM of 3 animals per transcript, but for proprotein convertases and secretory protein 6 animals per sample. ([1](Alvarez-Bolado *et al*., 1990) (rat), [2](Rius *et al*., 1991) (mouse), [3](Burgunder, 1994) (rat), [4](Ni and Jonakait, 1988) (mouse), [5](Thoss *et al*., 1995) (rat), [6](Lobo and Parnavelas, 1988) (rat), [7](Gambarana *et al*., 1990) (rat), [8](Van Eden *et al*., 1995) (rat), [9](Poulter *et al*., 1992) (rat), [10](Laurie *et al*., 1992) (rat), [11](Luo *et al*., 1996), [12](Wenzel *et al*., 1997), [13](Martin *et al*., 1998), [14](Marcinkiewicz *et al*., 1994), [15](Han and Morgan, 1999), [16](Burgunder and Young, 1990).

(see chapter 3). Of the gene groups containing 15 or more affected genes the most enriched (more than 2 times) groups were synaptic transmission, steroid metabolism, transmission of nerve impulse, and cell-cell signaling. The group cell-cell signaling included a large number of gene encoding neuropeptides. For a full description of these data see chapter 3 of this thesis. Here we report on an analysis and validation of the remarkable differences in neuropeptide gene expression.

RNA levels of neuropeptide genes were always higher in controls than in the mutants (Fig. 2). In the microarray analysis, levels of the somatostatin and substance P genes showed the largest differences (3 fold higher in the controls, Fig. 1A and 2). RNA levels of the dynorphin and enkephalin gene were 1.5 fold higher in the controls and cholecystokinin levels were also significantly higher (1.2 fold). The transcript levels of the neurotrophic gene BDNF were 1.5 fold higher. In contrast to the secreted neuropeptides, other synaptic signaling genes were not unaffected(Fig. 2). The only (minor) differences were observed for the genes encoding the neuropeptide-Y-receptor-1 (1.6 fold) and the $GABA_A$ receptor subunit $\alpha 5$ (1.8 times). Thus, 5 out of 5 neuropeptides showed marked differences in transcript levels, while 2 out of 22 other genes that have a role in synaptic signaling showed slight change in RNA levels.

qPCR confirms the differential expression of neuropeptide genes

For independent proof, we analyzed the transcript levels of the synaptic signaling genes also using quantitative PCR. The levels were measured relative to hypoxanthine phosphoribosyl transferase (HPRT, Fig. 1B). The cycle-threshold of a transcript relative to the control gene HPRT is an estimate of the relative amount of RNA in the cortex. Most neuropeptides had different RNA levels in the mutant, in a similar way as found on the microarray, although the absolute differences varied (Fig 1C). The transcript levels of dynorphin were 4 fold higher in the controls, the levels of enkephalin 2 fold and that of somatostatin and substance P 3 and 24 fold higher respectively (n=3). In contrast to the neuropeptides, the RNA levels for the corresponding receptors proprotein-convertases, most classical transmitter-receptors and classical transmitter synthesizing enzymes were not affected (Fig 2).

We also analyzed the RNA levels of enkephalin, dynorphin and substance P (data not shown) independently using Northern blotting, and confirmed their difference

in transcript levels. In conclusion, microarray analysis, qPCR and Northern blotting showed that neuropeptide genes had differences in transcript levels at E18, while almost all other synaptic signaling genes were not.

Selective loss of neuropeptide expressing cells cannot explain different mRNA levels.

The observed differences in neuropeptide mRNA levels in the neocortex might be caused by a selective loss or dysgenesis of certain neuropeptide expressing neurons. Therefore, we performed *in situ* hybridization on mutant and control animals at E18 for neuropeptide Y, somatostatin and somatostatin receptor 1 mRNAs (Fig. 3). The

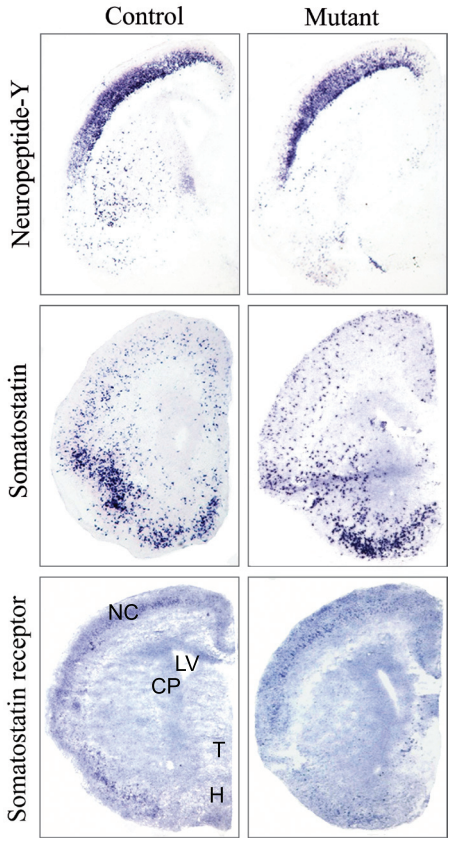

Figure 3. Distribution of neuropeptides and neuropeptide receptors in control and mutant mice. *In situ* hybridization on E18 control (first column) and mutant (second column) mice with probes against Somatostatin, Somatostatin receptor, and Neuropeptide Y. Control stainings with sense probes were empty (data not shown). Caudate putamen (CP), Hypothalamus (H), Lateral ventrical (LV), Neocortex (NC), and Thalamus (T). Staining for both neuropeptides (Somatostatin and Neuropeptide Y) was especially strong in the cortex; some staining for the neuropeptide receptors (Somatostatin receptor and Neuropeptide receptor (data not shown)) was also seen in the cortex. The localization of the staining in control and mutant mice is comparable.

expression patterns of these mRNAs were similar in mutants and controls and in line with earlier studies (Alcantara *et al*., 1998; Burgunder, 1994; Tong *et al*., 1997; Wulfsen *et al*., 1993). Somatostatin hybridization was present in the neocortex, but also highly expressed in the olfactory tubercle. Almost no expression was detected in the thalamus and hypothalamus. Hybridization for neuropeptide Y was detected in especially the cortex, but also in the olfactory tubercle and the caudate putamen. No hybridization signal was present in the thalamus and hypothalamus. For the somatostatin receptor low expression was found only in the neocortex. The sense probes did not produce signal on the sections for neither the neuropeptide nor the receptor transcripts (data not shown). These data indicate that a selective loss or dysgenesis of certain neuropeptide expressing neurons in the cortex cannot explain the lower levels of neuropeptide mRNAs in the mutant.

Neuropeptide genes are not differentially expressed at earlier stages

Genes regulated by synaptic activity should not be differentially expressed in the neocortex before E18, because few synapses have formed and no synaptic activity was detected before this stage (Konig *et al*., 1975; Verhage *et al*., 2000). To test this, we analyzed the transcript levels at E14 and E16 in the cortex of mutants and controls by qPCR (Fig. 4). Indeed, at E14 and E16 the transcript levels of tested neuropeptide were similar (n=3), although all transcripts were detectable at both these earlier stages. Hence, no differences in neuropeptide levels were detected in stages when no functional synapses are present.

In comparison to E18 (see also Fig. 1-2), the expression of all neuropeptide genes was lower at E14-16 (Fig. 4, left column) in controls. Therefore, it can be concluded that neuropeptide gene expression is induced between E14 and E18 in normal murine cortex, but this induction did not occur in the mutant. In contrast, differences in RNA levels of the munc18-1 itself were observed independent of embryonic age (Fig. 4).

Neuropeptide genes are not differentially expressed in sensory neurons

In contrast to other neurons, many sensory neurons do not depend on synapses to be activated, but are directly activated by the sensory receptors. Therefore, sensory neurons are expected to receive normal input in the *munc18-1* mutant. If synaptic input determines neuropeptide expression, the neuropeptide transcript levels should be similar in sensory neurons. To test this, we investigated the RNA levels of neuropeptides in the sensory neurons of the dorsal root ganglia (Fig. 5) at E18. In contrast to the neocortex, substance P transcript levels were similar in these ganglia and galanin and enkephalin had even higher levels in the mutant. The mRNA levels for

the control gene, GAPDH, were similar and munc18-1 mRNA was not detected in the mutant, as expected. Thus, differences in transcript levels of neuropeptides were not observed in neurons that are expected to have normal activity.

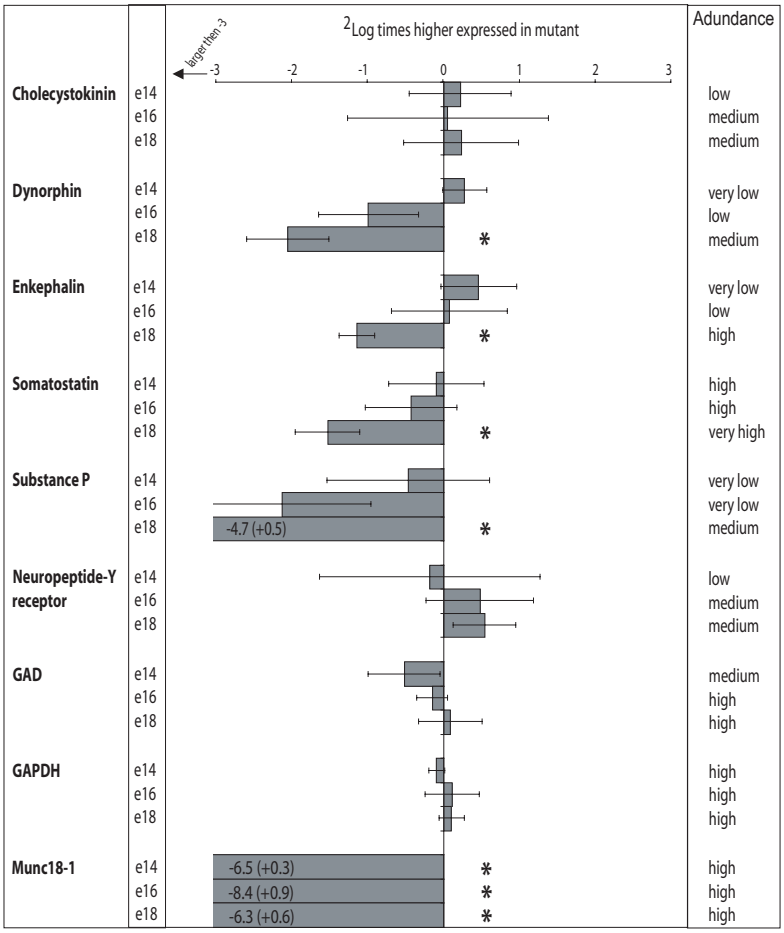

Figure 4: **RNA expression at E14, E16 and E18 in mutant relative to control mice.** RNA levels were quantified in mutant and control animals of embryonic day 14, 16 and 18. Each bar represents the average regulation in mutant relative to controls ± SEM (n=3). Significant differences (*) are calculated by Student's t-test (α=5%). An estimate of the abundance of a certain transcript (qPCR) is expressed as the difference between the cycle threshold of HPRT and the specific transcript (ΔCt value); very low: ΔCt > 4, low: 1<ΔCt<4, medium: -1 < ΔCt < 1, high: -4 < ΔCt < -1, very high: ΔCt < -4. Only the levels of the neuropeptides, dynorphin, enkephalin, somatostatin and substance P is significantly different at E18. Munc18-1 is differentially expressed at all developmental stages.

