Neuroligins Determine Synapse Maturation and Function

Dissertation
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der Georg-August-Universität Göttingen

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aus Echmiadzin, Armenien

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D7
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Koreferent: Prof. Dr. Michael Hörner

Tag der mündlichen Prüfung:
Dedicated to my Parents
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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-Amino-3- hydroxyl-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tryphosphate</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7 nitroquinoxaline-2, 3-dione disodium salt</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>DL-AP5</td>
<td>DL-2-Amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenedinitrilo-tetraacetic acid</td>
</tr>
<tr>
<td>eEPSCs</td>
<td>evoked excitatory postsynaptic currents</td>
</tr>
<tr>
<td>eIPSCs</td>
<td>evoked inhibitory postsynaptic currents</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td>et alters (and others)</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational acceleration, or gram</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>$G\Omega$</td>
<td>Giga Ohm</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo Hertz</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>$\mu$-</td>
<td>micro,-($10^{-6}$)</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>miniature excitatory postsynaptic currents</td>
</tr>
<tr>
<td>mIIPSCs</td>
<td>miniature inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleus ambiguus</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NH</td>
<td>Nucleus hypoglossus</td>
</tr>
</tbody>
</table>
Abbreviations

NL    Neuroligin
NMDA  N-methyl-D-Aspartate
N-terminal at the NH2-terminus of a protein
pA    pico Ampere
PAGE  Polyacrylamide gel electrophoresis
PBC   PreBötzinger complex
PBS   Phosphate buffer saline
PFA   paraformaldehyde
pH    Negative logarithm of H+ concentration
PDZ   Protein interaction domain, acronym for PSD-95, Dlg, ZO-1
ProSAP praline-rich synapse-associated protein
PSD   postsynaptic density
RT    Room temperature
s     second
SDS   Sodium dodecyl sulfate
sEPSCs spontaneous excitatory postsynaptic currents
sIPSCs spontaneous postsynaptic currents
SKO   Single knockout
sPSCs spontaneous postsynaptic currents
TKO   Triple knockout
Tris  Tris-hydroxymethyl-aminomethane
TTX   Tetrodoxin
VIAAT vesicular inhibitory amino acid transporter
vGlut vesicular glutamate transporter
WT    Wild type
5’    5 prime
1. Introduction

1.1 Neurotransmission

The mammalian brain is a highly specific neuronal network, where billion of neurons are connected through synapses, which are specialized intrinsic asymmetric contacts, mediating precise, rapid and efficient communication between neurons. Synapses are classified into two types: chemical and electrical. Most synapses in the nervous system are chemical. Typically they are consisted of presynaptic and postsynaptic compartments, separated by synaptic cleft. Presynaptic compartment usually is localized on axons, while postsynaptic specialization can be localized on dendrites, soma and in some cases on axon. Presynaptic specialization of the synapse is characterized by neurotransmitter-loaded synaptic vesicles at active zone and required for neurotransmitter release into synaptic cleft, whereas the postsynapse, which contains receptors, ion channels and associated proteins (post-synaptic density), is specialized for signal transduction. Cell adhesion molecules (CAMs) (Serafini, 1999; Yamagata et al., 2003) bridge the pre- and postsynaptic compartments of synapses in the central nervous system. They can promote differentiation of pre- and postsynaptic specializations and take a part in regulation of synaptic structure and function (Yamagata et al., 2003). They are several families of cell adhesion molecules that have been discovered (Brose, 1999; Missler et al., 2003; Scheiffele, 2003; Yamagata et al., 2003). These membranous proteins, present on pre- and postsynaptic membranes, are in a heterophilic or homophilic interaction with their partners on the opposite side of synaptic cleft.

Neurotransmitters are released in a process termed exocytosis. This process is initiated upon an arrival of an action potential at the presynaptic terminal, which generates depolarization of presynapse, leads to the opening of voltage gated Ca^{2+} channels at the active zones and Ca^{2+} ions entering into the presynaptic terminal (Zucker et al, 1993). The rise in Ca^{2+} concentration brings the readily releasable vesicles to fuse with the plasma membrane and release their
neurotransmitter into the synaptic cleft (Landis et al., 1988; Dresbach et al., 2001; Harlow et al., 2001). The transmitter molecules then diffuse across the synaptic cleft, bind to their specific receptors on the postsynaptic compartment of synapse and activate them, leading to the opening and or closing of ion channels.

1.2 Localization and function of the preBötzinger complex

The respiratory rhythm originates from special neuronal regions located in restricted areas in the reticular formation of the ventrolateral medulla. One of these regions is bilaterally localized preBötzinger complex (PBC), which contains all classes of respiratory neurons essential for respiratory rhythm generation (Smith et al., 1991; Connely et al., 1992; Schwarzacher et al., 1995; Feldman et al., 2003). PreBötzinger complex is located ventro-lateral to the nucleus ambiguus (NA), caudal to the retrofacial nucleus, and rostral to the anterior tip of the lateral reticular nucleus (Bregma-level -12.0 mm to -12.5 mm) (Gray et al., 1999; Guyenet and Wang, 2001; Wang et al., 2001) (Fig.1.1).

Figure 1.1 Schematic representation of the localization of preBötzinger complex in rat brainstem: On the left side a dorsal topview of the rat brainstem after removal of the cerebellum is shown. On the right side a sagittal section (top) and a transversal slice (bottom), which contains the PBC, are shown. Abbreviations: nucleus solitary tract (NTS), hypoglossal nucleus (XII), nucleus ambiguus, compact part (cNA), preBötzinger complex (PBC), principal nucleus of the inferior olive (IOp).
The nucleus ambiguus and principal nucleus of the inferior olive (IO\textsubscript{Pr}) help for anatomical identification of PBC. The neurons of PBC are immunoreactive for the neurokinin receptor neurokinin 1 (NK1) (Gray et al., 1999; Wang et al., 2001) and the destruction of neurons expressing NK1 leads to an ataxic respiration (Gray et al., 2001), which together with other experiments (Pierrefiche et al., 1998; Smith et al., 1991; Solomon et al., 1999) confirms the view that the preBötzinger complex plays a primary role in the respiratory rythmogenesis.

1.3 Neuroligins as cell-adhesion molecules

Neuroligins constitute a family of cell adhesion proteins that interact with presynaptic α- and β-neurexins and form heterotypic intercellular junctions. Neuroligin proteins have been identified in humans, rodents, chicken, drosophila melanogaster and Caenorhabditis elegans (Ichtchenko et al., 1995, 1996; Kwon et al., 2004; Gilbert et al., 2001; Bolliger et al., 2001; Paraoanu et al., 2005). Four members of neuroligin family are characterized in rodents and five genes coding for neuroligins have been identified in the human genome (Boucard et al., 2005, Ichtchenko et al., 1995, Song et al., 1999, Varoqueaux et al., 2004).

1.3.1 Structural features of neuroligins

All neuroligin isoforms have large extracellular acetylcholinesterase-like domain, which lacks catalytic activity and binds to neurexins. It is followed by transmembrane segment and shorter intracellular terminal PDZ binding motif. All neuroligins are subject to alternative splicing at two conserved splice sites, abbreviated to as A and B (Fig.1.2) that are distinct for neuroligin 1 and 2, while neuroligin 3 contains two types of alternatively spliced variants, one is similar to that present in neuroligin 1, and the second is a hybrid of sequences present in neuroligin 1 and neuroligin 2 (Ichtchenko et al., 1995, 1996). The existence of two alternatively spliced regions allows the generation of up to four different isoforms for each neuroligin gene (Ichtchenko et al., 1996; Boucard et al., 2005).
1.3.2 Expression of neuroligins

Neuroligins are expressed throughout brain with differential and overlapping distribution of different isoforms. As described in different studies, mRNAs of neuroligins 1-3 are expressed at low levels in newborn rat and mouse brain and their expression increases (2- to 3-fold) during postnatal development (Song et al., 1999; Varoqueaux et al., 2004). NL 1 is expressed in central nervous system and localized specifically at the excitatory synapses (Song et al., 1999; Prange et al., 2004), whereas NL 2 is concentrated at the postsynaptic membranes of inhibitory synapses (Varoqueaux et al., 2004, Graf et al., 2004) and presents also in other tissues such as pancreas, lung, endothelia, uterus and colon. NL 3 expression except neurons has also been found in different glial cells (Philibert et al., 2000). In newborn mice NL 4 is not detectable, and in adult mice, only 3% of the total NL proteins in brain are contributed by NL 4. In humans NL 4 is detected in heart, liver, skeletal muscle, pancreas, and at low levels in the brain. The existence of fifth neuroligin gene has also been reported (Bolliger et al., 2001; Jamain et al., 2003). NL 5 also referred as neuroligin 4Y because of its localization on the Y chromosome, differs from X linked NL 4 by only 19 amino acids.

1.3.3 Binding partners of neuroligins

There are some interaction partners for neuroligins that have been discovered. Extracellularly neuroligins bind to α- (Ichtchenko et., al 1995, 1996) and β-
neurexins (Boucard et al., 2005). The interaction between neuroligins and neurexins mediates trans-synaptic adhesion (Nguyen and Südhof, 1997). Postsynaptically, the intracellular tail of neuroligin isoforms binds to several PDZ domain-containing PSD-95, SAP-102, PSD-93 (Irie et al., 1997) and S-SCAM (Hirao et al., 1998) scaffolding proteins of glutamatergic postsynapses, which in turn interact with postsynaptic transmitter receptors, ion channels and signaling proteins (Hirao et al., 1998, Irie et al., 1997, Meyer et al., 2004). In contrast to glutamatergic synapses so far there are not direct experimental evidences for molecular composition of the postsynaptic specialization in GABAergic synapses and therefore the binding partners that are involved for targeting neuroligin 2 to inhibitory synapses are not clearly known (Varoqueaux et al., 2004). In Figure 1.3 are illustrated well known and proposed postsynaptic binding partners of neuroligins at glutamatergic and GABAergic postsynapses.

![Figure 1.3 Binding partners of neuroligins at glutamatergic and GABAergic synapses. This model shows many interactions of neuroligins with different proteins at glutamatergic postsynapse. In contrast, binding partners of neuroligin 2 at GABAergic postsynapses remain unknown. Lines indicate reported protein-protein interactions and dashed lines show proposed, most likely indirect, interactions.](image-url)
1.4 Neuronal disorders associated with dysfunction of neuroligins

Autism is a complex neuro-developmental disorder characterized by severe reciprocal social interaction and communication impairment, which is often associated with mental retardation and epilepsy. Autistic individuals have larger brains, although the cerebral cortex, hippocampus and amygdale are smaller (Herbert et al., 2003; Aylward et al., 1999). The male-to-female ratio is 4:1 in autism. Twin studies and high number of male autistic patients provide the evidence for a genetic origin of autism (Beaudet et al., 2002). Many studies support the hypothesis that mutations in human neuroligin genes NL 3 and NL 4 cause rare monogenic heritable forms of autism and mental retardation (Chih et al., 2004; Comolletti et al., 2004; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005; Talebizadeh et al., 2006), as are perturbances in the excitatory to inhibitory ratio and morphological aberrations in dendritic spines (Kaufmann et al., 2000; Hussman et al., 2001; Zoghbi., 2003). Therefore mice lacking neuroligin genes could be useful animal model for studying pathophysiologic mechanisms in autism.

1.5 The role of NLs in synaptogenesis and synaptic function in vitro

During development, synapse assembly is crucial highly controlled process in central nervous system that is initiated by contact formation between axon growth cone and target cell. Initial contact is followed by coordinated differentiation and maturation of the pre- and postsynaptic sites. Both processes seem to be driven by several classes of cell adhesion molecules (CAMs). Several recent studies performed in neuronal cultures have shown the potential role of neuroligins as CAMs and their binding partners in the formation and maturation of excitatory and inhibitory neuronal synaptic contacts (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004; Scheiffele et al.,
Overexpression of NLs in cultured neurons increases the number of synapses (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004) and knock-down of neuroligin 1, 2 and 3 expression either individually or collectively by RNAi causes reduction in both excitatory and inhibitory presynaptic terminals (Chih et al., 2005). For example, the data obtained from mouse hippocampal neurons (Levinson et al., 2005) demonstrate that neuroligins drive excitatory and inhibitory presynaptic contact formation and various members of neuroligin family are able to exert similar effects on formation of new synaptic contacts (Fig. 1.4). These studies demonstrate the possible involvement of these cell adhesion molecules, in particular NL 2, in controlling the balance between excitatory and inhibitory synapses. Simultaneous or individual overexpression and knock-down of neuroligins in cultured neurons leads to apparent changes in synaptic activity, which were explained by the dramatic effects of these manipulations on synapse numbers (Graf et al., 2004, Chih et al., 2005).

**Figure 1.4** DIV 5 hippocampal neurons transfected with HA-NL1, HA-NL2 or HA-NL3 and then fixed at DIV 8 have increased VGAT (A) and VGLUT(B) positive puncta as compared to GFP-transfected cells.
In vivo experiments on animals mutant for all known neuroligins may help to clarify the role of these proteins in synaptic function and different aspects of synapse development, including contact initiation, target recognition, synapse stabilization/maturation (Lise and El-Husseini, 2006).

1.6 Aims of the present study

The present work aims at investigating the role of neuroligins in synaptogenesis, and synapse function in the respiratory brainstem of mouse. For this purpose knockout mice were generated that had deletion of one, two or three neuroligins. The neuronal network of mouse respiratory brainstem, which is almost mature at birth time, is an appropriate system to investigate the role of neuroligins in vivo. The project is subdivided into two main steps. First step is the investigation of synaptogenesis and synaptic activity in NL 1-3 triple knockout mice, using molecular biological and electrophysiological approach. Because of early postnatal death of NL 1-3 triple KOs, experiments were performed on newborn mice. Second step contains detailed study of consequences of NL 2 deletion in synaptogenesis and synaptic transmission in brainstem respiratory network. For this, experiments were done in acute brainstem slices derived from neuroligin 2 knockout mice, using combined electrophysiological, immunohistochemical and biochemical methods.
2. Materials and methods

2.1 Electrophysiology

2.1.1 Animals

Experiments were performed on NL 1-3 and NL 2 knockout mice. To generate mice that are lacking NLs 1-3, exon sequences covering the translational start site and at least 380 bp of 5’ coding sequence of the respective genes were deleted by homologous recombination in embryonic stem cells. KOs lacking individual NLs and all combinations of double KOs (DKO 1/2, DKO 1/3 and DKO 2/3) were generated by interbreeding and were obtained at the predicted Mendelian frequencies. NL 1-3 and NL 2 KO mice were generated in and generously supplied by the lab of Prof. N. Brose. Animal experiments were carried out in accordance with the guidelines of the Ethics committee of the University of Göttingen.