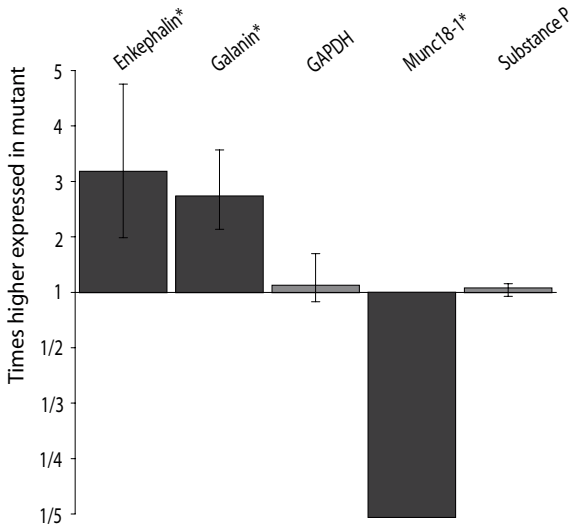

Figure 5. **Relative RNA levels of mutant dorsal root ganglia to control DRGs.** Quantification of RNA levels of several transcripts in dorsal root ganglia (DRG) at E18 by qPCR. Bars represent the average expression level in four mutant animals compared to four control animals ± SEM (of every animal several DRGs were pooled). Black bars represent significant and gray bars represent non-significant changes. Significant differences are calculated by Student's t-test (*, $\alpha = 5\%$). The levels of the neuropeptides enkephalin and galanin in the dorsal root ganglia is higher in mutant compared to control mice, while substance P and GAPDH are not changed.

Substance P protein levels are unaffected

We determined whether the protein levels of substance P, the most affected neuropeptide on RNA level, were also affected in the mutant using ELISA (enzyme immunoassay). Substance P levels were measured in the cortex (n=3) and in the whole brain, including the cortex (n=3). The substance P levels measured were standardized to the amount of Synaptobrevin 2 and GTP dissociation inhibitor (GDI) in the sample. These proteins were chosen because they have a low molecular weight and are still present after purification, and their protein levels were shown to be similar in the mutant compared to control animals (chapter 2). The peptide levels of Substance P in control and mutants were similar in both cortex and whole brain (Fig. 6), even with a trend to higher levels in the mutant animals.

In addition, we performed a Western blot for Substance P and Somatostatin (data not shown), which showed similar proteins levels in mutant and control animals for both neuropeptides. Thus, the protein levels for substance P and somatostatin are not affected in the mutant.

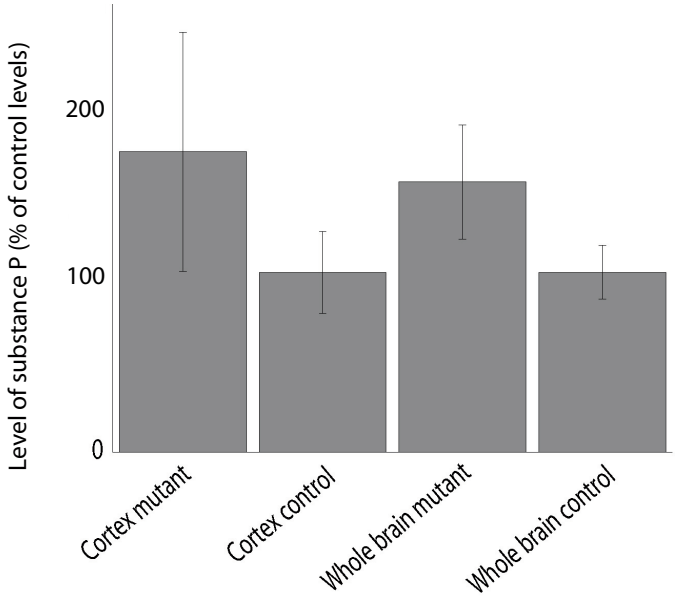

Figure 6. Protein quantification for the neuropeptide Substance P. Substance P levels in the cortex and whole brain were quantified using an enzyme immunoassay, the results were standardized to Synaptobrevin and GDI levels, which were quantified by Western blot (Bars indicate means ± SEM (n=3)). The protein levels in cortex and whole brain were not significantly different (α=5%).

DISCUSSION

In this paper we demonstrate differences in RNA levels of neuropeptide genes, but not neuropeptide receptors, neurotransmitter-producing enzymes, and neurotransmitter-receptors in a comparison at embryonic day 18 between normal mice and mutant mice that lack synaptic activity. These differences were not observed at earlier time points, E14 and E16, when functional synapses are rare, and also not in primary sensory neurons, which are expected to have a normal synaptic activity in the mutants. *In situ* hybridization indicated that the lower neuropeptide mRNA levels cannot be explained by a selective absence of neuropeptide expressing neurons in the mutant.

Methodological errors and apoptosis cannot explain the differential expression of neuropeptides

In this study, we used both microarray analysis and qPCR to analyze RNA levels of neuropeptides and differences were typically detected with both methods. The differences in transcript levels between mutant and control animals as measured by qPCR were usually larger than when measured by microarray analysis (see chapter 3 of

this thesis). In addition, qPCR was more sensitive than the microarray analysis, thereby generating a larger dynamic range to measure differences in transcript levels. For instance, the Arg-vasopressin receptor 1A, the substance P receptor and the serotonin receptor 1B were only detected by qPCR.
As descripted in chapter 3 of this thesis apoptosis cannot explain the specific changes in neuropeptide RNA levels. Moreover, imprecise dissection of the neocortex is also an unlikely explanation for differences in neuropeptide transcripts, because some of these genes are expressed mostly (>80 %) within the neocortex and not in the surrounding tissue, e.g. neuropeptide Y and somatostatin (see Fig 3). In conclusion, apoptosis and imprecise sampling in the mutant brain is not likely to explain the selective reduction in neuropeptide transcripts in the neocortex.

Neuropeptide gene expression is regulated by synaptic activity

Groups of genes involved in cell-cell signaling included the highest number of regulated genes, many of which coded for neuropeptides. In normal mice, the expression of many of these genes is induced around the time that the first functional synapses develop. In comparison, this increase does not occur in mutants, which do establish morphologically similar synapses, however, which do not transmit. A plausible explanation for these observations is that synaptic release does increase expression of neuropeptides between E16 and E18 but not in the silent *munc18-1* mutant. The observation that neuropeptide transcript levels in sensory neurons of the wild type and mutant are similar is in line with a synaptic activity-dependent induction of neuropeptide gene expression. Induction of neuropeptide expression is also observed in models for nerve injury (Ma and Bisby, 1998; Ma and Bisby, 1999) or hyperexcitation (Reibel *et al*., 2000). This induction may be explained by increased synaptic activity in both cases. An alternative explanation for the differences in neuropeptide transcrips in our mutant is that Munc18-1 itself acts as a transcriptional regulator. However, this is unlikely because of Munc18-1's subcellular localization, lack of DNA-binding motifs and currently available literature, which does not provide evidence in this direction.
Transcripts coding for synaptic released proteins were lower in the mutant, such as neuropeptides, BDNF and reelin. When the peptides are not released, protein levels in the cell may increase, which may feed backward and downregulate its expression. However, the expression may also only be increased when synaptic release occurs. The low reelin levels may cause the reduced maturation of synapses in the mutant (Bouwman *et al.*, 2004).
In contrast to the RNA level, the protein levels of the neuropeptide substance P were not significantly affected in the mutant. This observation may be explained by a different time course for the production of protein and RNA levels. Alternatively,

neuropeptide levels may remain high because they are not released in the mutant. The number of LDCVs in the mutant is about 70% of the number in control animals. However, the number of synapses is much lower in the mutant (25%; chapter 2). This indicates that synapses contain more LDCVs in the mutant. The lower release of LDCV in the mutant may lead to more LDCVs in the synapses. When vesicles are not used, a feedback mechanism on the transcription may reduce the expression of signaling molecules, but their protein levels will be relatively stable in the vesicles.

Transcript levels of neuropeptide receptors and prohormone convertases were not different in the mutant (Fig. 2). This may indicate that neuropeptide receptor gene expression does not depend on synaptic activity in a similar way as the neuropeptides. On the other hand, expression of neuropeptide receptors may lag behind expression of the neuropeptides and their induction may be part of a later phase. Proprotein convertases (PC1, and PC2), which cleave prohormones into the functional neuropeptides and are usually secreted together with the peptides, were unaffected in the mutant. This is in contrast with earlier research in the snail, *Lymnaea stagnalis* (Spijker *et al*., 2004b), where expression of neuropeptides and proprotein convertases is coupled upon physiological stimulation.

We anticipated that the expression of many synaptic genes would be altered in mutants that are unable to use their synapses. In contrast, most of the genes measured were unaffected and genes encoding neuropeptide receptors, classical transmitter receptors and converting enzymes were unaffected as well. Hence, the current results suggest that different biological principles exist to regulate expression of synaptic signaling genes. Whereas expression of neuropeptide genes appears to depend directly on the (abundance and) activity of synapses, other synaptic genes are regulated independent of this. Further genomic research is necessary to unravel the mechanisms behind these different principles. It can be expected that different control regions and transcriptional regulators orchestrate the induction of neuropeptide genes and of other synaptic genes. Such studies may also shed light on the question what the evolutionary advantage may be of the specific coupling between synaptic activity and neuropeptide gene expression.