2.1.2 Brain slice preparation

The preparation of transverse brainstem slices containing pre-Bötzinger complex (PBC) and hypoglossal nucleus (NH) followed the general procedure of making thin slices from the tissue of mouse central nervous system described in (Zhang et al., 1998). Postnatal day 0 (P0) to P5 male or female littermate mice were decapitated at C3-C4 spinal level. The whole brain was carefully removed from the cut-open skull, immediately transferred into ice-cold artificial cerebrospinal fluid (ACSF), which was already bubbled with carbogen (95% O₂ and 5% CO₂). The brainstem was separated from the cerebellum and forebrain. Transverse 200 µm-thick slices were cut using a vibratome slicer (752M Vibroslice, Campden Instruments, UK). Sectioning of the brainstem was done from the rostral to caudal part and fourth ventricle was used as a marker for the start of the region of interest. After sectioning, each slice was quickly placed into an incubation chamber containing aerated ACSF. Slices were kept at 28-30 ºC.
2.1.3 Solutions and drugs used for electrophysiology

The standard (Ringer) extracellular solution (Table 2-1) was used for brain slice preparations and electrophysiological recordings. It had a pH of 7.4 and an osmolarity of about 310 mOsm. The following tables (Table 2-2; 2-3) describe the compositions of different intracellular (patch-pipette) solutions. For recordings of spontaneous postsynaptic currents as an intracellular solution INK was used (Table 2-2) and to record evoked postsynaptic currents (ePSCs) the pipettes were filled with INLOW solution (Table 2-3).
Table 2-1. Extracellular solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration [mM]</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118</td>
<td>Roth</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
<td>Roth</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1</td>
<td>Roth</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>KCl</td>
<td>3</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1,5</td>
<td>Roth</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>Roth</td>
</tr>
</tbody>
</table>

Table 2-2. Intracellular INK solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration [mM]</th>
<th>Purchased from</th>
</tr>
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<tbody>
<tr>
<td>KCl</td>
<td>140</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CaCl₂x2H₂O</td>
<td>1</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>EGTA</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>MgCl₂x6H₂O</td>
<td>2</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Na₃GTP</td>
<td>0,5</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Na₃ATP</td>
<td>4</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Table 2-3. Intracellular INLOW solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration [mM]</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-Gluconat</td>
<td>140</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>EGTA</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>MgCl₂x6H₂O</td>
<td>2</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Na₃GTP</td>
<td>0,5</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Na₃ATP</td>
<td>4</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
Intracellular solutions mentioned above were adjusted to pH 7.2 with osmolarity about 310 mOSM.

Table 2-4. Drugs used for experiments

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration [µM&amp;mM]</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNQX</td>
<td>10 µM</td>
<td>Tocris</td>
</tr>
<tr>
<td>DL-AP5</td>
<td>10 µM</td>
<td>Tocris</td>
</tr>
<tr>
<td>Strychnine</td>
<td>1 µM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>1 µM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TTX</td>
<td>0.5 µM</td>
<td>Alomone labs</td>
</tr>
<tr>
<td>Muscimol</td>
<td>5 mM</td>
<td>Tocris</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5 mM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Glycine</td>
<td>5 mM</td>
<td>Roth</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5 mM</td>
<td>Roth</td>
</tr>
</tbody>
</table>

2.1.4 Electrophysiological recordings

All electrophysiological recordings were done on acute brainstem slices containing PBC and NH (Fig. 2.1). The slices were placed into the glass bottomed recording chamber and fixed by platinum wire with a grid of parallel nylon threads, to avoid of slice dislocation. The slice was continuously perfused with aerated extracellular solution during experiment, using a pump (Watson Marlow, 505LA). The brain slices were visualised under an Axioscope microscope (Zeiss, Germany) using a 5x objective. Somas of PBC neurons were identified under infrared gradient contrast illumination (C2400, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) with a 40x water immersion objective. Patch pipettes were pulled from borosilicate glass micropipettes (GC 150-10F, Clark Electromedical Instruments, UK) using a multistage puller (P87, Sutter Instrument Co., Novato, USA). When filled, they displayed resistance of 2-4 MΩ. Recordings were
performed using an Axopatch 200 amplifier (Axon instrument Inc., USA). In experiments was used voltage-clamp configuration of the “whole-cell patch-clamp” technique. The first step in this method is formation of a gigaseal, by touching the cell surface with pipette and applying gentle suction. After application of a short pulse of negative pressure to the electrode the patch of membrane under the pipette is ruptured, reaching the whole-cell configuration. The solution filling the pipette enters into the cell and equilibrates with the cell’s cytoplasm. After establishing of whole-cell configuration the recordings were performed at a holding potential of -70 mV.

The capacitance and series resistance was compensated 80 % according to manufacture recommendation. The membrane currents were filtered by a four-pole Bessel filter set at a corner frequency of 1 kHz and digitized at a sampling rate of 5 kHz using the DigiData 1200 interface (Axon Instrument Inc., USA). For correction of current measurements P/4 protocol was used. According to this protocol four leak-subtraction pulses were applied immediately before the main command step and leak currents were subtracted. All experiments were conducted at 33-35 ºC.

2.1.5 Capacitance and series resistance calculation

The passive properties of PBC neurons were estimated by determining membrane capacitance and series resistance for each recorded neuron before subsequent compensation. Capacitance and series resistance were calculated from the integral of the current transients induced by 20 mV hyperpolarizing voltage commands from a holding potential of –70 mV immediately after rupture of the cell membrane according to these formulas,

\[ C = \frac{I\tau}{20} \]

\[ Rs = \frac{\tau}{C} \]

where C is the capacitance, I is the current, \( \tau \) is the decay, Rs is the series resistance. There were no systematic differences of capacitance and series resistance between different genotypes.
2.1.6 Recordings of spontaneous and evoked postsynaptic currents

Evoked inhibitory and excitatory postsynaptic currents were recorded from hypoglossal motor neurons in presence of 10 µM CNQX, DL-AP5 or 1 µM bicuculline, 1µM strychnine, respectively. PSCs were evoked by 0.1 Hz field stimulations of axons near to preBötzing complex using bipolar platinum stimulating electrode (30 µM diameter, Degussa, Germany). The pipettes were filled with INLOW solution (Table 2-3). For each experiment was applied supramaximal stimulation strength, using an isolation unit IsoFlex (A.M.P.I.) with a custom-built power supply. Peak amplitudes were averaged from 25 consecutive responses. The failure rate was calculated from these 25 responses in each experiment. To monitor changes in input resistance, current responses to a -10 mV voltage steps (20 ms) from a holding potential of -70 mV were recorded before every fifth stimulus. In all experiments the distance between stimulation and recording electrodes was similar on all slices from different mice. Spontaneous inhibitory and excitatory postsynaptic currents were recorded from neurons of preBötzing complex at about 0 mV in presence of 10 µM CNQX, DL-AP5 or 1 µM bicuculline, 1µM strychnine, respectively. Spontaneous miniature inhibitory and excitatory PSCs were recorded as described above, but adding 0.5 µM tetrodotoxin (TTX) into bath solution. For these experiments as a pipette solution was used INK intracellular solution (Table 2-2). Signals with amplitudes of at least two times above background noise were selected and statistical significance was tested in each experiment. In all experiments, as mentioned above, was used bath application of drugs, but in some experiments drugs were applied directly in close vicinity to the patched neurons, using a glass pipettes filled with muscimol (5 mM), glycine (5 mM), glutamate (5 mM), or sucrose (300 mM). Patches with a series resistance of >20 MΩ, a membrane resistance of 0.8 GΩ or leak currents of >150 pA were excluded.
2.1.7 Ventilation recordings

Ventilation patterns were recorded by whole-body plethysmography. Unanaesthetized mice postnatal day 1 (P1) to P3 were placed in a closed chamber connected to a differential pressure transducer (CD15 Carrier Demodulator, ValiDyne). The analog signal of ventilation-related changes of air pressure was amplified and digitized using an A/D-converter (DigiData 3200, Axon Instruments). The coefficient of variation (CV) for ventilation was calculated according to this formula,

$$CV = \frac{S_d}{X}$$

where Sd is the standard deviation, X is the mean of the frequency.

2.1.8 Data analysis

Data acquisition and analyses was done using commercially available software: pClamp 6.0 and Axograph 4.6 (Axon Instruments Inc., USA), MiniAnalysis (SynaptoSoft, Decatur, GA), Prism 4 (GraphPad Software, San Diego, CA). All results are reported as mean±SEM. The statistical significance was evaluated with two-tailed unpaired Student’s $t$ test.

2.2 Fluorescence immunohistochemistry

2.2.1 Brain tissue preparation for immunohistochemistry

Postnatal NL2 KO mice (P5) were deeply anaesthetized with TBE (tribromoethanol) until they were unresponsive to painful stimuli. A thoracotomy was performed and animals were perfused through the aorta with 0.9% sodium chloride followed by 100 ml 2% paraformaldehyde in 0.1 M phosphate buffer. The whole brain was removed, post-fixed for 1 hour in the same fixative at 4 °C. The tissue was cryoprotected in 10-30% sucrose overnight at 4 °C. Afterwards it
was frozen by tissue freezing medium on dry ice and stored at -80°C. Series of transverse sections of brainstem with a thickness of 12 µm were cut using a cryoslicer. Each section was quickly placed on the slide. After sectioning the slides were kept at -80°C.

2.2.2. Solutions and chemicals

Table 2-5 Chemicals used for immunohistochemistry

<table>
<thead>
<tr>
<th>Substance</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Roth</td>
</tr>
<tr>
<td>KCl</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>Roth</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>Roth</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>NGS</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Mowiol</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>PFA</td>
<td>Roth</td>
</tr>
<tr>
<td>TBE</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

PFA 8% (for 1 L)
80g paraformaldehyde in 0.1 mM PB

PB (phosphate buffer for 2 L)
Buffer 1: 0.2 M NaH₂PO₄ (27.6 g NaH₂PO₄·H₂O)
Buffer 2: 0.2 M Na₂HPO₄ (71.7 g Na₂HPO₄·12H₂O)

PBS (for 1 L)
10 mM PB; 150 mM NaCl 2.7mM KCl,
(50 ml PB; 8.77 g NaCl; 200 mg KCl; 900 ml H₂O)
2.2.3 Antibodies used for immunofluorescence labeling

Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gephyrin, mouse</td>
<td>Synaptic system</td>
</tr>
<tr>
<td>ProSAP, rabbit</td>
<td>Kindly provided by Dr. TM Boecker</td>
</tr>
<tr>
<td>VGlut 1, guinea pig</td>
<td>Chemicon</td>
</tr>
<tr>
<td>VGlut 2, guinea pig</td>
<td>Chemicon</td>
</tr>
<tr>
<td>VIAAT, rabbit</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Anti-GP Alexa Fluor 488</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Goat Anti-mouse Alexa Fluor 555</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Goat Anti-rabbit Alexa Fluor 488</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Goat Anti-rabbit Alexa Fluor 555</td>
<td>Molecular probes</td>
</tr>
</tbody>
</table>

2.2.4 Immunofluorescence staining

Before starting the immunostaining, the slices were washed three times with PBS. The blocking of non-specific binding sites and permeabilisation of slices were done using 2 % NGS and 0.2-0.3 % Triton X-100 in phosphate-saline buffer (PBS) for 20-30 min at RT. Sections were incubated overnight at 4°C in primary antibodies dissolved in PBS containing 2 % NGS and 0.2-0.3 % Triton X-100. After incubation with primary antibodies the sections were washed 3 times for each 10 min and then incubated for 1 Hour at RT in the dark with species-specific fluorochrome-conjugated secondary antibodies, followed by three washing steps for 10 min each. Finally, sections were slightly air-dried and coverslipped with fluorescent mounting medium.
2.2.5 Data analysis

Sections from immunofluorescence staining were visualized by confocal laser scanning microscopy (Zeiss LSM510). Typically, stacks of 7-12 images (1024x1024 pixel) at a zoom factor 4 spaced by 0.38-0.42 µm were recorded, using a 63x oil-immersion objective. For quantitative analyses, the gain and offset were held constant across all images to allow for intensity comparisons. Images were then imported into the AnalySIS software (Soft-Imaging Systems) and puncta were quantified. For quantifications, thresholds were manually determined for each image prior to binarization, followed by a particle separation filter. The resulting image was added to the original, and particle detection was carried out to measure particle number, defined area, mean intensity, and integral intensity. Data analysis was done using commercially available software (Prism 4 Software, Graph Pad).

2.3 Biochemical methods

2.3.1 Solutions, antibodies used for biochemical procedures

**Homogenization buffer**
320 mM sucrose; 5 mM Hepes-NaOH (pH 7.4); 0.1 mM EDTA; proteases inhibitors (aprotinin, leupeptin)

**Stacking gel buffer (Upper Tris)**
0.5 M tris (pH 6.8); 0.4% SDS

**Separation gel buffer (Lower Tris)**
1.5 M Tris (pH 8.8); 0.4% SDS

**10x SDS-PAGE-running buffer (for 1 L)**
30.2 g Tris-HCl; 144g Glycine; 10 g SDS
Materials and methods

10x Transfer buffer (for 1 L)
31.2 g Tris-HCl; 144 g Glycine; 5 g SDS

1x Transfer buffer (for 100 ml)
10 ml 10x Transfer buffer; 20 ml methanol; 70 ml H2O

Ponseau-S solution (for 1 l):
2 g Ponceau-S; 50 ml Acetic acid; 950 ml H2O

10 x TBS buffer:
1.37 M NaCl; 200 mM Tris-HCl (pH 7.6)

1x TBS –Tween 20 buffer (for 1 L)
100 ml TBS; 900 ml H2O; 1 ml Tween 20

Blocking buffer:
5 % non-fat powder milk in TBS-Tween 20
2.3.2 Primary and secondary antibodies

Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;R α1, guinea pig</td>
<td>Kindly provided by Dr. JM Fritschy</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;R β2/3, mouse</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;R g2, guinea pig</td>
<td>Kindly provided by Dr. JM Fritschy</td>
</tr>
<tr>
<td>Gephyrin (3B11), mouse</td>
<td>Synaptic system</td>
</tr>
<tr>
<td>GluR1, rabbit</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GluR2/3, rabbit</td>
<td>Chemicon</td>
</tr>
<tr>
<td>NL 2, rabbit</td>
<td>Kindly provided by Dr. F. Varoqueaux</td>
</tr>
<tr>
<td>NMDAR1 (54.3), mouse</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>PSD-95, mouse</td>
<td>Abcam</td>
</tr>
<tr>
<td>Synaptophysin (7.2), mouse</td>
<td>Synaptic system</td>
</tr>
<tr>
<td>VGluT 1, guinea pig</td>
<td>Chemicon</td>
</tr>
<tr>
<td>VGluT 2, guinea pig</td>
<td>Chemicon</td>
</tr>
<tr>
<td>VIAAT, rabbit</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Tubulin, mouse</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Anti-GP HRP conjugated</td>
<td>Dianova</td>
</tr>
<tr>
<td>Goat Anti-mouse HRP conjugated</td>
<td>Dianova</td>
</tr>
<tr>
<td>Goat Anti-rabbit HRP conjugated</td>
<td>Acis antibodies</td>
</tr>
</tbody>
</table>

2.3.3 Protein extracts preparation

Brains from NL 2 KO mice were quickly removed from the cut-open skull, brainstem was cut and immediately frozen by immersion in liquid nitrogen and stored at -80ºC. The tissues of selected genotypes were homogenized in homogenization buffer with a glass Teflon homogenisator (homgen<sup>plus</sup>, Schütt) at
setting 1200 rpm. Afterwards the homogenate was aliquoted and stored at -20°C. The protein concentration was measured with the Lowry assay (see in section 2.3.1). For using the protein sample was resuspended in 3x loading buffer (see in section 2.3.1) and boiled at 100°C for 5 min. The boiling denatures the proteins, unfolding them completely.