ACKNOWLEDGEMENTS

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APPENDIX -Chapter 2

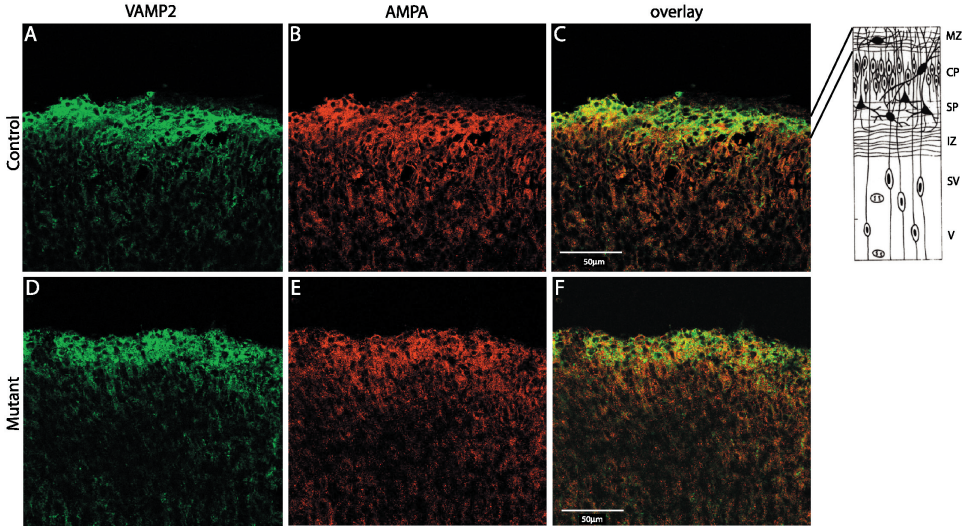

Fig. 3: Localization of pre- and postsynaptic proteins in neurons. Immunostaining for AMPA-receptor GluR2/3 shows receptors are targeted similar in wild type (B) and mutant (E). Staining for VAMP2 indicates that the localization of presynaptic proteins is unaffected in mutant (D) compared to wild type(A). The overlay of these two stainings (C,F) shows adjacent staining for the pre- and postsynaptic marker. The scheme to the side (Uylings *et al.*, 1990) showed what part of the cortex is represented in the pictures. Abbreviations: V, ventricular zone; SV, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; MZ, marginal zone.

REDUCED SYNTAXIN1 PROTEIN AND TRANSCRIPT LEVELS IN BRAINS OF *MUNC18-1* NULL MUTANT MICE

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Chapter 5

In preparation for publication

ABSTRACT

It is known that Munc18-1 binds to the SNARE-complex member Syntaxin1 with high affinity. This interaction may have a role in synaptic release. In addition, this interaction enhances the stability of Syntaxin1. Here we show that Syntaxin1 protein levels in the *munc18-1* null mutant neocortex, which lacks synaptic release, were reduced to 30% of control levels. However, the protein levels for several other synaptic proteins were unaltered, including the SNARE-complex members SNAP25 and Synaptobrevin2. Moreover, syntaxin1 transcript levels were reduced to 60% of control levels, whereas the transcript levels of other synaptic genes were unaltered. In primary sensory neurons, which do not depend on synaptic release for their input, Syntaxin1 protein levels were lower in the *munc18-1* mutant as well. However, transcript levels were unaffected in these neurons. Expression of munc18-1 in HEK-293 cells did not change the expression of any of the tested synaptic proteins, including Syntaxin1.

We conclude that syntaxin1 is the only core complex member of which transcript levels are regulated by input activity. This effect will reduce the syntaxin1 protein levels in the *munc18-1* mutant. The protein levels in this mutant are further reduced by a direct effect of the loss of *munc18-1*.

INTRODUCTION

Neurotransmitter secretion from synaptic vesicles in the mammalian brain depends on many interacting proteins. For this process the SNARE-complex, which is composed of three strongly interacting proteins (SNAP25, Synaptobrevin and Syntaxin1), is required. Several proteins have been implicated in the assembly and regulation of this SNARE-complex. Munc18-1, for instance, binds with high affinity to Syntaxin1 (Hata *et al.*, 1993) and is thought to regulate secretion via this interaction.

In addition to regulation by binding partners, Syntaxin1 levels have also been shown to be regulated by activity. For instance, prenatal sound stimulation increases the protein levels of syntaxin1 in the chick brainstem (Alladi *et al.*, 2002). A similar increase in syntaxin1 RNA levels was observed in the hippocampus after a working memory task by using *in situ* hybridization (Davis et al, 1998). In these papers only the expression of syntaxin1 was studied. Therefore, the effect on gene expression may be a more general effect. The levels of all synaptic proteins may be raised by an increase in the number of synapses. In addition, the stimulation protocols that were used in these papers may affect other processes than activity. As was shown in chapter 3 and 4 of this thesis often several genes of a functional group are regulated simultaneously. However, gene expression of syntaxin1 but not of other SNARE-complex members are affected by activity. For instance, the expression of syntaxin1 can be induced by overexpression

of P/Q-type calcium channels or ionomycin treatment of HEK-293 cells (Sutton *et al.*, 1999), whereas SNAP25 and synaptobrevin were unaffected. A similar effect was seen by induction of long-term potentiation in the dentate gyrus: syntaxin1 transcript levels increased, while the levels of other SNARE complex member were unaltered (Hicks *et al.*, 1997). These studies all show that the expression of syntaxin1 is very dynamic and enhanced activity showed an increase in syntaxin1 levels.

In the *munc18-1* mutant, which is severely impaired in all components of synaptic release (Verhage *et al.*, 2000; Voets *et al.*, 2002), Syntaxin1 protein levels are reduced to 30% of the amount present in control animals, while the levels of the other SNARE-complex members are unchanged. Munc18-1 was shown to stabilize Syntaxin1 protein levels, as was shown by pulse-chase analysis of syntaxin levels in *munc18-1* overexpressing HEK-293 cells (Toonen *et al.*, 2005). In the *munc18-1* null mutant Syntaxin1 is less stable in the absence of its binding partner Munc18-1. This reduction in Syntaxin1 protein levels however, may also be an effect of the lack of synaptic release in the mutant. Activity may, for instance, regulate syntaxin1 transcription.

This study aims to get insight into the role of synaptic release and the Munc18-1/Syntaxin interaction in syntaxin1 mRNA and protein expression. We measured protein and transcript levels of syntaxin1 in the *munc18-1* null mutant neocortex. This mutant is devoid of all synaptic activity and *munc18-1*, but has spontaneous action potentials. In order to make a distinction between effects of Munc18-1 and synaptic activity we measured syntaxin1 levels in mutant dorsal root ganglia (DRG). In contrast to mutant CNS neurons, mutant DRG neurons probably get input from the sensory cells, but both lack *munc18-1*. Protein levels were reduced in both the neocortex and the sensory DRG neurons. However, transcript levels were only reduced in the neocortex and not in the DRG neurons. These data suggest that synaptic release can adjust syntaxin1 gene expression. To test whether Munc18-1 can affect *syntaxin1* gene expression as well, we overexpressed *munc18-1* in HEK-293 cells. No significant effect of this overexpression was found on syntaxin RNA levels. Therefore, gene expression of *syntaxin1* most likely depends on input activity and Munc18-1 is mainly involved in stabilization of Syntaxin1 proteins levels. Both effects will contribute to the reduced syntaxin1 protein levels in the *munc18-1* null mutant.

EXPERIMENTAL PROCEDURES

Laboratory animals

Munc18-1 deficient mice were generated as described before (Verhage, *et al.*, 2000). Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous matings. The day a vaginal plug was observed was considered to be embryonic day 0 (E0). Embryos were harvested from mothers and rapidly sacrificed

after timed matings at E16 and E18. Experimental procedures were carried out with local regulatory approval for animal experimentation, according to the Dutch law for animal welfare.

RNA isolation

RNA was isolated from mutant and wild type animals by using the Trizol method (Invitrogen, San Diego, USA). The amount of RNA was measured using a spectrophotometer and Bioanalyzer (Agilent technologies, Palo Alto, USA). Approximately similar amounts of RNA were used in cDNA reactions or on a Northern gel. Genomic DNA was removed by a DNAse I treatment, which was apparently sufficient since genomic primers developed to detect an HPRT intron did not result in a PCR product.

qPCR

See chapter 3.

Northern

Total RNA was isolated from brains of E18 embryos by using Trizol (Invitrogen, San Diego, USA). The RNA (10 µg/lane) was subjected to electrophoresis (1.0% agarose, 0.04 M 3-[*N*-morpholino]propanesulfonic acid (pH 7.0), and 1.75% formaldehyde), transferred to a charged nylon membrane (Amersham, Uppsala, Sweden) and UV cross-linked (3 min). Sequential hybridizations were performed to cDNA probes for syntaxin1 and GAPDH, respectively. After prehybridization (at 65°C for 30 min; 0.5 M $NaHPO_4$ pH 7.0, 1 % BSA, 1 mM EDTA, 7 % SDS) hybridizations were performed overnight at 65°C in the same buffer now containing 0.2×10^6 cpm/ml probe, labeled with α-^{32}P dCTP to a specific activity of 2×10^9 cpm/µg, using the Random Primers DNA Labeling System (Roche, Basel, Switzerland). After hybridization, the membrane was washed twice in hybriwash 5 (at 65°C for 5 min; 40 mM $NaHPO_4$ pH 7.2, 1 mM EDTA, 5% SDS) and twice in hybriwash 1 (at 65°C for 15 min; 40 mM $NaHPO_4$ pH 7.2, 1 mM EDTA, 1% SDS) and then exposed up to 48 hr in a PhosphoImager screen (Fuji, Tokyo, Japan). Signals were analyzed with Aida 3.0 software.

Cortical cultures

Cortices were dissected from embryonic day 18 mice and collected in Hanks Buffered Salts Solution (HBSS) (Sigma, St. Louis, USA), buffered with 7 mM Hepes. After removal of the meninges, the cortices were incubated for 30 minutes in trypsinated HBSS at 37 °C. After washing the neurons were triturated with fire polished Pasteur pipettes, counted with a hemacytometer and plated in Neurobasal medium (Invitrogen, Carlsbad, USA)

supplemented with 2 % B-27 (Invitrogen), 1.8 % Hepes, 1 % glutamax (Invitrogen), 1 % Pen/Strep (Invitrogen) and 0.2 % β-mercaptoethanol. Low-density cultures were plated on poly-L-lysin coated glass coverslips at 25,000 cells/cm^2. For island cultures, neurons were plated at 6,000 cells/cm^2 on islands of rat glia. Glial islands were obtained by spraying a 0.25 mg/ml rat tail collagen solution (BD Biosciences, Bedford, USA) on glass coverslips. After drying and UV sterilization glial cells were plated at 600/cm^2. *Munc18-1* wild-type linked to enhanced green fluorescent protein (EGFP) via an internal ribosomal entry site (IRES) subcloned into pLenti-vectors and neurons were transduced as described (Naldini *et al.*, 1996), as control a vector with CRE linked to EGFP was used. The transduction efficiency was analyzed by counting fluorescent cells.