2.3.4 Protein concentration estimation

The total protein concentration was determined according to Lowry assay, using the total protein kit from Sigma with bovine albumin serum (Sigma-Aldrich, st. Louis MO) as a standard. At first a BSA standard curve with sample of known protein concentration was prepared. A series of dilutions (0, 25, 50, 100, 200, 300, 400µg/ml) were made in replicates of three with a final volume of 50 µl. Protein samples (1-3 µl) were diluted in SDS to 50 µl. Each protein concentration measurement was performed in triplicate. The following step was the transferring of standards and samples into microplate (96 well plate; Sarsdedt Newton Inc., Newton USA). The Lowry reagent (50 µl) was added to each well and incubated for 20 min at RT, which was followed by addition of 25 µl of Folin-Ciocalteu’s phenol reagent. The addition of this reagent leads finally to an intensive blue staining, which was measured by absorbance at a wavelength between 500 and 800 nm. All absorbance estimations were done using a Microplate reader (BioRad). Samples without protein were served as a reference. Analyses and statistics of the standard curve were performed using Sigma Plot software.

2.3.5 SDS-polyacrilamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a common biochemical method for protein separation. According to this method, proteins can be separated based on their molecular weight, as they move through polyacrilamide gel in response to an electric field. Protein samples before being subjected to electrophoresis were mixed with buffer containing SDS and β-mercaptoethanol. SDS mediates the disruption of three-dimensional structure of proteins by breaking non-covalent bonds and the loading
of proteins with negative charges. β-mercaptoethanol breaks disulfide bonds. Protein gel electrophoresis was performed using a minigel vertical apparatus. Glass-plate sandwich was built for preparation of the gel. The glass walls (10.5 x 10) were cleaned, sealed with silicone rubber band and clamped. After assembling the glass-plate sandwich of the electrophoresis apparatus, the stacking and separating gel solutions were prepared.

Table 2-7

<table>
<thead>
<tr>
<th>Solution</th>
<th>Separation gel (10%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA30</td>
<td>2.5 ml</td>
<td>0.325 ml</td>
</tr>
<tr>
<td>Tris buffer pH 8.8</td>
<td>1.875 ml</td>
<td></td>
</tr>
<tr>
<td>Tris buffer pH 6.8</td>
<td></td>
<td>0.787 ml</td>
</tr>
<tr>
<td>dH20</td>
<td>3.125 ml</td>
<td>1.525 ml</td>
</tr>
<tr>
<td>Themed</td>
<td>7.5 µl</td>
<td>3.7 µl</td>
</tr>
<tr>
<td>APS</td>
<td>40 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The separation gel was poured first and the surface was covered with isopropanol, which straightens the surface of the gel. After the gel polymerized (in 30-40 min), isopropanol was removed. The stacking gel was then poured over the separation gel, and the comb of 0.6 mm thickness was inserted. When the stacking gel was polymerized (in 15-20 min), the comb and the silicone rubber band were removed, and the gel was then placed into the gel electrophoresis apparatus and filled with running buffer. Shortly before loading, the samples were boiled. The molecular weight marker (for estimating the molecular weight of unknown proteins) and samples were loaded into the wells of stacking gel using a Hamilton Syringe (Hamilton Company; Reno, Nevada, USA). Power supply was attached and the gel electrophoresis was carried out at 80 Volt until the samples got stacked at the lower border of the stacking gel, and then at 150 Volt for 1-2 Hours.
2.3.6 Western blotting

The protein samples separated from SDS-PAGE can be transferred onto nitrocellulose membranes (Hybond ECL, Amersham), on the surface of which they are accessible to detection with specific poly- or monoclonal antibodies. Transfer of proteins from the gel onto nitrocellulose membrane was done using semi-dry blotting method. The transfer, nitrocellulose membrane and 6 sheets of Whatman filter papers of the same size as a gel were soaked with the transfer buffer for 15 min. The transfer stack was assembled from the anode to the cathode in the following order: 3 sheets of Whatman filter paper, transfer membrane, gel and 3 sheets of Whatman filter paper and during this procedure bubbles were removed. For protein transfer a constant current of 150 mA was applied overnight. Afterwards the blotted membrane was removed and stained with removable Ponceau-S stain for 2 min at RT in order to test the efficiency of protein transfer. It was then distained by washing few minutes in TBS-Tween. In order to inhibit non-specific binding sites of antibodies to proteins, the membrane was first incubated in blocking solution for 1 hour at RT. Afterwards the membrane was incubated with the primary antibody in appropriate dilution in the blocking solution overnight at 4°C. Each blot was stained in parallel for a reference protein (tubulin). After three washing steps for 10 min each with TBS-Tween, the membrane was incubated with HRP-conjugated antibody solution for 1 hour at RT, which binds to the heavy chain of primary antibody, followed by extensive washing steps. HRP coupled to the secondary antibody reduces the hydrogen peroxide and the resulting oxygen oxidizes the luminal, which releases the light. To visualize antigen-antibody reaction, enzymatic chemiluminescence’s detection reagents were used (AceGlow reagents; psqLab biotechnoligie GmbH). The detection reagents were mixed according to manufacturer’s protocol. Briefly, the mixture of Solution A and B (1:1) was poured over the membrane for 1 min at RT. The membrane was placed into dark chamber and the light was detected by a CCD camera, which captures the digital image of the western blot.
3. Results

Neuroligins Determine Synapse Maturation and Function

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2Center for Physiology and Pathophysiology and Center for the Molecular Physiology of the Brain Georg August University Göttingen D-37077 Göttingen Germany
3Department of Cell Physiology ND4 Ruhr University Bochum D-44780 Bochum Germany
4Department of Membrane Biophysics Max Planck Institute of Biophysical Chemistry D-37077 Göttingen Germany
5Center for Basic Neuroscience Department of Molecular Genetics Howard Hughes Medical Institute University of Texas Southwestern Medical Center Dallas, Texas 75390
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Summary

Synaptogenesis, the generation and maturation of functional synapses between nerve cells, is an essential step in the development of neuronal networks in the brain. It is thought to be triggered by members of the neurexin family of postsynaptic cell adhesion proteins, which may form transsynaptic contacts with presynaptic α- and β-neurexins and have been implicated in the etiology of autism. We show that deletion mutant mice lacking neurexin expression die shortly after birth due to respiratory failure. This respiratory failure is a consequence of reduced GABAergic, glutamatergic and glycinergic synaptic transmission and network activity in brainstem centers that control respiration. However, the density of synaptic contacts is not altered in neurexin-deficient brains and cultured neurons. Our data show that neurexins are required for proper synapse maturation and brain function, but not for the initial formation of synaptic contacts.

Introduction

Synapses are asymmetric intercellular contact sites specialized for temporally and spatially precise signal transmission in the brain. They are composed of a presynaptic compartment containing synaptic vesicles clustered around the transmitter release site, or active zone, and a postsynaptic compartment containing the transmitter receptor apparatus. During synaptogenesis in human brain development, about 10^11 nerve cells generate some 10^15 synapses to establish a complex but precise neuronal network.

Synaptogenesis involves two operationally defined and interdependent cell biological processes that are thought to be mediated by adhesion proteins (Barnett, 1995; Yamagata et al., 2003), the initial contact formation between an axonal growth cone and a target neuron, and the maturation of synaptic contacts through specific assembly and stabilization of pre- and postsynaptic proteins at the contact site. Adhesion proteins involved in the initial formation of synaptic contacts are thought to be encoded by large families of genes and to interact with each other in an isoform-specific manner according to a “lock-and-key” principle in order to allow for the cell-type specificity of synaptogenesis (Sperber, 1993). Adhesion proteins that mediate protein recruitment during synaptogenesis, on the other hand, additionally require specific intracellular binding sites for scaffolding proteins, presynaptic active zone components, and postsynaptic receptors (Garner et al., 2002; Kim and Sheng, 2004; Li and Sheng, 2003; Montgomery et al., 2004; Schiefele, 2003; Yamagata et al., 2003).

Several recent studies on the molecular mechanism of synaptogenesis indicated a role of neurexins (NLs) in the formation of synaptic contacts and their maturation (Chali et al., 2006; Chubykin et al., 2005; Dean et al., 2003; Fu et al., 2003; Graf et al., 2003; Levinson et al., 2005; Prange et al., 2004; Schiefele et al., 2003). NLs are type 1 transmembrane proteins and constitute a family of four (in rodents) or five (in higher primates and humans) postsynaptic cell adhesion proteins that interact with presynaptic α- and β-neurexins (NXs) (Boucard et al., 2006; Ichiheno et al., 1995; Song et al., 1999; Varoqueaux et al., 2004) via a large extracellular immunoglobulin-like domain. Intracellularly, NLs bind to several PDZ domain-containing scaffolding proteins, which in turn interact with postsynaptic transmitter receptors, ion channels, and signaling proteins (Ito et al., 1997; Meyer et al., 2004). Similarly, the intracellular domains of NXs, which are also type 1 transmembrane proteins, are indirectly associated with components of the presynaptic transmitter release machinery via interactions with PDZ domain-containing scaffolding proteins (Biederer and Südhof, 2000; Hata et al., 1996).

Expression of NLs in nonneuronal cells induces presynaptic specializations in contacting axons (Schiefele et al., 2000), and overexpression of NLs in cultured
neurons increases the number of synapses formed (Chin et al., 2006; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004), whereas knock-down of NL expression by RNAi leads to a reduction in synapse density (Chin et al., 2005).

Analogous effects were observed upon expression of t-Nx in neuronal cells (Graf et al., 2004; Nam and Chen, 2005). Overexpression and knock-down of t-Nx in cultured neurons causes dramatic changes in synaptic activity, which were explained by the dramatic effects of these manipulations on synaptic numbers (Chin et al., 2005; Graf et al., 2004). Accordingly, the trans-synaptic NL/Nx link is thought to be of key importance in the induction phase of synaptogenesis. t-Nx are thought to trigger synapse formation. In addition, t-Nx were suggested to also play a role in synapse maturation by recruiting scaffolding proteins, postsynaptic receptors, and signaling proteins to nascent synapses. That t-Nx are essential for proper brain function is documented by the fact that loss-of-function mutations in the human NL genes NLGN3 and NLGN4 cause autism and mental retardation (Chin et al., 2004; Comiello et al., 2004; Jaisam et al., 2005; Laumonnier et al., 2004; Talebizadeh et al., 2004).

To determine the role of t-Nx in synaptogenesis and synapse function in vivo, we generated and characterized knockout (KO) mice lacking individual or multiple t-Nx.

Results

Expression of t-Nx Genes in Rodents

The mouse genome contains at least four t-Nx genes: Nlgn1, Nlgn2, Nlgn3, and Nlgn4 (Ichikawa et al., 1996; S. Jernais, F.K., N.B., and T. Bourgeron, unpublished data). We employed quantitative Western blotting with isofom-specific antibodies to determine the absolute levels of the four t-Nx in the brains of newborn and adult mice. In newborn mice, Nlgn4 is not detectable (Table 1), whereas Nlgn1-3 are expressed at moderate levels (NL 1, 66 pg/mL; NL 2, 85 pg/mL; NL 3, 82 pg/mL). In adult mice, only Nlgn4 (14 pg/mL) of the total t-Nx protein in brain is contributed by Nlgn 4, while Nlgn1-3 are expressed at 10- to 30-fold higher levels than in newborn brain (NL 1, 113 pg/mL; NL 2, 170 pg/mL; NL 3, 322 pg/mL). In view of these low levels of Nlgn4, we conducted all subsequent studies on the characteristics of Nlgn1-3 in mouse brain.

The mRNAs of Nlgn1-3 are expressed at low levels in newborn rat brain and upregulated in parallel (2- to 3-fold) during postnatal development (Figure 1A), reflecting the developmental expression patterns seen for Nlgn protein levels in mouse and rat brain, as described above and in previous studies (Song et al., 1996; Varoqueaux et al., 2004). In situ hybridization experiments showed that in both the newborn and adult rat brain, the mRNAs of Nlgn1-3 are coexpressed in almost all neuronal populations (Figures 1B and 1C). Differential expression of Nlgn isoforms is only detectable in the newborn striatum and adult brain, hypothalamus, and thalamus, where relative Nlgn 1 mRNA expression levels are lower than those of Nlgn 2 and Nlgn 3 mRNAs (Figures 1B1 and 1C). In agreement with our previous studies on Nlgn mRNA and protein expression (Song et al.,

| Table 1. Expression Levels of Synaptic Proteins in NL1-3 Triple KO Mice |
|--------------------------|-------------------------|--------------------------|
| Presynaptic Proteins | Postsynaptic Proteins |
| **t-Catenin** | 79 ± 4 | 102 ± 3 |
| **N-cadherin** | 70 ± 2** | 112 ± 3 |
| **Munc13-1** | 103 ± 10 | 71 ± 16 |
| **RIM1A** | 92 ± 12 | 115 ± 11 |
| **PSD-95** | 82 ± 8 | 82 ± 8** |
| **SNAP-25** | 69 ± 10 | 97 ± 3 |
| **syntaxin 2** | 77 ± 3 | 99 ± 1 |
| **synaptophysin 1** | 49 ± 9 | 97 ± 4 |
| **synaptophysin 2** | 78 ± 3 | 83 ± 6 |
| **SNAP25** | 59 ± 10 | 77 ± 1** |
| **VAMP2** | 62 ± 4** | 106 ± 9 |
| **VGLUT2** | 67 ± 2 | 91 ± 4 |

Protein levels in newborn brain homogenates, expressed as percent of wild type levels ± SEM, are listed (Wild type, n = 3; NL1, n = 6; NL2, n = 4; NL3, n = 4; *p < 0.05; **p < 0.01; ***p < 0.001).

1999; Varoqueaux et al., 2004), areas rich in white matter exhibit only background labeling for NL mRNAs, indicating that oligodendrocytes and fibrous astrocytes express NL mRNAs at extremely low levels or not at all (Figures 1B and 1C).

Generation of NL KO Mice

To generate mouse KOs lacking Nlgn1-3, exon sequences covering the transialional start site and at least 380 bp of 5' coding sequence of the respective genes were deleted by homologous recombination in embryonic stem cells (see Figures S1A and S1B in the Supplemental Data available online). In all cases, this targeting strategy eliminates the respective signal sequences and a significant part of the extracellular cysteine-like domain and was therefore predicted to abolish NL expression completely. This was verified by Western blotting of brain homogenates from homozygous KOs using antibodies to the C termini of Nlgn1-3, which showed that neither full-length NLs (Figure S1C) nor truncated variants (data not shown) were expressed in the respective KO mice.

KOs lacking individual Nlgn1-3 and all combinations of double KOs were generated by interbreeding and were obtained at the predicted Mendelian frequencies. All single KOs, all types of double heterozygous mice, and triple heterozygous mice were viable and fertile and behaved normally in the cage environment. Likewise, all homozygous double KO combinations were viable. However, they showed drastically reduced reproduction rates and striking deficits in raising their offspring. As a consequence, breeding of triple KOs lacking Nlgn1-3 was extremely time consuming, even with very large breeding colonies. Nevertheless, triple KOs were obtained at the predicted Mendelian frequency. They had a slightly reduced body weight as compared to single and double KO littermates (Figure S2A), showed irregular and flat breathing movements as determined by whole-body plethysmography (Figures S2B and S2C), and died within 24 hr after birth. Nlgn4 levels were not upregulated in triple KO brains (Table 1).

Currently, subcellular localization data on Nlgn proteins in the brain are only available for Nlgn 1 and Nlgn 2. In the
rodent brain, the main NL1 splice variant is specifically localized to excitatory postsynaptic densities (Song et al., 1999; Chih et al., 2000), whereas the main NL2 splice variant is specifically localized to inhibitory postsynaptic specializations (Graf et al., 2004; Varoqueaux et al., 2004; Chih et al., 2000). However, both NL1 and NL2 alter the density of excitatory and inhibitory synapses when overexpressed in cultured neurons (Graf et al., 2004; Levinson et al., 2006). Some less abundant NL1 and NL2 splice isoforms are targeted to and induce inhibitory and excitatory synapses, respectively (Chih et al., 2000), and knock-down of individual NLs by RNAi or dominant-negative NLs cause comparable reductions of synapse numbers in neuron cultures (Chih et al., 2000; Nam and Chen, 2003). Moreover, the three NLs present in newborn brain, NL1-3, are coexpressed in almost all types of neurons (Figure 1B), and only the triple KO lacking NLs 1-3 but none of the single or double KOs showed a perinatally lethal phenotype. All these findings indicate a significant degree of functional redundancy among NLs 1-3, we studied the phenotype of NL 1-3 triple KOs in more detail.

Brain Cytoarchitecture and Synaptic Protein Expression in NL 1-3 Triple KO Mice

Histological analyses showed that the loss of NL expression in triple KOs had no effect on the gross cytoarchitecture of the newborn brain. The density and layering of neurons in olfactory bulb, cortex, and hippocampus of triple KOs were indistinguishable from those in triple heterozygous controls (Figure 2A). Even brain regions that are mature at birth, such as the brainstem, did not show evidence of cell loss (data not shown).

Analysis of total brain expression levels of selected synaptic marker proteins in triple KOs and wild-type controls by quantitative Western blotting revealed a complex pattern of changes (Table 1). The levels of all tested general, excitatory, and inhibitory synaptic vesicle markers were reduced in triple KO brains. Likewise, the levels of the soluble SNAP25 regulators complex 2 and α-SNAP were significantly lower in triple KOs as compared to controls, whereas the expression levels of the active zone proteins Munc13-1 and Rim12, the SNARE SNAP-25, and calbindin were unchanged. The Ca^2+ channel KCC2 showed reduced expression levels in triple KO brains, while the expression of gephyrin, β-dystroglycan, GABA_A receptor subunits α1 and δ/3, and glycine receptor α subunits, all markers for GABAergic/glycinergic postsynapses, was normal. Upon analysis of markers of glutamatergic excitatory postsynapses, only the expression levels of NMDAR1 were found to be reduced significantly in triple KOs. The levels of PSD-95 and of the glutamate receptor subunits GluR2/3 and NMDAR2A/B were similar in triple KO and control brains. The finding that the levels of integral components of synaptic contacts, such as the active zone markers Munc13-1 and
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Figure 2. Essential Role of NLs in Respiratory Function
(A) Average body weight of NL single (SKO), double (DKO), and triple (TKO) KO mice. (B) Representative ventilation traces and (C) averaged ventilation frequency of different NL mutant mice as measured by whole-body plethysmography. Numbers within the bar graphs indicate the number of mice tested for each genotype. Error bars indicate SEM.

RIM1/2, are not affected in the NL-1-3 triple KO whereas the expression levels of vesicle markers are reduced compatible with the notion that loss of NLs results in aberrant recruitment of synaptic proteins or vesicles but does not cause changes in the number of synaptic contacts (see below).

Synapse Formation and Function in Cultured NL-1-3 Triple KO Neurons

One of the best characterized NL interaction partners is the postsynaptic density protein PSD-95. It binds to NLs via its third PDZ domain (Vie et al., 1997), as well as to K⁺ channels (Kim et al., 1998), NMDA receptor subunits (Komar et al., 1999), and other scaffolding and signaling proteins at its two other PDZ domains and additional binding sites (Zhang et al., 2000; Li and Sheng, 2000; Montgomery et al., 2004; Schnell et al., 2004). PSD-95 also binds to stargazin, which in turn interacts with AMPA receptors (Fukakura et al., 2005; Schnell et al., 2002). Thus, PSD-95 represents an adaptor between synaptic cell adhesion and the postsynaptic signaling apparatus.

Experiments in neuron cultures showed that PSD-95, presumably through its interaction with NLs, and stargazin mediate the synaptic recruitment of glutamate receptors (Bolos et al., 2002; Fukakura et al., 2005; Levinson et al., 2006; Prange et al., 2004; Schnell et al., 2002). To examine the role of NLs in synaptogenesis and the regulation of the glutamate receptor complement at synapses, we studied glutamatergic synaptic transmission in cocultures of neocortical explants and dissociated neocortical target neurons from the same NL-1-3 triple KO mice by whole-cell patch-clamp recording at 13–15 DIV (Mohrmann et al., 1999). Cultures from triple heterozygous littermates served as controls and yielded data that were very similar to those obtained in preparations from an unrelated wild-type mouse strain (Figures 3AB–3B). In this system, the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs), the AMPA and NMDA receptor-mediated contributions to evoked excitatory postsynaptic currents (eEPSCs), and the specific loss of AMPA or NMDA receptors from postsynapses can be measured with high fidelity (Mohrmann et al., 1999) and can serve as readouts for the effects of NL deletion on synaptogenesis and synaptic receptor recruitment. Neither the frequency nor the amplitude of AMPA mEPSCs differed significantly between neurons obtained from triple KOs and heterozygous controls (Figures 3A, 3C, and 3D). These results are in line with recent electrophysiological data obtained in RNAi knock-down experiments on cultured neurons, where a strong reduction of NL-1-3 expression had little effect on AMPA mEPSC frequency or amplitude (Chih et al., 2006). In subsequent experiments, we examined the ratio of evoked NMDA receptor- and AMPA receptor-mediated eEPSCs at the same set of synapses. No significant differences in AMPA eEPSCs, NMDA eEPSCs, or their ratio were found between triple KO neurons and heterozygous control neurons in this culture preparation (Figures 3B and 3E). In contrast, NMDA receptor-mediated, but not AMPA receptor-mediated, synaptic transmission onto CA1 pyramidal cells appears to be perturbed in acute hippocampal slices from NL-1 KO animals (A. Drachby and H. Isacsson, unpublished data).

The electrophysiological features of NL-1-3 triple KO cortical neurons agreed with the morphological characteristics of hippocampal and cortical neurons from these mutants. Immunocytochemistry double staining of the synaptic markers synaptophysin and PSD-95 revealed no differences in synapse densities of neuron cultures from triple KO and control hippocampus (Figures 3A and 3B). Moreover, the ultrastructure of synapses formed by NL-1-3 triple KO neurons was indistinguishable from the triple heterozygous control situation, as assessed by morphometric electron microscopic analysis of cortical explants at DIV 15 (Figures 3C and 3D). These electrophysiological and morphological findings indicate that the initial formation of synaptic contacts does not depend on NLs.

Respiratory Failure in NL-1-3 Triple KO Mice

Because of the apparent synaptogamy of NL overexpression in cultured neurons (Chih et al., 2006; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2006; Nam and Chen, 2006; Prange et al., 2004; Schnell et al., 2003), the progressive role of NLs in the synaptic recruitment of glutamate receptors (Chih et al., 2006; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2006; Nam and Chen, 2006; Prange et al., 2004; Schell et al., 2003), we had initially predicted that KO of all NLs in mouse brain neurons would...
result in decreased synapse numbers and/or aberrant glutamatergic synaptic transmission, but our experimental approaches to test these predictions in cultured neurons failed to uncover phenotypic changes in NL triple KO neurons (Figures 3 and S3). A reason for this finding may be that phenotypic changes induced by NL deletion can be obscured in neuron culture preparations.

We therefore turned to the most obvious phenotypic characteristic of the triple KOs, their early postnatal death, which is most likely caused by respiratory failure, as NL 1-3 triple KOs show flat and irregular breathing movements (Figure 2). We examined the function of the brainstem respiratory network consisting of the pre-Bötzinger complex (PBC) and the hypoglossal nucleus (NH) in acute brainstem slices from triple KO mice by whole-cell patch-clamp recordings (Müller et al., 2003; Zhang et al., 2006). Because this type of analysis required large numbers of triple KO animals and it is practically impossible to obtain sufficient numbers of NL 1-3 triple KOs and wild-type control littersmates, we followed a breeding strategy using mice that were heterozygous for one NL allele (mostly NL 3, some NL 1, no NL 2) and heterozygous for the other. As a consequence, single KO (NL 3 or NL 1, no NL 2) and double KO littersmates (NL 1 and NL 3) had to serve as controls in these experiments. For statistical purposes, the datasets from these two control groups of animals were pooled separately and are referred to as single KO and double KO below. In all cases, the pooled data from single KOs approached wild-type control levels, which were measured in a wild-type line with a similar mosaic genetic background as the NL 1-3 triple KO (data not shown). Essentially, this statistical analysis underestimates phenotypic changes contributed by the KO of NL 1 or NL 3.

We first recorded voltage-activated Na⁺, K⁺, and Ca²⁺ currents in the PBC, which is essential for generating a normal respiratory rhythm. Neither Na⁺ nor K⁺ or Ca²⁺ current densities were significantly altered in NL 1-3 triple KO neurons of the PBC (Figure 6A). To test whether synaptic function is impaired in the brainstem of triple KOs, we next analyzed synaptic transmission in the PBC. Spontaneous post-synaptic currents (sPSCs) measured in whole-cell patch-clamp recordings from PBC neurons revealed a dramatically reduced frequency of total sPSCs as well as of pharmacologically separated GABAergic and glycineergic sPSCs in NL 1-3 triple KOs as compared to single KOs, while sPSC amplitudes were less affected (Figures 4A–4F). Different double KO combinations caused a similar but less pronounced phenotype. Analysis of the amplitude distribution of GABAergic and glycineergic sPSCs showed two distinct populations in single KOs with mean amplitudes of 35 and 100 pA, which most likely correspond to miniature and action potential driven events, respectively (Figure 4F). Deletion of two NLs decreased the high-amplitude population of GABAergic and glycineergic sPSCs by more than 60%, while the number of sPSCs with lower amplitude was only moderately changed (c.20%). In triple KOs, the number of GABAergic and glycineergic sPSCs with lower and higher amplitude were decreased by 85% and 95%, respectively. In an additional series of experiments, we analyzed miniature
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GABAergic and glycinergic mPSCs (mPSCs) in PBC neurons directly. While the frequency of GABAergic/glycinergic mPSCs was reduced by 60% in triple KO cells as compared to single KO control cells, the amplitudes of GABAergic/glycinergic mPSCs were similar in all tested genotypes (Figures 4J–4L).

To examine the effects of the NL1-3 deletion on glutamatergic synaptic transmission in the respiratory brainstem, we monitored pharmacologically isolated spontaneous glutamatergic postsynaptic currents in PBC neurons. In comparison to data from single KO slices, the frequency of glutamatergic sPSCs in PBC neurons was decreased in NL1-3 triple KO slices (Figures 5A and 5B). The amplitudes of glutamatergic sPSCs were similar in all tested genotypes (Figures 5C and 5D). In order to detect differential effects of the NL 1-3 triple KO on glutamatergic and GABAergic/glycinergic synaptic transmission, we determined the relative contribution of glutamatergic sPSCs and GABAergic/glycinergic sPSCs to the total synaptic events in a subset of experiments. In all single KOs, most events were GABAergic/glycinergic sPSCs (80%), and glutamatergic sPSCs contributed only 20% of the total synaptic activity (Figure 5E). This ratio is very similar to that observed in respiratory brainstem slices from newborn wild-type mice (Messier et al., 2003). Deletion of two or three NL1s dramatically changed the balance between glutamatergic and GABAergic/glycinergic sPSCs, with glutamatergic sPSCs contributing 70% and GABAergic/glycinergic sPSCs contributing 30% of all synaptic events in NL1-3 triple KOs (Figure 5F). This change did not result from an increase of total glutamatergic activity, but was rather a consequence of the strong decrease in GABAergic/glycinergic sPSC frequency. When we analyzed miniature glutamatergic mPSCs in PBC neurons directly, we observed a 80% reduction in mPSC frequency in triple KO cells as compared to single KO controls, whereas the amplitudes of glutamatergic mPSCs were similar in all tested genotypes (Figures 5H–5J).

Finally, we examined electrically evoked synaptic transmission in the NH. For this purpose, we stimulated neurons in the vicinity of the PBC and measured synaptic transmission to NH neurons by whole-cell patch-clamp recordings in the presence of CNQX for GABAergic/glycinergic ePSCs, or bicuculline/muscimol for the analysis of glutamatergic ePSCs. Compared to slices from single KOs, the average amplitude of GABAergic/glycinergic ePSCs was strongly decreased in NH neurons from NL1-3 triple KOs, whereas GABAergic/glycinergic ePSC amplitudes in double KO neurons were not changed significantly (Figures 4G and 4H). Despite the fact that we used supramaximal stimulation, the failure rate of GABAergic/glycinergic ePSCs was strongly increased in NL1-3 triple KO neurons as compared to controls (Figure 4I). In contrast to GABAergic/glycinergic
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Figure S, Impaired Glutamatergic Neurotransmission in the Respiratory Centre of the NL 1-3-Trio KO

A) Representative recordings of pharmacologically isolated spontaneous glutamatergic post-synaptic currents (pSCs) in brainstem PRC neurons. Stacks were prepared from littermate NL single (SKO), double (DKO), and triple KO (TKO) mice.

B) and C) Frequency (B) and amplitude (C) of glutamatergic pSCs in PRC neurons.

D) CNQX-sensitive glutamatergic fraction of pSCs, expressed as percent of total pSCs in PRC neurons of NL KO mice.

E) Amplitude distribution of spontaneous glutamatergic pSCs in PRC neurons of NL-KO mice.

F) and G) Reduced evoked excitatory neurotransmission in hypoglossal neurons from NL-KO mice. Sample traces of evoked glutamatergic pSCs (F) and the mean amplitudes of evoked glutamatergic pSCs in response to extracellular stimuli (G) are given.

H) Representative recordings of pharmacologically isolated spontaneous glutamatergic mEPSCs in PRC neurons.

I and J) Frequency (I) and amplitude (J) of glutamatergic mEPSCs in PRC neurons.

Numbers within the bar graphs indicate the number of mice tested for each genotype. Error bars indicate SEM.

sPSCs, the amplitudes of glutamatergic pSCs were not significantly different in NH neurons from single, double, or triple KOs (Figures S8 and S9), and failure rates were similar in all tested genotypes (0.9% ± 2.3%; n = 13, 5.0% ± 5.6%; n = 13, and 4.8% ± 1.2%; n = 5, for single, double, and triple KOs, respectively).