Electrophoresis and Immunoblotting

DRGs were collected at E18 and homogenized in SDS-PAGE loading buffer (66 mM Tris/HCl, pH 6.8, 3 % [w/v] SDS, 5 % [v/v] glycerol, 2 % [v/v] β-mercaptoethanol, and 0.001 % [w/v] bromophenol blue). Cortices were isolated for brains for E18 animals, homogenized and SDS-PAGE loading buffer was added. SDS-PAGE was performed with all DRGs of one embryo per lane, 10µg protein of cortex-material, using gradient gels (6-15 %) in Tris-glycine electrophoresis buffer. Proteins were transferred to PVDF membranes at 100 V for 1 hr and detected with either secondary antibodies coupled to horseradish peroxidase and enhanced chemiluminescense or alkaline phosphatase-conjugated secondary antibodies and enhanced chemifluorescence. Quantification was performed on a Fluor S MultiImager (BIO-RAD, Tokyo, Japan).

Antibodies

Monoclonal antibody against munc18-1 was purchased from Transduction Laboratories (Lexington, KY). The monoclonal anti-syntaxin-1A/B (HPC-1) was purchased from Sigma (St. Louis, MO). The polyclonal anti-VCP (K331; Sugita and Sudhof, 2000) was as described previously. The horseradish peroxidase-conjugated secondary antibody against mouse IgG was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA), and the peroxidase-conjugated secondary anti-rabbit IgG from Sigma. The ECL-substrate (Super Signal West Dura Extended) was purchased from Pierce (Rockford, IL). The alkaline phosphatase-conjugated secondary mouse and rabbit IgG and IgM and ECF-substrate were purchased from Amersham (Buckinghamshire, UK).

RESULTS

Syntaxin1 protein levels are reduced in the neocortex of the *munc18-1* null mutant

Syntaxin1 levels are reduced with 70% in the whole brain of the *munc18-1* null mutant compared to control animals (Toonen *et al.*, 2005). To analyze the role of synaptic activity and the Munc18-1/Syntaxin1 interaction on *syntaxin1* expression, we examined the protein levels of Syntaxin in the neocortex of *munc18-1* null mutant mice, because other brain areas but the neocortex are degenerated at E18 in the mutant. Syntaxin1A and Syntaxin1B levels were approximately 70% lower in the mutant neocortex, while the levels of the other SNARE-complex proteins, Synaptobrevin/VAMP2 and SNAP25, and other presynaptic proteins, like Synaptotagmin and VCP were unaffected (Figure 1 and chapter 2 of this thesis). In conclusion, Syntaxin1 is the only SNARE-complex member of which protein levels are reduced.

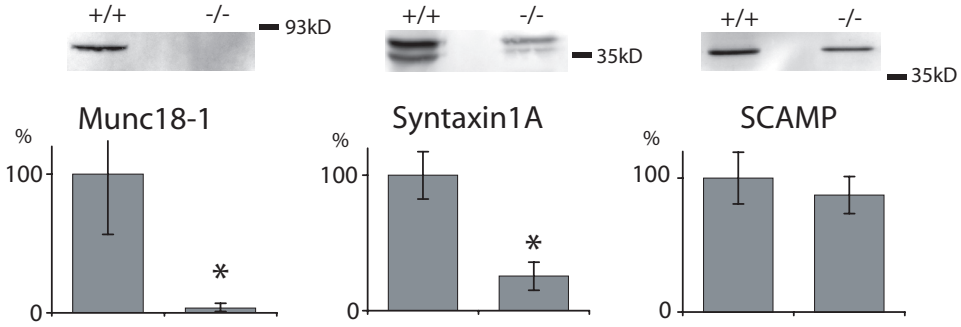

Figure 1: Reduced syntaxin-1 protein levels in the neocortex from *munc18*-1-deficient mice. Quantification of immunoblot analyses with specific antibodies against Syntaxin1 (monoclonal), Munc18-1 (monoclonal) and SCAMP (polyclonal) of protein levels in control (+/+) and mutant (-/-) mice in the cortex of E18 embryos. Results are represented as mean percentage of control levels ± SEM (n=3). Example blots are also shown (top). Differences were analyzed using a Student's *t*-test (α=5%). The protein levels of both Munc18-1 and Syntaxin1 were significantly different (*).

Syntaxin1 mRNA expression is lower in mutant mice

To test whether a lower mRNA expression causes the observed changes in Syntaxin1 protein levels, we analyzed *syntaxin1* transcript levels in the cortex of control and *munc18-1* null mutant mice by using quantitative PCR (qPCR) at several embryonic stages. Syntaxin1 levels were 1.7 times lower at E18 in the mutant compared to control animals, whereas the transcript levels of other pre- and postsynaptic proteins, like DOC2a/b, NARP, SNAP25, stargazin, synapsin2, synaptotagmin1, VAMP2 and VCP, were not significantly changed (Figure 2A; α=5%). The lower syntaxin1 expression in the mutant relative to control animals was confirmed using Northern blotting (Figure 2B)

and microarray analysis (chapter 3 of this thesis). The transcript levels of rabphilin3A another presynaptic gene were 1.6 times lower in the mutant.
Earlier in development, at E14 and E16, none of the synaptic protein transcripts, including syntaxin1, were differentially expressed.

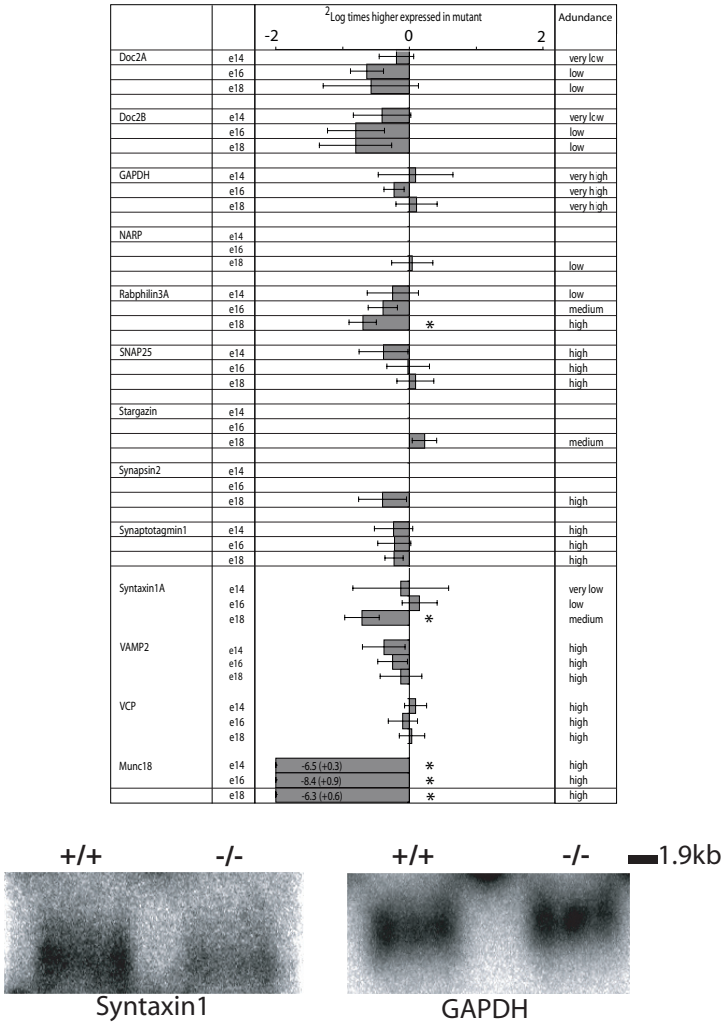

Figure 2: Quantification of expression levels of synaptic proteins in control and mutant mice. A: RNA levels were quantified in mutant and control animals of embryonic day 14, 16 and 18. Each bar represents the average regulation in mutant relative to controls ± SEM (n=3). Whether the differences are significant is tested by the Student's *t*-test (*, α=5%). An estimate of the abundance of a certain transcript is obtained by the difference between the cycle threshold of HPRT and the specific transcript (ΔCt value) of the qPCR: very low: ΔCt>4, low: 1<ΔCt<4, medium:-1<ΔCt <1, high:-4<ΔCt<-1, very high: ΔCt <-4. Transcript levels increase during development. The expression of only syntaxin1A and rabphilin3A is significantly different at E18. Munc18-1 is differentially expressed at all developmental stages. B: Northern blot analysis of the RNA levels of syntaxin1 and GAPDH in control (+/+) and mutant mice (-/-) at embryonic day 18 (per lane approximately 10 µg was loaded). Using northern blot, the same expression level is seen as in qPCR (n=2).

Syntaxin1 protein levels are lower in mutant sensory neurons

The lower protein amount of Syntaxin1 in the *munc18-1* mutant may be the direct result of the absence of *munc18-1* or the consequence of the complete absence of synaptic activity in these animals. Therefore, we tested protein and transcript levels of syntaxin1 in sensory neurons. Unlike cortical neurons, these neurons most likely have normal input, because they are activated by sensory receptors on their peripheral ends and

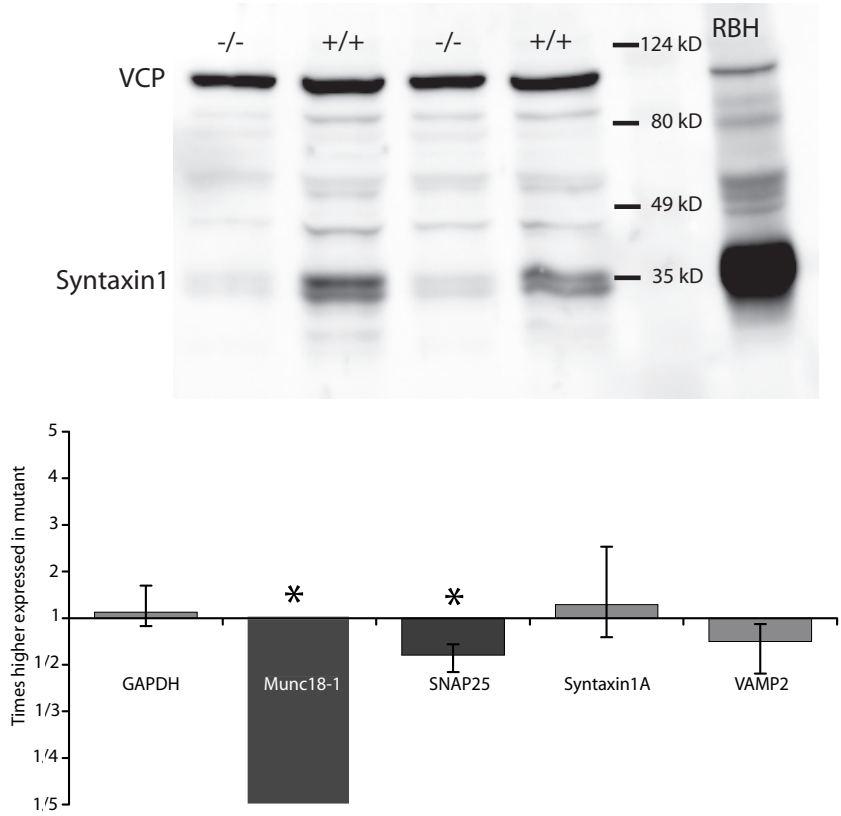

Figure 3: Quantification of syntaxin-1 protein and RNA levels in DRG's of *munc18*-1-deficient mice. Immunoblot analyses with specific antibodies against Syntaxin1 (monoclonal) and VCP (polyclonal) shows the protein levels in control (+/+) and mutant (-/-) mice in the cortex of E18 embryos (per lane all DRG of 1 animal was loaded). B: RNA levels of SNARE complex proteins were quantified in DRGs of E18 mutant and control animals. Each bar represents the average regulation in mutant relative to controls ± SEM (n=3). Significant differences are calculated by Student's *t*-test (α=5%). Black bars indicate significant different RNA levels (*) and gray bars represent non-significant changes.

thus do not depend on synaptic release. These neurons, like cortical neurons, cannot secrete neurotransmitter on their postsynaptic cells, because they lack *munc18-1*. We measured protein levels and mRNA levels in dorsal root ganglia (DRG) at E18. Syntaxin1 protein levels were much lower in mutant DRG (Figure 3A), similar to the neocortex. The transcript levels of syntaxin however were not significantly different, as were the levels of VAMP2 and GAPDH mRNA. In conclusion, in DRG neurons only Syntaxin1 protein levels are reduced, whereas the transcript levels are similar in mutant and control animals.