Synaptogenesis in the Brainstem of NL 1-3

Triple KO Mice

The neuronal network of the respiratory brainstem in mice is almost mature at the time of birth, with most synaptic contacts already established. It can therefore serve as an ideal system for the analysis of the consequences of NL deletion on synaptogenesis in the intact brain of newborn NL 1-3 triple KOs.

To examine whether the striking functional deficits in the respiratory brainstem of NL 1-3 triple KOs reflect aberrant synaptogenesis, we performed a detailed morphometric analysis of synapse types and densities at the light microscopic and ultrastructural level. Immunofluorescence staining for neurite sections for the presynaptic marker synaptophysin 1 showed no differences in the overall density of synaptophosphatase terminal in the PBC, the NH, and the neighboring inferior olive when images from control and NL 1-3 triple KO mice were compared. However, the ratio between the number of glutamatergic terminals as assessed by VGlut1/2 immunoreactivity and the number of GABAergic/glutamatergic terminals as assessed by VAAAT immunoreactivity was slightly increased in the PBC and inferior olive, but not in the NH of triple KOs (Figures S6A and S6B). This change was due to a very subtle alteration in the absolute numbers of glutamatergic and GABAergic/glycinergic synapses, which was not statistically significant and became only apparent when the ratios between the numbers of the two types of synapses were analyzed.

Using immunofluorescence staining for inhibitory postsynaptic markers in the PBC, we found a 30% reduction in the number of postsynaptic clusters containing GABAAR clusters but no significant changes in the number of postsynaptic clusters containing gephyrin or GlyR subunits (Figures S6C and S6D). Moreover, the staining intensity of individual GABAAR clusters appeared to be reduced in triple KO preparations (Figure 6D). Ultrastructural analysis of the PBC revealed synaptic specializations whose numbers were similar in NL 1-3 triple KO and control samples (Figures S6E and S6F).

In a final series of experiments, we performed double labeling experiments in order to determine the number of matched and mismatched pre- and postsynaptic glutamatergic (staining for VGlut1/VGlut2 and PSD-95) and GABAergic/glycinergic compartments (staining for VAAAT and gephyrin) in control and NL 1-3 triple KO PBC (Figure 7). We had to use an alternative fixation method for these double staining experiments, i.e., formaldehyde instead of methanol (see Experimental Procedures). As a consequence, the datasets of the single (Figures 6A-6D) and double staining experiments (Figure 7) are not directly comparable. In the case of both, glutamatergic and GABAergic/glycinergic structures, the number of “empty” pre- or postsynaptic structures was high in the newborns PBC but very similar in control and triple KO. Likewise, the number of pre- and postsynaptically double labeled glutamatergic and
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GABAergic/glycinergic synapses was almost identical in control and triple KO PBC. Together with our morphological data on cortical explants and cultured hippocampal neurons from NL 1-3 triple KO brains (Figure 3), these findings indicate that NLs do not control the initial formation of synaptic contacts in the brain as synapse numbers remain unchanged in the absence of NLs. Consequently, the reduced expression levels of synaptic vesicle markers in NL 1-3 triple KO brains (Table 1) are unlikely to be due to prominent changes in synapse numbers, but may rather reflect aberrant protein equipment of synaptic vesicles or changes in total vesicle numbers.

Discussion

The present data show that NLs are essential for proper synapse and brain function but not for the formation of synaptic contacts per se. Loss of NLs results in a dramatic decrease in spontaneous GABAergic/glycinergic activity (Figure 4) and moderately reduced spontaneous glutamatergic activity (Figure 5) in the respiratory brainstem, which causes respiratory failure (Figure 2). The altered GABAergic/glycinergic activity in the respiratory brainstem of triple NL 1-3 KO mice appears to be partly due to altered postsynaptic recruitment of GABA Receptors (Figures 6C and 6D), but a presynaptic dysfunction may also contribute (Table 1). Similarly, a combination of post- and presynaptic perturbations likely underlies the glutamatergic dysfunction in NL 1-3 triple KO brains. Interestingly, NLs influence the balance between glutamatergic and GABAergic/glycinergic transmission in the respiratory brainstem without affecting the total number of synapses (Figures 4-5). Triple NL 1-3 KO mice exhibited an increased ratio of glutamatergic versus GABAergic/glycinergic synapses (Figures 6A-D) and an increase in the ratio of spontaneous glutamatergic versus GABAergic/glycinergic synaptic activity (Figure 6E) in the respiratory rhythm generating brainstem network.

Synaptogenesis and Synaptic Function in NL 1-3 Triple KO Mice

None of our analyses on NL 1-3 triple KO mice yielded data that would indicate a significant role of NLs in the initial formation of synaptic contacts in vivo. Irrespective of the preparation or brain region tested, synapse numbers were not affected by NL loss (Figures 3A, 3B, 6, and 7). Even the change in the ratio between the number of glutamatergic synapses and GABAergic/glycinergic synapses that we observed in the brainstem of NL 1-3 triple KO mice (Figures 6A and 6B) was caused by only very

nucleus (NH) of NL 1-3 triple KO mice plotted as the percentages of glutamatergic and GABAergic/glycinergic synapses observed in the control (Ctrl) (P < 0.001). The total number of synapses was not significantly different in the two experimental groups.

(A) Representative micrographs of the PBC area of control (Ctrl) and NL 1-3 triple KO (TKO) sections after triple labeling for GABAergic/glycinergic (stained for VAAT, red), glutamatergic (stained for VGAT1/2, green), and all presynapses (stained for synaptophysin 1, blue).

(B) Number of GABAergic/glycinergic (white) and glutamatergic (black) synapses in the PBC, inferior olive (ION), and hypoglossal nucleus (NH) of NL 1-3 triple KO mice plotted as the percentages of glutamatergic and GABAergic/glycinergic synapses observed in the control (Ctrl) (P < 0.001). The total number of synapses was not significantly different in the two experimental groups.

(E) and (F) Ultrastructural analysis of the PBC showing synaptic specializations in NL 1-3 triple KO (TKO) and control (Ctrl) preparations.

(F) Quantification of synapse numbers as identified by ultrastructural analysis.

Scale bars, 10 μm (A and D), 250 nm (E). Error bars indicate SEM.
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**Figure 7. Properly Aligned Pre- and Postsynaptic Specializations in the Respiratory Centers of the NL 1-3 Triple KO**

- The left panel shows representative micrographs of the PBC area of control (Ctrl, n = 4) and NL 1-3 triple KO (TKO, n = 2) sections after double labeling for glutamatergic presynapses (stained for VGAT, green) and excitatory postsynapses (stained for PSD-95, red). The right panel shows representative micrographs of the PBC area of control (Ctrl, n = 4) and NL 1-3 triple KO (TKO, n = 4) sections after double labeling for GABAergic/glycineergic presynapses (stained for VAAT, green) and inhibitory postsynapses (stained for gephyrin, red).

- The left panel shows the quantification of isolated and co-localized VGAT/PSD-95 puncta in the PBC of control (Ctrl, white) and NL 1-3 triple KO mice (TKO, black). The right panel shows the quantification of isolated and co-localized VAAT and gephyrin puncta in the PBC of control (Ctrl, white) and NL 1-3 triple KO mice (TKO, black).

The total number of synapses was not significantly different in the two experimental groups. Scale bars, 30 μm. Error bars indicate SEM.

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subtle and statistically insignificant changes in absolute GABAergic/glycineergic (decrease) and glutamatergic synapse numbers (increase) in the range of 15%, without affecting the total synapse number. These findings are not entirely congruent with the effects of NL knockdown in cultured neurons (Chih et al., 2005) and indicate that NLs determine functional parameters of synapses by regulating the recruitment of synaptic proteins (e.g., GABA$\text{\textsubscript{A}}$ receptors; Figures 5C and 6D) or organelles (e.g., synaptic vesicles; Table 1), rather than the generation of synapses per se.

At first glance, our electrophysiological studies in brainstem seem to indicate that deletion of NLs 1-3 affects inhibitory synaptic transmission more strongly than excitatory transmission. However, as mentioned above, the necessity to produce large numbers of NL 1-3 triple KO mice forced us to use single NL 1 and NL 3 KO mice as well as NL 1/3 double KOs as lentiminate controls in our electrophysiological analyses (note that for electron microscopic [Figures 5E and 6F] and Western blot studies [Table 1], wt mice with a similar mosaic genetic background were used as controls, and for light microscopic studies [Figures 3A, 3B, 6A–6D, and 7], mostly triple heterozygous lentiminate controls were used; see Experimental Procedures). Given that the main splice isoforms of both NL 1 (Song et al., 1999; Chih et al., 2006) and NL 3 (F.V. and S. Jamain, unpublished data) are preferentially localized to glutamatergic synapses, our approach is therefore likely to result in an underestimation of phenotypic changes in glutamatergic synapses of NL 1-3 triple KOs. A biological reason for a preferential effect of the NL 1-3 triple KO on GABAergic/glycineergic synapses may be that glutamatergic synapses, in contrast to GABAergic/glycineergic synapses, contain multiple transsynaptic cell adhesion and signaling systems that interact with intracellular scaffolding proteins (Garner et al., 2002; Kim and Sheng, 2004; Li and Sheng, 2003; Montgomery et al., 2004; Schoffeniels, 2003; Yamagata et al., 2003). These may act in parallel with the NL/NX system and may therefore be able to partially mask the effects of NL loss. This type of compensation is likely to be much less efficient in GABAergic/glycineergic synapses.

Nevertheless, our analysis of glutamatergic transmission did reveal significant functional changes in NL 1-3 triple KOs. Most notably, the frequency of glutamatergic sPSCs in the PBC of NL 1-3 triple KOs was reduced, while ePSC amplitudes were similar in all NL KO combinations (Figures 5A–5C), and the number of glutamatergic synapses was unaltered in the brainstem of NL 1-3 triple KOs (Figures 6A–6D, and 7). Evoked glutamatergic ePSC amplitudes in NH were slightly reduced in NL 1-3 triple KOs (Figures 5F and 5G), but cultured cortical neurons from NL 1-3 triple KOs showed no evidence of changes in glutamatergic transmission (Figure S3). Interestingly, our analysis of miniature glutamatergic mPSCs revealed a strongly reduced mPSC frequency but normal mPSC amplitudes in the PBC of NL 1-3 triple KOs (Figures 6H–6J). These findings, together with all other data on brainstem morphology and glutamatergic transmission, indicate that the absence of NLs 1-3 leads to the functional shut-down of a significant number of glutamatergic synapses, hence the strong reduction in sPSC and mPSC frequency (Figures 5B and 5I) with no change in sPSC and mPSC amplitudes (Figures 5C and 5J) or synapse numbers (Figures 6 and 7). This shut-down of synapses may not only be caused by postsynaptic changes but may also be due to a presynaptic dysfunction, a notion that is supported by the finding that expression levels...
of synaptic vesicle proteins are reduced in NL 1-3 triple KO brains (Table 1).

Spontaneous GABAergic/glycinergic sPSC amplitudes in the PBC and evoked GABAergic/glycinergic sPSC amplitudes in NH were both strongly reduced in NL 1-3 triple KOs (Figures 4E and 4H). Together with the observation that the number of postsynaptic clusters containing GABA<sub>B</sub> or Gly receptors were reduced while the number of postsynaptic clusters containing gephyrin or Gly receptors were lower than 1000 (Figures 6C and 6D) and the numbers of inhibitory synapses as determined by double labeling for VTA and gephyrin (Figure 7) were unaltered in the brainstem of NL 1-3 KOs, these data indicate that GABAergic synaptic contacts are formed initially but do not function properly in the absence of NLSs, possibly due to a deficiency in the recruitment of receptors to GABAergic synapses. As seen with glutamatergic synapses, GABAergic/glycinergic mPSC frequencies are reduced but mPSC amplitudes are unaltered in NL 1-3 triple KO PBC (Figures 4J-4L). This observation likely reflects a functional shut-down of a subpopulation of GABAergic synapses due to the loss of postsynaptic receptors (Figures 6C and 6D) and/or a presynaptic dysfunction, such as a partial loss of synaptic vesicles or their aberrant equipment with proteins (Table 1).

The dramatic decrease in overall spontaneous synaptic activity in the respiratory brainstem of NL 1-3 triple KOs (Figures 4A, 4B, 5A, and 5B) is likely to be caused by the combined glutamatergic and GABAergic synaptic dysfunction. GABAergic and glycinergic transmission in the newborn respiratory brainstem is still largely excitatory (Schiller and Zhang, 2000). Consequently, a combined reduction in glutamatergic and GABAergic synaptic transmission will result in reduced excitatory drive and overall spontaneous synaptic activity. Changes in neuronal excitability do not appear to contribute to the decreased network activity in NL 1-3 triple KOs, as neuronal Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> currents were normal in these mice (Figure S4). Given that NLSs do not regulate initial synaptogenesis per se, the increase in glutamatergic versus GABAergic/glycinergic synapse ratios observed in the PBC and in the brainstem of NL 1-3 triple KOs may partly be due to homeostatic adaptations that occur as a consequence of the strongly reduced network activity.

Redundancy among NLSs
At first glance, the fact that all single NL KOs as well as all combinations of NL double KOs are viable whereas NL 1-3 triple KOs die shortly after birth may indicate a significant degree of functional redundancy among NLs 1-3, which even extends to the differentially localized dominant splice isoforms of NL1 and NL2. However, an analysis of NL 2 distribution in NL 1 single KO neurons showed that the main NL 2 splice variant is not recruited to glutamatergic synapses when NL 1 is absent (P.V. and N.B., unpublished data), demonstrating that these NLs do not simply replace each other ecotopically if one of them is eliminated. In view of these findings, we propose that the perinatal lethality of the NL 1-3 triple KOs is due to a synthetic lethal effect of the combination of the three NL single KOs, where subtle mutant synaptic phenotypes accumulate to cause the failure of essential brain networks, such as the respiratory system, and death.

NL KO Mice versus NL Knock-Down and Overexpression in Cultured Neurons
The present study shows that NLSs are dispensable for the initial formation of synapses in cultured neurons (Figure 3) and in the intact brain (Figures 6 and 7). This finding is in agreement with data obtained in x-NK KOs, where loss of all three x-NLSs has very little effect on overall synapses densities (Mistler et al., 2003) but, as mentioned above, does not entirely agree with results obtained in experiments involving NL overexpression or RNAi knock-down of NLs in cultured neurons (Dean et al., 2003; Gnst et al., 2004; Chih et al., 2005; Levinson et al., 2006; Nam and Chen, 2006; Prange et al., 2004; Schieflfler et al., 2005). To a certain degree, the discrepancy between the present data on NL 1-3 triple KOs and the data obtained in NL overexpression experiments can be formally dismissed because the overexpression data document that NLs are sufficient to trigger synaptogenesis in vitro whereas our present KO study documents that they are not necessary for this process in vivo. However, even in view of these considerations, certain aspects of the discrepancy between the present data on NL 1-3 triple KOs and the data obtained after RNAi knock-down of NLSs in cultured neurons remain. For example, even the knock-down of a single NL causes strong reductions in synapse numbers of cultured neurons in vitro (Chih et al., 2005) whereas KO of all NLS expression has no effect on synapse numbers in cultured neurons in vitro (Figure 3) or in the intact brain in vivo (Figures 6 and 7).

We propose that one explanation for this discrepancy may be an indirect and activity-dependent homeostatic effect rather than a direct effect of NL RNAi knock-down on synaptogenesis in cultured neurons. Such activity-dependent homeostatic regulation of synapse densities has been described in numerous studies (Turrigiano and Nelson, 2004) and occurs even when activity changes are very small. For example, synaptophysin 1-deficient neurons, which show no electrophysiologically measurable functional deficit (McMahon et al., 1996), generate fewer synapses than wild-type neurons in coculture (Tans and Geda, 2000), indicating that even subtle functional differences between neurons cause a competition situation, in which functionally disadvantaged neurons maintain their synapses less efficiently. A similar situation may arise in the published cell culture experiments involving RNAi knock-down of NLSs (Chih et al., 2005), where knock-down after plasmid transfection results in a mixed population of transfected and untransfected neurons that differ with respect to their NL expression and that are therefore also likely to differ functionally with regard to synaptic transmission. Neurons with different NL expression levels in this type of mixed culture compete with each other for the same target neurons. Differences in the numbers of synapses formed by these neurons may then be secondary to differences in synaptic efficiency and synapse maintenance rather than caused by a genuine synaptogenic effect of NLSs. The notion that the decrease in synapse density after knock-down of NLSs in cultured neurons (Chih et al., 2005) may indeed be due to an activity-dependent homeostatic effect rather than an interference with a genuine synaptogenic role of NLSs is supported by the fact that knock-down of NLSs leads to a reduction in
the number of glutamatergic synapses without affecting the frequency or amplitude of spontaneous glutamatergic synaptic events. This finding indicates that knock-down of NLs in cultured neurons affects mainly large and inactive synapses, which might even be unique to cultured neurons.

An alternative explanation for the discrepancy between the present data on NL 1-3 triple KO s and the data obtained after RNAi knock-down of NLs in cultured neurons (Chih et al., 2006) could be that off-target effects of the RNAi constructs cause perturbations of synapse stability. A recent publication showed that retraction of synapses and dendritic spines are induced by such off-target effects of RNAi (Alvarez et al., 2008). However, Chih et al. (2006) employed state-of-the-art RNAi control experiments in their study, and their findings are therefore very unlikely to be due to off-target artifacts.

It is also possible that the difference between the present study and the published cell culture experiments on NLs, which indicated that NL levels in neurons determine synapse densities (Chih et al., 2005; Dean et al., 2003; Gherini et al., 2006; Kharchenko et al., 2006; Nam and Chen, 2006; Prange et al., 2004; Scheiffele et al., 2007), are due to specific compensatory effects in the NL 1-3 triple KO s in vivo, e.g., through upregulation of other cell adhesion systems. However, we regard this possibility to be unlikely for the following reasons. First, the expression of NL 4, which would be the ideal compensator of a loss of NLs 1-3, is not upregulated in NL 1-3 triple KO s (Table 1). Second, dramatic homeostatic compensatory changes in postsynaptic protein expression are also known to occur in cultured cells in vitro (Eilers, 2003). If the loss of NLs in the triple KO mice were compensated by other synaptic cell adhesion processes, this occluding an effect of NL deletion on synaptogenesis, the same compensatory mechanisms should be relevant in cultured neurons, because the time that typically passes between overexpression or knock-down of NLs and the subsequent analysis of cultured neurons (5-6 days) is similar to the time that passes between neurogenesis and synaptogenesis in the developing brain in utero. Likewise, functional redundancy among different synapse cell adhesion systems is a rather unlikely explanation for the finding that the KO of NL s has no effect on synaptogenesis in the brain, while RNAi knock-down of NL s reduces synapse densities in cultured cells, because the same synaptic adhesion systems are likely to operate in the two preparations.

A final possibility that needs to be considered is the context of a possible role of NLs in synaptogenesis and in view of the discrepancies between our data on NL 1-3 triple KOs and the published literature on the putative synaptogenic function of NLs in cultured neurons is that the deletion of NLs in mice does not prevent initial synaptogenesis but rather delays it. While such an effect may have been missed in our analysis of synapse densities in the brainstem, which is almost mature at birth, analyses of the immature hippocampus of newborn NL 1-3 triple KO pups also failed to detect altered synapse densities (data not shown). These data indicate that even in early phases of synapse formation the lack of NLs does not affect synapse numbers in the intact brain.

NLs and Autism

Autism is a developmental disorder that is defined behaviorally by aberrant language acquisition, perturbed social interactions, and repetitive or ritualistic behaviors. Moreover, mental retardation and epilepsy are often associated with autism. The most consistent neuroanatomical finding in autistic patients is a tendency to unusually large brains with a disproportionate contribution of white matter to the increased brain volume (Herbert, 2003). In addition, functional abnormalities such as aberrant information processing or disturbed neuronal connectivity have been postulated to contribute to the autistic phenotype (Herbert, 2006; Pellegrino and Lauter, 2004).

Studies on affected twins and the disproportionately high number of male patients with autism led to the consensus that autism is one of the most heritable psychiatric diseases. Indeed, loss-of-function mutations in the human NL genes NLGN3 and NLGN4 cause rare monogenic heritable forms of autism (Chih et al., 2004; Comioli et al., 2004; Jhamra et al., 2003; Laumonnier et al., 2004). Consequently, the NL 3 KO mice generated in the course of the present study could become a useful genetically defined animal model of autism. The phenotypic changes in NL triple KO s described here are compatible with current hypotheses about the functional deficiencies in the brains of autistic patients, according to which "autism might be caused by an imbalance between excitation and inhibition in key neural systems" (Pellegrino and Lauter, 2004). However, detailed behavioral analyses will be required to assess the extent to which NL 3 KO mice model the spectrum of behavioral and cognitive phenotypes that are typically associated with autism in humans.

Experimental Procedures

Absolute NL Protein Quantification

Brains from newborn and adult mice were homogenized in 320 mM sucrose, and protein concentrations were adjusted to a final concentration of 2 mg/ml. Defined amounts of NL-T1,-NLs-1,-NL2,-NL3, or NL4 fusion proteins, including the epitopes of the isoform-specific antibodies to the different NLs, were run on SDS-PAGE gels in parallel with the brain homogenates. Fusion proteins and native NLs were detected on Western blots with isoform-specific polyclonal antibodies raised against 14 amino acid peptides, followed by an 14C-labeled secondary antibody. After exposure to Biomax film (Kodak), bands were scanned and signal strength analyzed by densitometry (Elite).
homogenized in 320 mM sucrose and adjusted to a final protein concentration of 2 mg/mL. 20 μl per lane was run on SDS-PAGE gels and blotted onto nitrocellulose membranes. Several proteins (Table 1) were detected with specific primary antibodies and fluorescence-labeled secondary antibodies and quantified on an Odyssey fluorescence reader (Li-Cor). Each blot was stained in parallel for a reference protein (actin or tubulin) and protein levels were expressed as the ratio over the reference protein.

Immunostaining and Light Microscopic Quantification
Braains from newborn control and NL-1-3 triple KO pups were removed and either frozen by immersion in N2-cooled isopentane at -80°C or immersion-fixed overnight in 4% paraformaldehyde, cryoprotected in sucrose, and frozen on dry ice. Serial frontal cryo-

Electrophysiological Recordings in Brainstem Slices
All electrophysiological analyses were performed on thalamus neurons of mice whose genotype was unknown to the experimenter. Acute slices containing the pre-Bötzinger complex (PBC) and hypoglossal nucleus (NII) from newborn littermate mice were used for whole-cell recordings. The bath solution in all experiments consisted of (in mM) 118 NMD, 3 KCl, 1.5 MgCl2, 25 NaHCO3, 1 Na2HPO4, 5 glucose, pH 7.4, saturated with 95% O2 and 5% CO2 and held at 28°C-30°C. Evoked glutamatergic and GABAergic glycineergic PSCs were recorded from hypoglossal neurons in the presence of 1 μM strychnine and 1 μM bicuculline or 10 μM CNX, respectively. PSCs were evoked by 0.1 Hz field stimulations of axons of interneurons close to the PBC using a bipolar platinum electrode. An isolation unit BioFlex (A.M.P.L.I.) with a custom-built power supply was used to apply currents of supramaximal stimulation strength (around 100 μA current near the slice as confirmed by current measurements). The patch electrode for evoked glutamatergic and GABAergic glycineergic PSCs measurements contained (in mM) 140 KCl (guanidinium glutamatergic PSCs), 1 CaCl2, 10 EGTA, 2 MgCl2, 4 Na2ATP, 0.5 Na3GTP, 10 HEPES, pH 7.3. Peak amplitudes of PSCs were averaged from 25 consecutive responses. To monitor changes in input resistance, current responses to a -10 mV voltage step (20 ms) from a holding potential of -70 mV were recorded before every fifth stimulus. In all experiments the distance between the stimulation and recording electrodes was similar between slices of different genotypes. Spontaneous GABAergic glycineergic and glutamatergic PSCs were recorded from neurons of the PBC at a 0.2 Hz renewal potential of about 0 mV in 10 μM CNX or 1 μM strychnine and 1 μM bicuculline, respectively. Signals of amplitudes of at least two times above background noise were selected, and statistical significance was tested in each experiment. Spontaneous GABAergic glycineergic and glutamatergic PSCs were recorded as described above, but in the presence of 0.5 μM tetrodotoxin (TTX). Signals with amplitudes of at least two times above background noise were selected, and statistical significance was tested in each experiment. There were no significant differences in noise levels between different genotypes. Voltage-activated currents were measured from neurons of the PBC with patch electrodes containing (in mM) 110 CsCl, 30 TEA-Cl (for Na+ and Ca2+ current), or 140 KC (for K+ current). 1 CaCl2, 10 EGTA, 2 MgCl2, 4 Na2ATP, 0.5 Na3GTP, 10 HEPES, pH 7.3, and with 0.5 μM tetrodotoxin and 200 μM CdCl2 for blocking Na+ and Ca2+ current in the bath solution. After establishing the whole-cell configuration, membrane capacitance, series resistance, and membrane resistances were estimated from current transient induced by 20 mV hyperpolarization voltage commands from a holding potential of -70 mV. The serial resistance was compensated by 80%, and patches with a series resistance of <30 MΩ, a membrane resistance of >10 GΩ, or leak currents of >100 pA were excluded. The membrane currents were followed by a four-

Electrophysiological Recordings in Dissociated Cortical Neurons
Cultures of thalamus and dissociated neocortical target neurons of newborn NL-1-3 triple KO and triple heterozygous littermates were prepared as described (Mohrmann et al., 1998). After 2 weeks in vitro, recordings were performed in neurons from NL-1-3 triple KO and triple heterozygous animals. AMPA receptor-mediated miniature postynaptic currents (AMPA mPSCs) were recorded at -60 mV holding potential in the presence of 1 mM Mg2+, 20 μM bicuculline methiodide, and 1 μM TTX. To increase the frequency of AMPA mPSCs an elevated K+ concentration (30 mM) in the extracellular solution was used. Glutamate receptor-mediated PSCs were evoked by local extracellular stimulation using wide-

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Supplemental Data
The Supplemental Data for this article, including four Figures, can be found online at http://www.neuron.org/cgi/content/full/51/6/744/DC1.

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Supplemental Data

Neuroligins Determine Synapse

Maturation and Function

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Figure S1. Targeting Strategy and Detection of the NL 1, NL 2, and NL 3 Deletion Mutations

(A) Representation of the targeted region of the murine NL genes, targeting vectors, and mutated genes resulting from homologous recombination. The location of the probes used for Southern analysis of genomic DNA is indicated by white bars. In all three cases, exons (ex) sequences covering the respective translational start site and 546 bp, 380 bp, and 574 bp of 5' coding sequence of NL 1, NL 2, and NL 3, respectively, were targeted for deletion by homologous recombination in embryonic stem cells.

(B) Southern blot analysis of genomic DNA from different NL genotypes. DNA was digested with Bgl II (for NL1 and NL3) or EcoR1 (for NL2). Bands representing the wild type (WT) and KO alleles are indicated on the left. Arrows indicate triple KO genotypes.

(C) Western blot analysis of adult brain homogenates from different NL genotypes. Blots were probed with isoform-specific polyclonal antibodies to the C-termini of the indicated NLs. NL 1 and NL 3 comigrate at about 116 kDa while NL 2 is slightly smaller.
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Figure S2. Normal Brain Cytoarchitecture in the Absence of NLs

Micrographs of Nissl-stained sections taken at the level of the olfactory bulb (A), the neocortex (B) and the hippocampus (C) illustrate the normal organization of the newborn brain in the NL 1-3 triple KO (TKO) as compared to the control triple heterozygous (Ctrl) brain. Scale bar: 180 μm (A), 20 μm (B), 60 μm (C).
Figure S3. Intact Glutamatergic Synaptic Transmission in Cultured Neocortical Neurons of NL 1-3 Triple KOs

Representative example traces of AMPA receptor-mediated miniature mPSCs (A) and pharmacologically isolated AMPA and NMDA receptor evoked ePSCs (B) in wild type (WT), littermate triple heterozygous control (THz) and triple KO (TKO) neurons. Neither the mean frequency (C) nor the mean amplitude (D) of AMPA mPSCs differ significantly between neurons from the different experimental groups. Furthermore, no significant differences in AMPA ePSCs, NMDA ePSCs nor in their ratio (E) were observed. Numbers within the bar graphs indicate the number of neurons tested for each genotype.
Figure S4. Unaltered Voltage-Activated Channel Function in NL 1-3 Triple Kos

A

SKO
DKO
TKO

Na⁺ current density (pA/pF)

1 nA
4 ms

SKO 12
DKO 12
TKO 7

B

SKO
DKO
TKO

K⁺ current density (pA/pF)

2 nA
20 ms

SKO 2
DKO 9
TKO 5

C

SKO
DKO
TKO

Ca²⁺ current density (pA/pF)

100 pA
20 ms

SKO 16
DKO 17
TKO 24

The left panels show representative whole-cell recordings of voltage-activated Na⁺- (A) K⁺- (B) and Ca²⁺-currents (C) obtained from neurons in the PBC. The right panels show the corresponding bar diagrams of averaged current densities in NL 1-3 KO and control mice. Numbers within the bar graphs indicate the number of mice tested for each genotype.
3.1 Ventilation patterns measured by whole-body plethysmography

In contrast to early postnatal death of NL 1-3 triple KO mice, NL 2 KO mice are viable, fertile and behaviorally normal in the cage environment. However, using whole-body plethysmography method we observed changes in the breathing pattern that did not interfere with survival of the mice. For this recordings postnatal day 1 (P1) to P3 mice were used. The respiratory rhythm was measured as frequency and coefficient of variation of ventilation. The sample traces (Fig. 3.1A) show irregular respiration pattern in neuroligin 2 mutant mice. The coefficient of variation of ventilation (CV) was significantly decreased in NL 2 KO mice (0.12±0.018 in control mice to 0.28±0.06 in KOs; p<0.001; Fig 3.1C), whereas the ventilation frequency was slightly reduced as compared to control mice (3.2±0.26 Hz in control animals to 2.6±0.32 Hz in KOs; ns; Fig. 3.1B). These results indicate that the lack of single neuroligin gene has no significant, but influencing role on breathing of animals at P1-P3 day.