Activity and *munc18-1* expression do not increase syntaxin1 expression in HEK-293 cells

To examine whether syntaxin1 expression is regulated by munc18 directly, HEK-293 were transfected with *munc18* and *syntaxin1*. The syntaxin1 transcript and proteins levels were undetectable in HEK-293 cells (Figure 4), indicating that Munc18-1 cannot induce endogenous syntaxin1 gene expression in HEK-293 cells. However, the regulatory machinery that can induce syntaxin1 expression in neurons may not be present in HEK-293 cells. Therefore, a better experiment would be to transduce neurons of control animals with *munc18-1*. We performed a pilot experiment, where *munc18-1* was co-expressed with EGFP via an internal ribosomal entry site. Transcript levels of syntaxin1, VAMP2, SNAP25 and GAPDH were not different as compared to control neurons (data not shown). However, from this experiment no conclusions can be drawn, because only about 5% of the cells had detectable EGFP levels. Using qPCR, differences as small as 1.4 times can be measured, which means that syntaxin1 levels in the 5% transfected neurons should be upregulated 28 times to be detectable.

In addition, to test whether syntaxin1 is regulated by activity, we treated HEK-293 cells with ionomycin, to stimulate calcium influx. Using PCR or Northern blot to analyze syntaxin1 transcript levels, syntaxin1 transcript levels in stimulated or unstimulated HEK-293 cells were undetectable. Sutton et al (1999) showed an induction of syntaxin1A transcription in HEK-293 cells after ionomycin stimulation, however we are unable to reproduce their results.

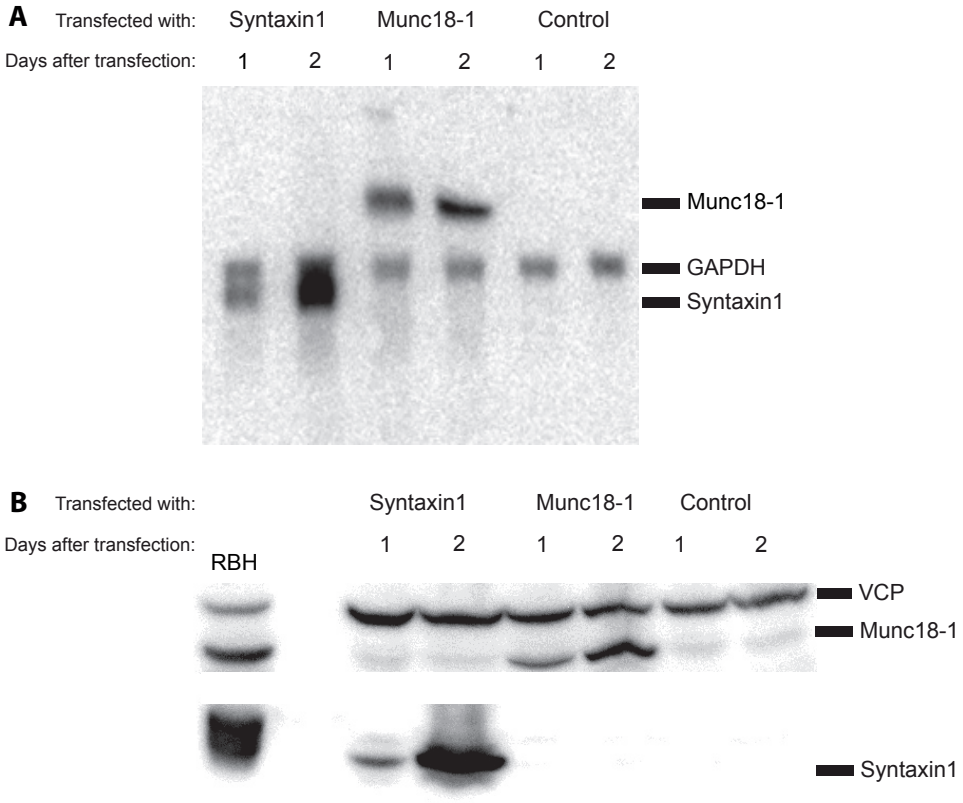

Figure 4: Quantification of syntaxin1 RNA and protein levels in HEK293 cells overexpressed with *munc18-1*. HEK293 cells were transfected with *syntaxin1* and *munc18-1*. The transfection was successful, because the transfected samples contained RNA and protein of the transfected gene. A: Northern blot analysis of transcript levels in HEK293 cells. Only GAPDH was endogenously expressed in HEK293 cells. Expression of *syntaxin1* or *munc18-1* did not induce expression of the other gene. B: Western blot showing that expression of syntaxin1 or munc18-1 does not effect the expression of the other protein.

DISCUSSION

In this paper we studied the role of synaptic release and interaction between Munc18-1 and Syntaxin1, in the regulation of *syntaxin1* expression. We made use of *munc18-1* null mutant mice that lacks synaptic release and are therefore synaptically silent. We found lower syntaxin1 RNA and protein levels at E18, whereas the expression of several other synaptic proteins was unaffected. At earlier time points, when synapses are not yet functional in the cortex, no difference was detected. Lower Syntaxin1 protein levels were also observed in mutant sensory neurons, which do not depend on synaptic activity for their input. Differential transcription levels of syntaxin1 were not observed in these neurons. In cortical cultures containing low percentage of *munc18-1* transfected

cells, syntaxin1 transcript levels were not significantly increased. Transfection of HEK-293 cells with *munc18-1* did not influence syntaxin1 gene expression.

Syntaxin1 is the only SNARE-complex member regulated by activity

In this paper, protein and transcript levels for all three SNARE-complex members were studied under several conditions. In these experiments only transcript levels of syntaxin1 were altered, and SNAP25 and VAMP levels were unaffected. This is in line with some other publications in which syntaxin1 was reported to be affected by an increase in activity, whereas the other SNARE-complex members were unaltered (Hicks *et al.*, 1997; Sutton *et al.*, 1999). Since all three SNARE-complex members are needed for synaptic release, the amount of Syntaxin1 may be rate-limiting when the amount of syntaxin1 is lower than the levels of the other core complex members. In the qPCR experiment transcript levels of syntaxin1 were shown to be lower than the other SNARE-complex members. Therefore, the reduction in syntaxin1 levels may contribute to the abolishment of synaptic release in the *munc18-1* mutant and be a way for neuronal networks to regulate secretion capacity in a use-dependent manner.

The role of synaptic release and munc18-1 in the regulation of syntaxin1 expression

The lower Syntaxin1 protein levels in mutant mice may in part result from decreased stability in absence of its binding partner Munc18-1 (Toonen *et al.*, 2005). However, we show here that the lower protein levels may partly be an effect of reduced gene expression. Synaptic release may cause this change in *syntaxin1* gene expression, because syntaxin1 was only differentially expressed at E18, when synapses are functional, and not earlier in development. In addition, in sensory neurons, which are probably active in the mutant, no effect on transcript levels was observed. Expression of *munc18-1* in HEK 293 cells did not induce syntaxin1 transcript levels. These four findings indicate that synaptic release may regulate the transcription of syntaxin1 via the influx of calcium (see Sutton *et al.*, 1999). Syntaxin1 RNA levels are most likely not affected by Munc18-1 directly, because syntaxin1 transcript levels were similar in mutant and control sensory neurons, which do not depend on synaptic release for their activity. Moreover, *munc18-1* overexpression in neurons and expression in HEK-293 cells did not change the expression of syntaxin1, although these data were not conclusive.

In conclusion, *syntaxin1* gene expression is most likely regulated by input activity, this effect will reduce the protein levels in the *munc18-1* mutant. In addition, the protein levels of syntaxin1 are further reduced by a direct effect of the loss of *munc18-1*, because Munc18-1 enhances the stability of Syntaxin1.

GENERAL DISCUSSION

Chapter 6

DISCUSSION

The aim of the studies described in this thesis was to elucidate the role of synaptic transmitter release in the development of the nervous system. Three main questions were investigated: First, how does synaptic activity influence initial brain development and synapse formation. Second, which genes are (dis-)regulated under different conditions of synaptic release during development. Third, is the supply of synaptic proteins dependent on synaptic transmission? This chapter briefly summarizes our main findings and discusses the results obtained.

Using the *munc18-1* null mutant mouse, which is severely impaired in all forms of synaptic release, we were able to analyze the role of synaptic release in development **(chapter 2)**. We found no differences in overall cortical structure, and no changes in synaptic protein levels and munc18 targeting in these animals. In addition, immature synapses were found both in the mutant and control animals from developmental day E16 on. However, the number of synapses was lower in the mutant at E16. The number of synapses increased in the control animals, but did not increase over time in mutant mice. In addition to synapses, synapse-like structures (with vesicles present on both sites of the electron dense stained membrane) were observed by EM in the neocortex of mutant and control mice. These 'multivesicular structures' were present at similar levels in E16 mutant and control animals, but at E18 their number was higher in the mutant compared to control animals. These results imply, that immature synapses do form without synaptic release, but cannot mature and therefore may form multivesicular structures instead.

The regulation of RNA levels under conditions of defective synaptic release was studied in **chapter 3**. In the *munc18-1* null mutant, gene expression was studied using micro-array analysis and quantitative PCR. Generally, the expression of most genes was unaffected in the mutant. At E18, genes with higher transcript levels in the mutant are of specific functional classes and may have a function in glial cells, immune cells or lysosomes. Most genes having lower levels in the mutant were assigned to a function in neuronal transmission, and were not regulated at E14 and E16. These data indicate that synaptic release is necessary to increase the expression of several components required for synaptic transmission. In **Chapter 4** the relation between the usage of the synapse and the expression of synaptic proteins was studied. Neuropeptides were found to have lower RNA levels at E18, when comparing the *munc18-1* null mutant versus control, whereas the levels of several synaptic release-related genes, such as neuropeptide receptors, were unaffected. At earlier time points E14, E16, when synapses are not yet functional in the cortex, no difference was detected in the transcript levels of the neuropeptide genes. RNA levels of neuropeptides were similar in primary sensory neurons, which are probably active in the *munc18-1* mutant. Thus, these data strongly

support the idea that synaptic release is involved in the regulation of the expression of neuropeptide genes.

The regulation of the expression of the Munc18-1 binding partner syntaxin1 was studied in **chapter 5**. Previously, Syntaxin1 protein levels were shown to be lower in whole brain isolations of *munc18-1* mutant relative to controls. We found lower levels of syntaxin1 mRNA and protein at E18 in the neocortex of the *munc18* null mutant when synaptic release is absent. However, at earlier time points in embryonic life, no difference was observed. In sensory neurons, which do not need Munc18-1 to be active, Syntaxin1 protein levels were lower in the mutant compared to control animals. However, transcript levels were unaffected in these sensory neurons. This indicates that syntaxin1 expression can be regulated by input-activity-dependent gene expression and that Syntaxin protein levels may be stabilized by Munc18-1.

1 - The role of synaptic release in neuro-development and gene expression

Deletion of synaptic release initially leads to normal brain development. Neurons can migrate to the correct location and are able to form axons, dendrites and synapses with normal structure in the absence of synaptic release. However, the number of synapses is lower in the *munc18-1* mutant. Therefore, synaptic release may play a role in synapse maturation and/or stabilization, which is one of the final steps of the development of neuronal connections. In this thesis, we have shown that during synapse development some proteins with a synaptic function are regulated by activity. This activity-dependent increase of synaptic proteins may be involved in functional maturation of the synapse. Both the increase in synapse number and the increase in synaptic proteins may either be caused by input or output activity, because almost every neuron is both pre-and postsynaptic. The DRG data in chapter 4 indicate that the expression of neuropeptides may be regulated by input activity.

Besides increasing the number of synapses during embryonic development, after birth activity may act as a limiting factor, as was indicated in a recent article (Flavell *et al.*, 2006).

In addition to genomic effects, activity may influence processes such as translation, protein localization and posttranslational modification. These processes may be important for network development as well. Examples of these are Synaptic vesicle protein 2 (SV2) and Rab3A that are first localized in neurites. Later, these proteins appear in nerve terminals (Confaloni *et al.*, 1997; Stettler *et al.*, 1994). In addition, NCAM is recruited to the cell surface in an activity-dependent manner (Kiss *et al.*, 1994) and eye opening induces a rapid dendritic localization of PSD-95 in central visual neurons (Yoshii *et al.*, 2003). Moreover, incorporation of receptors in the post-synaptic membrane depends on activity (see for review Barish *et al.*, 1998). Also translation can be regulated

in an activity-dependent way. Some synaptic transcripts are polyadenylated in response to activity and this will most likely lead to an increase in translation rates (Du and Richter, 2006). All these effects are consistent with the idea that only active synapses will become reinforced.

A - Processes involved in the activity-dependent regulation of genes

Which processes underlie activity-dependent regulation of gene expression is not completely understood, although there are some clues. During early development, neurons express basal levels of several activity-dependent proteins, such as receptors and synaptic vesicle proteins. The synapse may only be consolidated when gene expression of both pre- and post-synaptic are coordinated. The frequency of synaptic release in the growth cones may be one of the factors that define whether a synapse should be formed. A certain frequency may increase the expression of specific genes in one cell but not in another; this frequency-dependent regulation of gene expression has been shown for cell adhesion molecules (Itoh *et al.*, 1997).

The influx of calcium as a result of synaptic release, has been shown to be an important second-messenger for activity-dependent gene expression (see for review Barish *et al.*, 1998). The activity-dependent effects of calcium may require the activation of a large variety of Ca^{2+}/CaM-dependent effector molecules, like Ca^{2+}/CaM-dependent protein kinases (see for review Bito, 1998). These kinases may, for instance, phosphorylate CREB, which will lead to CRE-dependent transcription. In addition, calcineurin is required for the activity-dependent transcription via CREB (Hahm *et al.*, 2003). Calcineurin is less expressed in the *munc18-1* mutant than in control animals (chapter 3) and this reduction may be responsible for some of the differentially expressed genes in the mutant. Impey *et al.* studied which genes are a target for CREB (2004). Some of the CREB targets found in the article were affected in the mutant, for instance the levels of VGF, synaptotagmin 7, shank and cyclin G were changed. However, also some CREB targets were unaffected in the mutant like VAMP2, GKAP and ephrin A5.

In addition to CREB-dependent regulation of expression, several other pathways may be involved in this process, for instance the MAPK-pathway, which is essential for the translational (Kelleher *et al.*, 2004) and transcriptional component of LTP (Davis *et al.*, 2000). In conclusion, most likely several pathways are involved in the regulation of transcription and a central role for calcium seems plausible.

B - Differences in synaptic release-dependent gene expression during development and adult plasticity

Several genes were differentially expressed during both development and synaptic plasticity (See for review Lynch, 2004). However, some differentially expressed genes during synaptic plasticity were not affected in our study on developing neurons,

including cell adhesion molecules and neurotransmitter-receptors. For instance, LTP induced by BDNF leads to an induction of other transcripts then we have seen. Following BDNF-LTP the transcript levels of Narp, neuritin, CARP, TIEG and ARL4L are co-upregulated with Arc (Wibrand *et al.*, 2006). In the *munc18-1* mutant we have analyzed the RNA levels of NARP, TIEG and ARL4 relative to control animals, all were unchanged. In addition, we did not find a difference in the expression of NMDA receptors subunits in the *munc18-1* mutant, whereas it has been shown that the expression is affected during synaptic plasticity as well as in other studies on developmental expression of the NMDA receptor (Williams *et al.*, 1993; Roberts and Ramoa, 1999). However, the developmental NMDA subunit switch may occur at a later embryonic age than we performed this experiment. In general, some of the activity-dependent gene expression may occur only after birth, because the expression of some genes may depend on the higher activity levels that occur only after birth. Immature synapses only receive a low frequency of action potentials. This low frequency firing may stimulate the expression of some proteins and in this way may strengthen the synapse. High frequency and phasic firing is correlated with consolidation of terminals, synapse stabilization, and myelination (see Itoh *et al.*, 1997). High frequency firing may lead to substantial gene expression. Indeed the largest change in transcription is seen after birth (Stead *et al.*, 2006), confirming the idea that the higher activity levels after birth may lead to larger changes. Firing frequency may explain the differences in the expression during development and plasticity as well. In the mutant the expression of neuropeptides was lower relative to control animals, whereas the expression of the neuropeptide receptors was similar. This difference may be explained by the fact that the pre-synapse is established earlier than the post-synapse. In conclusion, the difference in activity-dependent expression during development and plasticity may be a matter of precise moment of development and a difference in robustness in action potential firing.

2 - The role and regulation of LDCV release in brain development

A – The importance of LDCV release for normal and munc18-1 mutant brain development

Several studies have shown that neurotrophins may strengthen the synaptic transmission in the CNS (see Vicario-Abejón, *et al.*, 1998; Martínez *et al.*, 1998). For instance, in the late phase of LTP neurotrophins are involved, as has been shown by pretreating brain slices with a TrkB-IgG (Kang *et al.*, 1997). Furthermore, for this LTP phase, gene expression is required. This gene expression may in turn be regulated by neurotrophins, since they are known to be involved in activity-dependent gene expression (Frey *et al.*, 1998; Messaoudi *et al.*, 2002). The gene expression induction by NGF was studied in PC12 cells (Lee *et al.*, 2005). This study shows some overlap with our study (cytochrome P450 and ATPase are changed in both studies), however no change in neuropeptide and

synaptic release proteins expression is seen in the PC12 cells. In our mutant still the reduction in other neurotrophins (especially BDNF) may be responsible for the changes in transcription.
In conclusion, in the *munc18-1* mutant activity-dependent reduction in neurotrophins, may be the cause of the lower expression of synaptic compounds, since the gene expression of some neurotrophins is decreased.

B – The role of synaptic activity on the expression of genes involved in release of LDCVs and synaptic vesicles

In the *munc18-1* null mutant we found a reduced expression of several genes involved in synaptic release. In particular, genes involved in LDCV release were regulated, some of these genes are involved in synaptic vesicle release as well. However, none of these genes were solely involved in synaptic vesicle release. For instance, synaptotagmin7, SNAP23 and Doc2 levels were differentially expressed in the *munc18-1* mutant compared to control animals and are only involved in LDCV release (Shin *et al.*, 2002; Chen *et al.*, 2000; Charvin *et al.*, 1999). RNA levels of syntaxin1 that is involved in both synaptic vesicle release and LDCV release (Foran *et al.*, 1996) are lower in the mutant. In contrast, the levels of SNAP25 and synapsin1, which are only involved in synaptic vesicle release but not in LDCV release (Foran *et al.*, 1996; Navone *et al.*, 1984), were similarly expressed in mutant and control animals. In conclusion, only genes involved in LDCV release were found affected in the *munc18* mutant, whereas genes, which have a function in synaptic vesicle release only are unaltered. In conclusion, particularly genes involved in LDCV release are susceptible to regulation, in contrast to genes that are involved in synaptic vesicle release.

3 – Other effects of *munc18-1* deletion

In chapter 2 we describe that for the maintenance of neuronal networks, synaptic activity may be essential. Because neuronal networks in the mutant cannot be maintained, lower brain areas go into apoptosis. However, recent data show that deleting *munc18-1* in Purkinje cells leads to apoptosis of these neurons (Heeroma *et al.*, 2004). Therefore, the degeneration of brain areas other than the cortex, may be a direct effect of deletion of *munc18-1* and not of synaptic activity.
The proposed relation between secretion and neuronal maintenance was tested in low-density cultures, organotypic cultures and *in vivo*, by conditional, cell-specific inactivation of the *munc18-1* gene (Heeroma *et al.*, 2004). This study shows first, the lack of synaptic activity cannot explain the degeneration in *munc18-1* mutants. Second, trophic support delays but cannot prevent degeneration, and finally, a cell-intrinsic, yet unknown function of Munc18-1 is essential for prolonged survival.