3.2 Synaptic transmission in brainstem preBötzinger complex from neuroligin 2 mutant mice

Considering the fact that mice lacking neuroligin 1-3 genes die after birth within few hours most likely caused by breathing disturbances, we found quite reasonable to examine the function of the brainstem respiratory network, which is essential for normal breathing. The neuronal network of respiratory system is almost mature at the time of birth, which was a perfect system for us to investigate the function of already established many synapses. We examined the synaptic function of brainstem respiratory network performing experiments in acute brainstem slices of NL 1-3 triple KO newborn mice consisting of preBötzinger complex (PBC) and hypoglossal nucleus (NH), using whole-cell patch clamp recordings. The whole data set from these recordings showed the strong impairment of synaptic activity in the respiratory brainstem of NL 1-3 KO mice (Varoqueaux, Aramuni, et al., 2006).
These results turned us to observe the role of single neuroligin gene in synaptic function. For this purpose the mice lacking neuroligin 2 were used for further experiments. Viability of these mice gave us a possibility to perform experiments at different postnatal ages. For different experiments postnatal day 0 (P0) to P5 pups were used. Before experiments the genotypes were unknown to experimenter.
3.2.1 Decreased spontaneous postsynaptic current in brainstem PBC neurons of NL 2 mutant mice

NL 2 being a one of the heterotypic cell-adhesion molecules of neuroligin family has its unique important role in synaptic activity, despite the structural similarities to other members of this family (Ichtchenko et al., 1996, Irie et al., 1997). To test weather synaptic function is impaired in the brainstem of NL 2 KO mice, we first thought to examine the total spontaneous synaptic transmission using whole-cell patch clamp recordings. Recording the spontaneous postsynaptic currents from PBC neurons of control and NL 2 KO mice, we found significant reduction in both sPSC frequency (5.22 ± 0.077 Hz in control to 1.7 ± 0.082 Hz in NL 2 KO mice; p<0.0001; Fig. 3.2B) and amplitude (91.8 ± 0.97 pA in control cells to 55.8 ± 0.83 pA in KOs; p<0.0001; Fig. 3.2C) in NL 2 mutant mice compared to controls.

3.2.2 Impaired inhibitory synaptic transmission in NL 2 KO mice

3.2.2.1 Spontaneous inhibitory postsynaptic current

We next analyzed inhibitory (GABAergic/glycinergic) synaptic transmission in brainstem acute slices, containing PBC neurons. As we had initially predicted according to many previous studies (Varoqueaux et al., 2004, Chih et al., 2005, Prange et al., 2004) that neuroligin 2 is localized to inhibitory synapses in mammalian brain, inhibitory synaptic function in mice lacking NL 2 was strongly impaired. Recordings of spontaneous GABAergic/glycinergic postsynaptic currents were performed in the presence of 10 μM CNQX and 10 μM AP5, in order to block glutamatergic synaptic transmission. The deletion of NL 2 gene dramatically decreased the frequency of inhibitory spontaneous PSCs by more than 60 % (6.2 ± 0.07Hz in control to 1.95 ± 0.07 Hz in NL 2 KO in mice; P<0.0001; Fig. 3.3B), and the amplitude was diminished about 30 % (85.06 ± 0.58 pA in control to 59.6 ± 1.18 pA; P<0.0001; Fig. 3.3C).
**3.2.2.2 Miniature spontaneous inhibitory postsynaptic currents**

The following step was to analyze the properties of pharmacologically isolated spontaneous miniature GABAergic/glycinergic postsynaptic currents (minis) in the preBötzinger complex in acute brainstem slices from NL 2 mutant mice using whole-cell recordings in the presence of 10 μM CNQX, 10 μM AP5 and 0.5 μM tetrodotoxin (a Na⁺ channel blocker, which blocks action potential dependent network activity). Similar to frequency and amplitude of inhibitory sPSCs, the deletion of neuroligin 2 gene caused a dramatic decrease in frequency (1.38 ± 0.11 Hz in control to 0.29 ± 0.079 Hz in KO cells; p<0.0001; Fig. 3.4B) and moderate reduction in amplitude (61.58 ± 2.08 pA in control to 42.75 ± 2.41 pA in KO mice; p<0.0001; Fig. 3.4C) of spontaneous miniature GABA/glycinergic
Figure 3.3 Impaired spontaneous GABA/glycinergic postsynaptic currents in NL 2 mutant mice. Representative recordings (A) of pharmacologically isolated spontaneous inhibitory postsynaptic currents in PBC neurons. Frequency (B) and amplitude (C) of sIPSCs in brainstem preBötzinger neurons are significantly decreased in NL 2 mutant mice compared to control mice. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.

postsynaptic currents. In these experiments the strong decrease of frequency and amplitude of spontaneous minis in NL 2 KOs as compared to control cells could indicate general defect in the presynaptic terminal function and altered properties of postsynaptic receptors.
3.2.2.3 Hypertonic sucrose application

For eliciting hypertonic responses (Rosenmund and Stevens, 1996, Schlüter et al., 2004) sucrose was ejected to the neuron with 500 mbar for 500 ms. We recorded miniature inhibitory synaptic transmission induced by 300 mM sucrose in PBC neurons of littermate control and NL 2 KO mice in presence of 10 µM CNQX, 10 µM AP5 and 0.5 µM TTX. Hypertonic sucrose directly stimulates the secretory apparatus independent of Ca$^{2+}$ (Rosenmund and Stevens, 1996). Deletion of neuroligin 2 moderately decreased the frequency of miniature inhibitory postsynaptic currents induced by 300 mM sucrose.
Figure 3.5 Release of GABAergic/glycinergic mPSCs by application of hypertonic sucrose. (A) Representative traces of miniature GABAergic/glycinergic postsynaptic currents after direct application of 300 mM hypertonic sucrose with 500 mbar for 500 s. After sucrose application the total frequency of mIPSCs and duration of synaptic responses (B) is diminished in NL 2 mutant compared to control mice. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.

sucrose (Fig. 3.5A, B). Comparing the frequency of mIPSCs induced by hypertonic sucrose between control and NL 2 mutant cells, we saw that the averaged frequency after sucrose application was decreased 14 % in KO mice
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(13.06 ± 0.48 Hz in control to 11.31 ± 0.54 Hz in NL 2 KO mice; p<0.01). Analyzing the frequency of sucrose mediated mIPSCs during three minutes of recording we found the following: the duration of whole-cell synaptic responses elicited by hypertonic sucrose was shorter in NL 2 KO mice, except this the response to sucrose started earlier in NL 2 KO cells compared to controls.

3.2.3 Deletion of NL 2 causes severe reduction of GABAergic synaptic transmission

3.2.3.1 Decreased spontaneous GABAergic PSCs in NL 2 KO mice

The important role of GABAergic and glyciner gic inhibitions in stabilizing the rhythmic network activity is indisputable (Richter and Spyer, 2001), malfunction of which shows caused the early postnatal death of NL 1-3 triple KO mice. In accordance with this we examined the role of NL 2 in inhibitory synaptic transmission and observed its importance in inhibitory function, but arose then the question whether GABAergic or glyciner gic inhibition is affected in mice lacking neuroligin 2. To solve this question we performed recordings of GABAergic sPSCs in PBS neurons in brainstem acute slices having 10 µM CNQX, 10 µM AP 5 and 1 µM strychnine in extracellular solution. Deletion of NL 2 dramatically decreased the frequency of GABAergic sPSC by more than 80% from 6.12 ± 0.11 Hz in control to 0.94 ± 0.16 Hz in NL 2 deficient mice (p<0.0001; Fig. 3.6B), while the amplitude was reduced 28 % in NL 2 KOs mice (54.22 ± 0.54 pA in control cells to 39.27 ± 1.7 pA in KO cells; p<0.0001; Fig. 3.6C).

3.2.3.2 Impaired spontaneous miniature GABAergic PSCs in NL 2 KO mice

To examine GABAergic minis we performed experiments in PBC neurons in presence of additional 0.5 µM tetrodoxin (TTX) in extracellular bath solution. Similar to frequency and amplitude of GABAergic sPSCs (Fig. 3.6B, C), the frequency of GABAergic miniature postsynaptic currents was reduced about
Figure 3.6 Impaired spontaneous GABAergic neurotransmission in the respiratory brainstem of the NL 2 KO mice.
(A) Representative traces of pharmacologically isolated spontaneous GABAergic postsynaptic currents in brainstem PBC neurons. Slices were prepared from littermate control and NL 2 KO mice. The lack of neuroligin 2 leads to strong reduction of frequency (B) and amplitude (C) of GABAergic sPSCs in PBC neurons. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.

80 % (1.07 ± 0.13 Hz in control cells to 0.23 ± 0.03 Hz in KOs cells; p<0.0001; Fig. 3.7B) and the amplitude was diminished 29 % in neuroligin deficient mice as compared to controls (60.53 ± 2.08 pA in control to 43.06 ± 1.7 pA in NL 2 mutant mice; p<0.0001; Fig. 3.7C).
3.2.3.3 Application of GABA\textsubscript{A}-receptor agonist muscimol by pressure ejection

The reduction in amplitude of spontaneous and miniature GABAergic postsynaptic currents caused by NL 2 deletion was one of the reasons to perform further experiments with direct application of GABA\textsubscript{A}-receptor agonist muscimol. To block glutamatergic and glycinergic postsynaptic currents these experiments were carried out in presence of 10 µM CNQX, 10µM AP5 and 1µM strychnine (in extracellular solution), respectively. TTX (0.5 µM) was added into bath solution to block action potential network activity. Direct pressure application of the 5 mM muscimol with 500 mbar for 500 ms to the brainstem neuron elicited different responses in control and NL 2 mutant mice (Fig.3.8A, B). The response evoked
after muscimol application was decreased in NL 2 KO mice by more than 60% (6.22 ± 0.51 nA in controls to 2.30 ± 0.6 nA in KOs; p<0.0001; Fig 3.8B) as compared to controls.

### 3.2.4 Glycinergic synaptic transmission is affected in NL 2 KO mice

#### 3.2.4.1 Deletion of NL 2 affects spontaneous glycinergic postsynaptic currents

As mentioned above the glycine also plays an important role in rhythmic network activity. Regarding to this and as well as to our results of ventilation recordings by whole-body plethysmography we next examined the glycinergic synaptic transmission in the same brain area. The recordings of spontaneous glycinergic postsynaptic currents were carried out in presence of 10 μM CNQX, 10μM AP5, and in order to inhibit GABAergic postsynaptic currents we added 1μM bicuculline into the bath solution. The frequency and amplitude of spontaneous glycinergic postsynaptic currents were decreased markedly. In NL 2 KO mice the
frequency of glycineergic sPSCs compared to frequency of spontaneous GABAergic postsynaptic currents (Fig. 3.6 A, B) was affected less, about 60 % (7.38 ± 0.14 Hz in control to 2.76 ± 0.14 Hz in mutant mice p<0.0001; Fig. 3.9B), whereas in both cases the amplitude reduction was almost the same about 29 % (68.97 ± 0.89 pA in controls to 48.72 ± 1.03 pA; p<0.0001; Fig. 3.9C; 3.6C).

### 3.2.4.2 Miniature glycineergic postsynaptic currents in NL 2 deficient mice

Next we analyzed the glycineergic minis in NL 2 KOs, adding 10 µM CNQX, 10µM AP5, 1 µM bicuculline and 0.5 µM tetroxod in into extracellular solution. The comparison between GABAergic and glycineergic minis showed the following differences. The frequency of glycineergic mPSCs was decreased 60 % (1.07 ± 0.05 Hz in control to 0.4325 ± 0.05 Hz in mutant cells; p<0.0001; Fig. 3.10B), while the frequency of GABAergic minis (Fig. 3.7B) was diminished 20 % more.
In mice lacking NL 2 the reduction of amplitude of glycinergic mPSCs compared to amplitude of miniature GABAergic postsynaptic currents (Fig. 3.7C) was 10 % less (55.56 ± 1.21 pA in control to 46.25 ± 1.85 Hz KO mice; p<0.0001; Fig. 3.10C).

![Image of Figure 3.10 Decreased miniature glycinergic PSCs in PBC neurons of NL 2 KO mice.](image)

3.2.4.3 Direct application of glycine to the postsynaptic neuron

To test whether function of postsynaptic glycine receptors is depressed in mutant mice, we recorded the response to direct pressure application of glycine (5 mM) with 500 mbar for 500 ms to the PBC neuron in the presence of 10 µM CNQX, 10 µM AP5, 1 µM bicuculline and 0.5 µM tetrodotoxin. Pressure ejection of glycine elicited different response in all genotypes (3.11A, B). The synaptic response to
glycine was decreased about 40 % from 6.15 ± 0.25 nA in control to 3.74 ± 0.45 nA in neuroligin 2 mutant mice; (p<0.0001; Fig.3.11B), whereas the synaptic response caused by direct application of GABA<sub>A</sub> – receptor agonist muscimol was reduced by more than 60% (Fig. 3.8B).

Thus, these data revealed reduction of spontaneous and miniature glycinerergic postsynaptic currents, but taken together all our experiments performed in brainstem PBC neurons indicate that deletion of NL 2 impairs GABAergic synaptic transmission stronger, than glycinerergic synaptic transmission.

Figure 3.11 Direct pressure application of glycine elicits different responses in control and NL 2 KO neurons. (A) Sample recordings and (B) synaptic responses in PBC neurons from littermate control and NL 2 KO mice in presence of 0.5µM tetrodotoxin, bicuculline, CNQX and AP5 after application of 5mM glycine. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.
3.2.5 Unaltered excitatory synaptic transmission in PBC of mice lacking NL 2

In many recent studies performed in neuronal cell cultures and brain tissues has been shown the role of different neureligins in synaptogenesis and synaptic function. Despite some discrepant experimental results gotten by different research groups, there is a main outcome suggesting the potential role of NL 1 at excitatory and for NL 2 at inhibitory synapses.

3.2.5.1 Unaltered spontaneous excitatory postsynaptic currents in NL 2 KO mice

Recordings of excitatory postsynaptic currents in preBötzinger complex of NL2 deficient mice showed completely different picture as compared to GABAergic/glycinergic synaptic transmission. Our experiments with NL 1-3 triple KO mice revealed that deletion of neureligins brings to significant changes in glutamatergic transmission, most probably evoked by lack of neureligin 1 and 3, but not by NL 2, which is concentrated at inhibitory synapses (Varoqueaux et al; 2004; Prange et al; 2004, Chih et al; 2005). As another functional evidence for this may serve our data obtained in acute brainstem slices from NL 2 KO mice, which show unchanged spontaneous and miniature excitatory postsynaptic currents performed in mice lacking NL 2. For this purpose we first examined spontaneous EPSCs in PBC neurons in presence of 1µM bicuculline and 1µM strychnine in extracellular solution to block GABAergic and gycineric, synaptic transmission, respectively. Analyzes of spontaneous excitatory postsynaptic currents revealed no differences in frequency (0.97 ± 0.05 Hz in control to 0.87 ± 0.07 Hz in mutant cells; non significant; Fig. 3.12B) and amplitude (32.81 ± 0.49 pA in control to 31.43 ± 0.6 pA in NL 2 KO mice; ns; Fig. 3.12C) between control and NL 2 deficient mice.
Results

Figure 3.12 Deletion of NL 2 has no effect on spontaneous EPSCs. Sample traces (A) of sEPSCs in control and NL 2 KO mice in presence of 1µM bicuculline and 1µM strychnine. Frequency (B) and amplitude (C) of spontaneous excitatory postsynaptic currents remained unchanged in neuroligin 2 mutant mice compared to controls. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.