4 - Future directions

This thesis focused on the role of activity in the development of neuronal networks and the regulation of gene expression by secretory activity. Some questions remain to be answered. For instance, the question what the role of activity is in neuronal network formation, has not been completely answered. Using the synaptic silent *munc18-1* mutant we made some progress. However, it now has been shown that the degradation of the lower brain area is caused by a cell-intrinsic yet unknown function of Munc18-1 and not only by lack of activity. Therefore, other synaptic vesicle release devoid mouse models, like the *munc13-1/2* double mutant, should be used to study this question further. However, this model has drawbacks as well, since most likely not all neurons in this mutant are silent and LDCV release is not impaired. LDCV release is another form of activity that has most likely a substantial role in network development, especially via the release of neurotrophins. The role of LDCV release in developing and mature networks could be studied in inducible mutants for certain genes, like for instance an inducible SNAP23 mutant. As SNAP23 is important for LDCV release, this form of synaptic release is most likely impaired in this mutant. Comparing control animals with the SNAP23 mutant at RNA and morphological level may reveal the role of LDCV release on gene expression and network formation.

Another question discussed in this thesis is, which genes are regulated by activity. From the *munc18-1* mutant data we have many candidate genes, which are probably regulated by activity. Other models lacking activity, like the *munc13-1/2* double mutant and the inducible *munc18-1* mutant may be used to validate the activity-dependent expression of these genes. In addition, the sensory neurons of the DRG, which do not depend on synaptic release for their activity but lack *munc18-1*, can be analyzed by microarray technology. The analysis of DRGs will reveal which genes are regulated directly by the removal of *munc18-1* and not by activity. In the current study we might have missed some of the genes regulated by activity. First, in this thesis we studied the gene expression differences in the total cortex, which is composed of several cell types. Second, we only analyzed the expression of approximately 25 % of genes coded in the mouse genome. Studying single cell cultures may reveal other activity-dependent genes, for instance *munc18-1* mutant cell cultures and a more extended microarray may be used to analyze the expression of more genes. In addition, the question should be answered whether activity regulates gene expression at other levels than transcription, for instance by influencing translation or posttranslational modification. Therefore, the *munc18-1* mutant cortical protein levels should be analyzed.

To conclude, we have shown that immature synapses can form without synaptic release and that synaptic release is necessary to increase the expression of several neuronal transmission related genes. Using several mutants and proteomics analysis of the release machinery we can shed more light on the function of synaptic release and LDCV release in brain development.

ABBREVIATIONS

ACh:	Achetylcholine
Arc:	Activity-regulated cytoskeleton protein
ARL:	ADP-ribosylation factor-like protein
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
BDNF:	Brain-Derived Neurotrophic growth Factor
CAMK:	Calmodulin-dependent protein kinase
CARP:	Cbl-related protein
ChAT:	Choline-acetyltransferase
DRG:	Dorsal root ganglia
EDTA:	EthyleneDiamineTetraacetic Acid
Elisa:	Enzyme immunoassay
E16:	Embryonic day 16
E18:	Embryonic day 18
GDI:	GDP dissociation inhibitor
GABA:	Gamma-aminobutyric acid
GAD:	Glutamic acid decarboxylase
GAPDH:	Glyceraldehyde-3-phosphospate dehydrogenase
GO:	Gene orthology
HPRT:	hypoxanthine phosphoribosyl transferase
LDCV:	Large dense-core vesicle
LGN:	Lateral geniculate nucleus
LTP:	Long-Term Potentiation
MAPK:	Mitogen-activated protein kinase
Munc18-1	Mammalian homologue of *Caenorhabditis elegans* UNCoordinated locomotion-18
NARP:	Neuronal activity-regulated pentraxin
NCAM:	Neural cell adhesion molecule
NGF:	Nerve growth factor
NMDA:	N-methyl-D-aspartatic acid
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NPY:	Neuropeptide Y
NT:	Neurotrophin
PBS:	Phosphate Buffered Saline
PC:	Proprotein convertase
qPCR:	Real-time quantitative PCR
SAM:	Significance Analysis of Microarrays
SCAMP	Secretory carrier membrane protein
SDS:	Sodium Dodecyl Sulfate
SEM:	Standard error of the mean
SSC:	Sodium chloride Sodium Citrate
SNAP-25:	synaptosomal associated protein of 25 kD
SNARE:	Soluble N-ethylmaleimide-sensitive fusion (NSF) protein attachment protein (SNAP) receptors)
SV:	Synaptic vesicle protein
TIEG:	TGF-beta-induced immediate early gene-1
Trk:	Tyrosine kinase
TTX:	Tetrodotoxin
VAMP:	Vesicle-associated membrane protein

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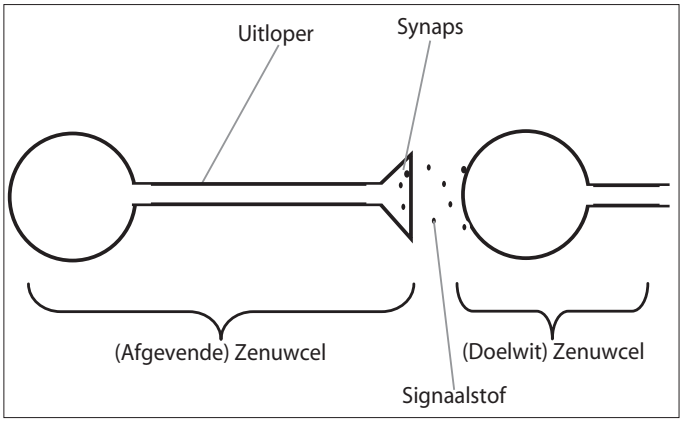
NEDERLANDSE SAMENVATTING

Studies naar de rol van synaps activiteit in synaps ontwikkeling en gen expressie

Onze hersenen worden geïnformeerd over vele processen in en buiten ons lichaam en sturen vele andere processen aan. Voor veel van deze processen zijn specifieke gebieden (centra) in de hersenen aanwezig. Tussen deze hersencentra bevinden zich verbindingen om informatie samen te brengen en om activiteiten van het lichaam op elkaar af te stemmen. Zo regelt een bepaald centrum in de hersenen tijdens het spreken de bewegingen van de mond en de tong. Een ander hersengebied bepaalt welke woorden je moet (of wilt) uitspreken. In weer een ander deel komt de informatie binnen die meldt dat de tong beweegt of die aangeeft dat de thee zoet is. Het lichaam is in staat goed te functioneren, omdat hersencentra onderling verbonden zijn en dus nauwkeurig met elkaar samenwerken.

Binnen zo'n centrum is een heel netwerk van zenuwcellen (neuronen) verantwoordelijk voor de totstandkoming van een bepaald proces. De verbindingen tussen de zenuwcellen binnen een hersencentrum, maar ook tussen hersencentra onderling, worden gevormd door uitlopers van de cellen. Als je je realiseert dat de hersenen bestaan uit 100 miljard zenuwcellen en dat iedere cel vele uitlopers heeft, dan is duidelijk dat hier sprake is van een immens netwerk.

Zenuwcellen kunnen met elkaar communiceren door het afgeven van bepaalde signaalstoffen (figuur 1). De afgifte van deze signaalstoffen gebeurt in daarvoor gespecialiseerde delen van de uitlopers, de zgn. synapsen, waarvan we er 60 biljoen hebben.



Figuur 1. Zenuwcellen kunnen signaalstoffen afgeven, die worden herkend door de doelwitcel.

Om de juiste zenuwcellen en centra met elkaar te verbinden, moet de ontwikkeling van het zenuwstelsel strak worden gereguleerd. Bijvoorbeeld moeten de afgevende cel en de doelwitcel elkaar kunnen herkennen. Hoe daarvoor wordt gezorgd is nog niet helemaal duidelijk. Wel weten we dat dit een zeer complex proces is. Een proces dat niet alleen voor de geboorte plaatsvindt, maar ook daarna. In dit proefschrift proberen we antwoord te geven op de vraag hoe deze stap in de ontwikkeling van de hersenen verloopt.

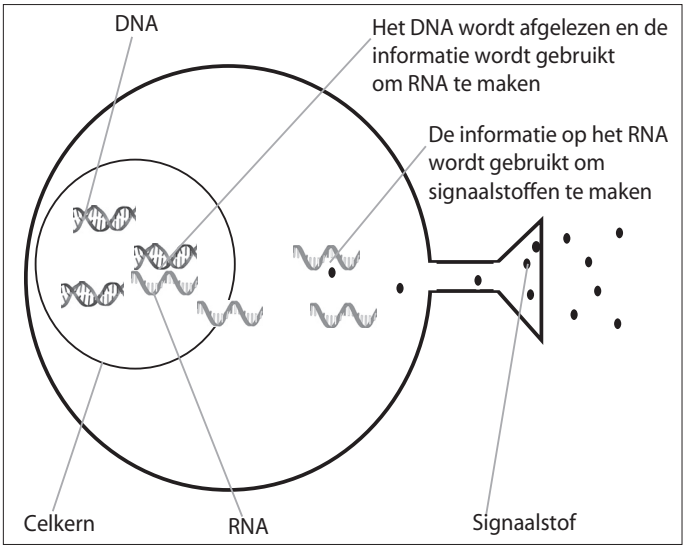
Al vroeg in de ontwikkeling geven zenuwcellen signaalstoffen af. Er werd altijd aangenomen dat deze signaalstoffen ervoor zorgen dat de uitlopers hun weg vinden naar de juiste zenuwcel en dat signaalstoffen zorgen voor het aanmaken van de juiste synapsen. Echter, uit ons onderzoek blijkt dat de signaalstofafgifte bij geen van deze processen een rol speelt. In dit onderzoek hebben we gebruik gemaakt van een genetisch gemodificeerde muis die deze signaalstoffen niet afgeeft. In deze zgn. mutante muis vinden de uitlopers gewoon hun weg en worden synapsen gevormd. Wel speelt de signaalstof een rol bij de functie van de synaps; zonder signaalstof is er geen communicatie tussen de zenuwcellen.

Het vormen van synapsen is één belangrijke stap, maar het behoud ervan is minstens zo belangrijk. Vele synapsen moeten namelijk jaren mee, sommige zelfs ons hele leven. De afgifte van signaalstoffen zou door zenuwcellen gebruikt kunnen worden om elkaar als partners te herkennen en daarmee de juiste synapsen te vormen. Als synapsen worden gevormd tussen verkeerde cellen, zijn deze instabiel en gaan verloren. De vraag of signaalstofafgifte betrokken is bij synaps-stabilisatie hebben we beantwoord in dit proefschrift (Hoofdstuk 2). In hoofdstuk 2 laten we zien dat in een mutante muis zonder signaalstofafgifte minder synapsen en synapsen met een rare vorm aanwezig zijn. De rare vorm van de synapsen, die we vinden in de mutant zijn waarschijnlijk synapsen die worden opgeruimd. Deze gegevens suggereren dat signaalstofafgifte inderdaad een stabiliserende werking op de synaps heeft.

Vervolgens hebben we de vraag gesteld waardoor stabilisatie afhankelijk is van de afgifte van signaalstoffen. Het zou kunnen dat de afgifte van signaalstoffen ervoor zorgt dat de doelwitcel de synaps in stand houdt, bijvoorbeeld door een bepaald stabiliserend eiwit gaan maken als hij meer signaalstof krijgt van de afgevende cel. Zenuwcellen moeten zich aanpassen aan verschillende omstandigheden, zoals het hart zich aanpast aan hoe actief je bent. Als je hardloopt, hebt je een hogere hartslag dan wanneer je slaapt. De afgifte van signaalstoffen in de hersenen zou voor deze aanpassing en de synaps-stabilisatie belangrijk kunnen zijn.

De informatie hoe een bepaald eiwit gemaakt moet worden ligt op het DNA, in de celkern. Als er veel van dat eiwit nodig is, wordt de informatie op het DNA vaker afgelezen (figuur 2). De betreffende informatie van het DNA wordt tijdens het aflezen gekopieerd op een drager, het zogenaamde boodschapper RNA. Dit RNA wordt van

de celkern naar de rest van de cel gebracht en wordt daar gebruikt om eiwit te maken. De hoeveelheid boodschapper RNA voor een bepaald eiwit is dus een maat voor de hoeveelheid eiwit die op dat moment wordt gemaakt.



Figuur 2. Informatie hoe een eiwit gemaakt wordt ligt op het DNA. Voor ieder eiwit is er een gen op het DNA. Dit gen wordt afgelezen en er wordt RNA gemaakt. Voor ieder eiwit is er dus ook een specifiek RNA. De informatie op het RNA wordt vervolgens gebruikt om een eiwit te maken.

In hoofdstuk 3 hebben we gekeken hoe de hoeveelheid RNA voor bepaalde eiwitten verandert als er geen sprake is van afgifte van signaalstoffen. Hiervoor hebben we de hoeveelheid RNA in cellen van onze mutante muis vergeleken met die van een normale muis. We laten zien dat vooral de hoeveelheid RNA van eiwitten die een rol spelen in de synapsvorming verandert. Dit bevestigt de hypothese dat afgifte van een bepaalde signaalstof er tijdens de hersenontwikkeling voor zorgt dat de juiste eiwitten worden gemaakt en dat die op hun beurt de betrokken synapsen stabiliseren.

In hoofdstuk 4 hebben we onderzocht wat het effect is van de afgifte van signaalstoffen op hun eigen aanmaak. Vooral de hoeveelheid RNA van één groep van signaalstoffen in de hersenen, de neuropeptiden, is lager als er geen afgifte is van deze of andere signaalstoffen. In zenuwcellen die geen afgifte van signaalstoffen nodig hebben om toch actief te kunnen zijn, vinden we deze verlaging in neuropeptide hoeveelheden niet. Dit duidt erop dat de afgifte van signaalstoffen inderdaad de hoeveelheid neuropeptide RNA reguleert. Dit effect is ook nog behoorlijk specifiek, want de RNA hoeveelheden van andere groepen signaalstoffen zijn dan niet of nauwelijks veranderd.

In hoofdstuk 5 hebben we gekeken naar drie andere eiwitten die in de synaps aanwezig

zijn. Deze eiwitten zijn betrokken bij het afgeven van signaalstoffen. Van maar één van deze eiwitten, Syntaxin1, zijn RNA hoeveelheden verlaagd in de mutant terwijl de andere twee onveranderd zijn. Vervolgens hebben we gekeken of ook de eiwit hoeveelheden van Syntaxin1 veranderd zijn in de mutante muis. Aangezien RNA wordt gebruikt om eiwitten te maken verwacht je dat als er minder RNA is er ook minder eiwit is. Inderdaad waren ook de eiwit hoeveelheden lager. Dat zou kunnen betekenen dat ook de Syntaxin1 hoeveelheden worden gereguleerd door signaalstofafgifte. Dat is echter waarschijnlijk maar voor een deel waar. Als we namelijk in de mutant kijken naar zenuwcellen die wel actief zijn (ook zonder afgifte van signaalstoffen) dan zijn de RNA hoeveelheden van Syntaxin1 hetzelfde als in normale muizen, maar de eiwit hoeveelheden zijn wel lager. Dit komt waarschijnlijk door het eiwit (munc18-1) dat weggehaald is in de mutante muis. Syntaxin1 kan aan dit eiwit binden. Als munc18-1 niet aanwezig is zoals in de mutant, dan zou Syntaxin1 makkelijker door enzymen kunnen worden afgebroken. In de cel zijn altijd enzymen aanwezig die eiwitten afbreken, zo worden oude eiwitten weer opgeruimd. Als een eiwit in zijn eentje door de cel zwerft kunnen de enzymen het makkelijker afbreken. Dit laat zien dat de veranderingen die we zien in de mutant niet alleen komen doordat er geen signaalstofafgifte is, maar dat de afwezigheid van Munc18-1 ook veranderingen in eiwit en RNA hoeveelheden geeft.

Een belangrijke conclusie uit mijn proefschrift is dat het ontbreken van de afgifte van signaalstoffen de hoeveelheden RNA en eiwitten kan beïnvloeden. Signaalstofafgifte is dus is naar alle waarschijnlijkheid via vele andere moleculen betrokken bij de ontwikkeling van functionele synapsen in de hersenen.

EN UITERAARD DEED IK DIT NIET ALLEEN.....

Afgelopen jaren heb ik van velen praktische en/of emotionele steun gekregen. Mijn promotor Matthijs, bedankt dat je mij hebt geholpen een zelfstandig onderzoeker te worden. Alhoewel ik jouw werkschema niet zou kunnen volhouden, was het zeker een stimulerend voorbeeld. Je enthousiasme voor wetenschap werkte aanstekelijk. Ik hoop dat ik inmiddels jouw manier van data op een duidelijke manier presenteren goed heb afgekeken. Guus, dank je dat je op het juiste moment mijn onderzoek een zet in de goede richting hebt gegeven. Daarnaast hartelijk dank voor de gastvrijheid op jouw lab, waarvan ik nog steeds met veel plezier gebruik van maak. Mijn co-promotor Sabine, ik denk nog met veel plezier terug aan het kliederen met lijm om dammetjes te bouwen voor de array-kleuringen. Iets minder fijn vanwege de kou, maar zeker niet minder leerzaam waren de dagen tussen kerst en nieuwjaar, toen je me inwijdde in de wereld van de quantitative PCR.

De eerste OIO-jaren heb ik doorgebracht op het RMI. De sfeer was er altijd erg goed. Vooral de vrijdag-middagen waren echt een begrip. Bij deze wil ik met name Peter, Marten, Rea, Leo, Joke, Ceriel, Niki, Julia, Marjan, Patrick, Johanneke, Wouter, Dorien, Hikke, Robert, Anita, Tom, Ria, Joost, Wout en Bert bedanken voor de hulp en/of mentale steun. Tineke, dank je voor de nuttige snoeptripjes en niet alleen omdat ze eindigden met een lekkernij.

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Ontspanning is toch minstens zo belangrijk als je deze berg beklimt. Zo werkte ik met enige regelmaat met Daniëlle aan mijn stapel kleren, ik hoop dat er nog vele winkel

uitstapjes mogen volgen. Daniëlle dank je voor de mooie omslag. Maar uiteraard ook dat je mijn paranimf wilde zijn en voor alle steun en toeverlaat. Jeroen, dank je voor alle gezellige etentjes. Afra, Arnoud, Arjen, Marten en Juriaan, even mijn zinnen verzetten was heel belangrijk, de klimavondjes, tochten en avondjes uit waren een welkome afwisseling.
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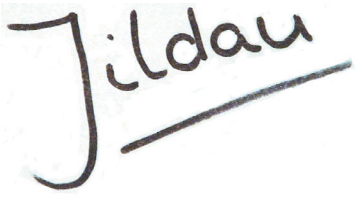

P.S. Uiteraard is er altijd de mogelijkheid dat je iemand gaat vergeten. Bij deze wil ik iedereen bedanken die op welke manier dan ook deze afgelopen jaren geholpen heeft mijn proefschrift tot een goed einde te brengen!

CURRICULUM VITAE

Jildau Bouwman werd geboren op 18 februari 1976 in Heemskerk. In 1988 begon ze aan haar VWO opleiding aan het Berlingh College in Beverwijk, waar ze in 1994 haar diploma haalde. Datzelfde jaar startte ze aan de studie Biologie aan de Vrije Universiteit Amsterdam. Ze deed twee stages, een met Dr. R van Kesteren over synaps formatie in *Lymnaea stagnalis* en de tweede met Prof Dr. A. B. Brussaard over de veranderingen in $GABA_A$-subunit samenstelling in de hersenen van een pas bevallen rat. In 1999 studeerde ze af en begon in maart aan haar promotie onderzoek onder begeleiding van Prof Dr. M Verhage en Prof. Dr. J.P.H. Burbach aan het Rudolf Magnus Institute for Neuroscience in Utrecht. In 2002 werd dit onderzoek voortgezet aan de vrije Universiteit Amsterdam. Dit proefschrift beschrijft dit onderzoek. Jildau werkte op dit moment als post-doc bij de afdeling Moleculaire celfysiologie van professor Westerhoff. Ze onderzoekt in samenwerking met verschillende andere onderzoekers hoe de cel in de praktijk zijn functie regelt. Waarschijnlijk gebeurt dit op verschillende gen expressie niveaus (bv. trancriptie, RNA degradatie, en/of metaboliet concentraties) afhankelijk van de perturbatie waar de cel zich aan aan moet passen.

CURRICULUM VITAE (English version)

Jildau Bouwman was born on February 18th 1976 in Heemskerk, the Netherlands. In 1988, she started grammar school at the 'Berlingh college' in Beverwijk where she graduated in 1994. That year she started to study Biology at the Vrije Universiteit Amsterdam. She did two internships one with Dr. R. van Kesteren on synapse formation in *Lymnaea stagnalis* and the second with Prof. Dr. A.B. Brussaard on changes in the $GABA_A$ subunit in the rat brain after giving birth. In 1999 she graduated and started in March of that year with her PhD research under supervision of Prof. Dr. M Verhage and Prof. Dr. J.P.H. Burbach at the Rudolf Magnus Institute for Neuroscience in Utrecht. In 2002, the research was continued at the vrije Universiteit Amsterdam. This research is described in this thesis. Jildau is currently working as post-doc at the department of Molecular Cell Physiology, which is headed by Prof Dr. H. V. Westerhoff. Together with several other researchers she studies how the cell actually regulates its function. Probably this occurs at several gene expression levels (i.e. transcription, RNA degradation and/or the metabolite concentration) depending on the perturbation to which the cell has to adapt.

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