3.2.5.2 NL 2 deficient mice have unchanged spontaneous miniature EPSCs

We next monitored pharmacologically isolated spontaneous excitatory postsynaptic currents, blocking action potential dependent network activity. Experiments were performed in brainstem PBC neurons in presence of 1µM bicuculline, 1µM strychnine and 0.5 µM tetrodoxin in extracellular solution. The frequency of mEPSCs was identical with 0.77 ± 0.07 Hz in control and 0.77 ± 0.08 Hz in NL 2 deficient mice (ns; Fig. 3.13B). The amplitude of mEPSCs was slightly increased from 34.97 ± 0.83 pA in control and 37.62 ± 0.81 pA in mutant mice (ns; Fig. 3.13C).
3.2.5.3 The effect of pressure applied glutamate

In this series of experiments we recorded the response to extracellular pressure applied glutamate (5mM). The experiments were performed in presence of 1μM bicuculline, 1 μM strychnine and 0.5 μM tetrodotoxin, which were applied into bath solution. Direct ejection of glutamate (500 mbar, 500 ms) to the postsynaptic PBC neuron in contrast to responses evoked by muscimol or glycine application (Fig. 3.8A, B; Fig.3.11A, B), did not provoke decrease of synaptic response in NL 2 KO mice; rather the response to glutamate in NL2 mutant mice was increased from 1.42 ± 0.28 nA in control to 2.29 ± 0.49 nA in NL 2 deficient mice (ns; Fig. 3.14B).
3.3 Evoked neurotransmitter release in NH of NL 2 deficient mice

After our observation that GABAergic/glycinergic synaptic transmission in brainstem PBC neurons is strongly impaired and glutamatergic transmissions is not affected in mice lacking NL 2, we next examined electrically evoked synaptic responses in completely different group of respiratory brainstem neurons. These experiments were performed stimulating the neurons in the vicinity of the preBötzinger complex and measuring the synaptic transmission in hypoglossal motor neurons by whole-cell patch-clamp recordings. The distance between stimulation and patch electrodes was kept similar on all slices. For these recordings the highest stimulation strength (10 mA) was used.
3.3.1 Lack of neuroligin 2 slightly decreases electrically evoked synaptic transmission in hypoglossal neurons

To analyze evoked neurotransmission in NH, first we recorded evoked postsynaptic currents. In contrast to synaptic transmission measured in brainstem PBC neurons, electrically evoked neurotransmission in hypoglossal neurons was less decreased. The amplitude of evoked synaptic responses was reduced from 491.3 ± 105.9 pA in control to 300.9 ± 70.04 pA in NL 2 mutant mice was (ns; Fig. 3.15B).

Figure 3.15 Electrically evoked synaptic responses in brainstem hypoglossal neurons.
(A) Sample recordings of ePSCs in hypoglossal neurons from control and neuroligin 2 KO cells during field stimulation of axons from preBötzinger complex. Amplitude (B) of ePSCs is decreased in KO as compared to control mice. Deletion of NL 2 significantly increases the failure rate (C) of ePSCs. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.
Despite the fact that we used supramaximal stimulation (10mA), the failure rate of evoked postsynaptic currents was increased in NL 2 KO neurons as compared to controls (0.2% ± 0.2% in control to 6.57% ± 3.67% in NL2 mutant mice; p<0.01; Fig. 3.15C).

3.3.2 Electrically evoked inhibitory neurotransmission is affected in neuroligin 2 mutant mice

Recordings of evoked inhibitory postsynaptic transmission were performed in the NH in presence of 10 µM CNQX and 10 µM AP 5. Deletion of NL 2 caused 57% reduction in the amplitude of eIPSCs from 94.70 ± 15.63 pA in control mice to 40.56 ± 15.57 pA in KOs (p<0.01; Fig. 3.16B). Similar to recordings of evoked PSCs in NH, for these experiments also maximal stimulation length (10 mA) was used. The failure rate was markedly diminished in neuroligin 2 mutant mice (1.66 % ± 1.04% in control to 29.60 % ± 19.46 % in NL 2 KO cells; p<0.01; Fig. 3.16C).
These results show that the lack of NL 2 leads to stronger impairment of inhibitory synaptic transmission in the preBötzinger complex, than in hypoglossal motor neurons. Nevertheless, deletion of NL 2 impairs the GABAergic/glycinergic synaptic function in neuronal network of respiratory brainstem.
3.3.3 Evoked EPSCs is not different between control and NL 2 KO mice

Finally, we examined electrically evoked glutamatergic synaptic transmission in respiratory brainstem. Recordings were done in hypoglossal motor neurons of littermate control and NL 2 mutant mice, having a 1 µM bicuculline and 1 µM strychnine in extracellular solution, in order to block inhibitory synaptic transmission. Analyzing the amplitude of electrically evoked synaptic responses, we detected almost no change between control and NL 2 mutant mice (453.7 ± 116.3 pA in control to 392.8 ± 90.3 pA; ns; Fig. 3.17B), which means that excitatory synapses in respiratory brainstem are functioning normally. The very small failure rate was similar in all tested genotypes (0.33 % ± 0.33% in control to 0.31% ± 0.31% in NL 2 KO mice; ns; Fig.3.17C). These data are in agreement with results obtained from PBC neurons that show no change in amplitude of spontaneous and miniature glutamatergic postsynaptic currents in control and NL 2 knockout mice.
Figure 3.17 Unaltered evoked excitatory neurotransmission in NH of NL 2 KO mice. (A) Sample recordings of pharmacologically isolated evoked excitatory postsynaptic currents (eEPSCs) in brainstem hypoglossal neurons from littermate control and NL 2 KO mice. Unchanged amplitude (B) and failure rate (C) of eEPSCs in hypoglossal neurons from control and NL 2 mutant mice in response to extracellular stimuli. Numbers within bar graphs indicate number of cells and mice tested for each genotype. Error bars indicate SEM.
3.4 Synaptic protein expression and synaptogenesis in brainstem of NL 2 KO mice

3.4.1 The expression of synaptic proteins in brainstem of NL 2 KO mice

To determine the consequences of NL 2 deletion on the expression of different synaptic proteins in brainstem we performed Western blot analyses using brainstem homogenates of littermate control and NL 2 mutant mice at postnatal day 5. The expression of all tested general (synaptophysin, as presynaptic marker), excitatory (VGlut 1/2, PSD-95) and inhibitory (VIAAT, gephyrin) synaptic markers were similar in littermate control and neuroligin 2 KO mice (Fig. 3.18). Likewise, the expression levels of inhibitory (GABAaR a1, GABAaR b2/3, GABAaR g2, GlycR) and excitatory synaptic markers (NMDAR 1, GluR 1 and GluR 2/3) were unchanged in mice lacking NL 2 as determined by Western blot analyses (Fig. 3.18).

![Western blot analysis](image-url)

Figure 3.18 Unaltered synaptic proteins expression in NL 2 KO mice.

Expression patterns of inhibitory (VIAAT, Gephyrin, GABAaR a1, GABAaR b2/3, GABAaR g2, GlycR) and excitatory (VGlut2, PSD-95, NMDAR 1, GluR 1, GluR 2/3) synaptic markers as determined by Western blotting of control (WT) and NL 2 KO (KO) mouse brainstem homogenates at postnatal day 5. Tubulin is used as a loading control.
The finding that the loss of neuroligin 2 does not cause changes in the expression level of excitatory and inhibitory synaptic markers is in agreement with data obtained from morphological experiments (see below).

3.4.2 Synaptogenesis in respiratory brainstem of NL 2 KO mice

3.4.2.1 Unaltered GABAergic/glycinergic synapse numbers in preBötzinger complex of NL 2 KO mice

As described above, our electrophysiological experiments revealed strong impairment of inhibitory synaptic activity in respiratory brainstem of NL 2 KO mice and to examine whether this functional change in mutant animals is due to aberrant synaptogenesis, we performed immunofluorescence double labeling in preBötzinger complex of control and neuroligin 2 KO mice at the postnatal day 5. In order to determine the number of matched and mismatched pre- and postsynaptic GABAergic/glycinergic specializations in control and NL 2 KO mice, the brain sections containing preBötzinger complex were costained for VIAAT (marker for inhibitory presynapses) and gephyrin (inhibitory postsynaptic marker) (Fig.3.19A). Quantification of isolated VIAAT-labeled immunoreactive puncta revealed almost similar number of GABAergic/glycinergic presynaptic structures in control and NL2 KO PBC (20.03 ± 2.09 in control mice to 21.31 ± 4.26 in KOs; ns; Fig.3.19B). The number of GABAergic/glycinergic presynaptic postsynaptic structures was decreased about 27% from 33.8 ± 5.75 in control to 24.52 ± 2.49 in NL 2 KO PBC, (ns; Fig.3.19B), which was not statistically significant. Quantification of colocalized puncta showed that the number of GABAergic/glycinergic synapses is identical in preBötzinger complex of control and NL 2 KO mice (11.1 ± 1.66 in control mice to 10.9 ± 2.2 in KOs; ns; Fig.3.19B). Moreover, analyzing the staining intensity we did not observe any difference between control mice and NL 2 KOs.

These experiments indicate that in the absence of neuroligin 2 the GABAergic/glycinergic synapse numbers remain unchanged.
Figure 3.19 Inhibitory pre- and postsynaptic specializations in PBC neurons. 
(A) Representative micrographs of the PBC area of control (Control, n=5) and NL 2 KO (KO, N=5) sections after double labeling for inhibitory presynapses (stained for VIAAT, green) and inhibitory postsynapses (stained for gephyrin, red). (B) The quantification of isolated and colocalized VIAAT and gephyrin puncta in the PBC of control (Control, white) and NL 2 KO mice (KO, black). Scale bars, 10 µm. Error bars indicate SEM.
3.4.2.2 Deletion of NL 2 leads to the slight increase of glutamatergic synaptic specializations

In a final series of experiments, we performed double immunofluorescence staining for glutamatergic specializations in control and NL 2KO preBötzinger complex in order to examine the role of NL 2 on glutamatergic pre- and postsynaptic compartments. The brain sections containing PBC were colabeled for VGluT 1/2 (markers for glutamatergic presynapses) and ProSAP1 (glutamatergic postsynaptic marker) (Fig.3.20A). Quantification of isolated VGluT 1/2 puncta and ProSAP 1 puncta in PBC revealed no significant changes in the number of pre- and postsynaptic compartments. The number of pre- and postsynaptic structures was slightly increased in NL 2 KO mice as compared to controls from 22.29 ± 3.35 in control to 32.33 ± 6.23 in KO PBC and from 36.98 ± 4.88 in control to 43.57 ± 6.09 in NL 2 KO mice (ns; Fig.3.20B), respectively. Likewise, the deletion of NL 2 caused a slight increase of glutamatergic synapse numbers, which was not statistically significant. The staining intensity was similar in all tested genotypes. Analyzing the ratio between the number of glutamatergic terminals as assessed by VGluT1/2 immunoreactivity and the number of GABAergic/glycinergic terminals as assessed by VIAAT immunoreactivity, we found that it was increased in the PBC of NL 2 KO mice, which was not statistically significant (3.21). This alteration could occur due to small changes in the absolute numbers of glutamatergic and GABAergic/glycinergic synapses. These morphological data obtained from respiratory brainstem of NL 2 KO mice show again that neuroligins are not essential for synapse formation.
Figure 3.20 Glutamatergic pre- and postsynaptic specializations in PBC neurons. 
(A) Representative micrographs of the PBC area of control (Control, n=5) and NL 2 KO (KO, N=5) sections after double labeling for glutamatergic presynapses (stained for VGluT1/2, green) and glutamatergic postsynapses (stained for ProSAP, red). (B) The quantification of isolated and colocalized VGluT1/2 and ProSAP puncta in the PBC of control (Control, white) and NL 2 KO mice (KO, black). Scale bars, 10 µm. Error bars indicate SEM.
Figure 3.21 Altered ratio of glutamatergic versus GABAergic/glycinergic terminals in the preBötzinger complex of NL 2 KO mice. Number of GABAergic/glycinergic (white) and glutamatergic (black) synapses in the PBC of NL 2 KO mice plotted as the percentages of glutamatergic and GABAergic/glycinergic synapses observed in the control (Ctrl).
4. Discussion

Neuroligins are present at postsynaptic terminal and together with their presynaptic α- and β-neurexin binding partners form asymmetric intercellular junctions. Several data obtained from cultured neurons suggest the essential role of neuroligins in synapse formation and synaptic function (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004; Scheiffele et al., 2000). In this present project to determine the role of neuroligins in synaptogenesis and synapse function in vivo, we used neuroligin triple knockout mice (NL 1-3 TKO). For detailed investigation of inhibitory synaptic activity, mice lacking neuroligin 2 (NL 2 KO) were used.

4.1 Neuroligin triple knockout mice

NL triple KO mice have impaired postnatal survival. They die after birth within few hours due to respiratory problems, whereas all single and double KOs are viable. Neuroligin 1-3 triple KOs display irregular and flat breathing movements as determined by whole-body pletismography. They have slightly reduced body weight as compared to single and double KO littermates.

The present data obtained from respiratory brainstem of neuroligin triple KO mice show that all isoforms of neuroligins are not essential for formation of initial synaptic contacts and play an important role in synaptic function. The deletion of all three neuroligins leads to severe decrease in spontaneous inhibitory activity and moderate reduction in spontaneous excitatory activity in the respiratory brainstem, which causes respiratory failure.

4.1.1 Synaptogenesis in mice lacking NLs 1-3

Our detailed morphometric analyses of synapse types and densities at light microscopic and ultrastructural level in hippocampus and neocortical cultured neurons, and respiratory brainstem slices derived from NL 1-3 triple KO mice did not reveal the significant role of neuroligins in initial formation of synaptic contacts. The immunoflourescense double staining of the synaptic markers
synapsin and PSD-95 showed the same synapse densities in control and neuroligin 1-3 triple KO hyppocampal cultured neurons. In addition, the ultrastructural studies of neuron cultures from control and neuroligin triple KO cortex demonstrated no abnormalities in synaptic structure. Different synaptic parameters such as a length of the postsynaptic density (PSD), width of the synaptic cleft and the number of docked vesicles were similar in control and neuroligin 1-3 triple KO mice. In vivo ultrastructural analyses of PBC containing respiratory brainstem slices showed similar numbers of synaptic specializations in NL 1-3 triple KO and control samples. Likewise, the number of pre- and postsynaptically double labeled glutamatergic and GABAergic/glycinergic synapses was almost identical in mutant and control mice. Interestingly, the ratio between the number of glutamatergic synapses and GABAergic/glycinergic synapses was increased in NL 1-3 triple KO mice, which was caused by only small and statistically insignificant changes in absolute GABAergic/glycinergic (decrease) and glutamatergic synapse numbers in the range of 15 %. The imbalance between excitation and inhibition caused by loss of neuroligins supports the hypothesis that neuroligins contribute to the homeostatic mechanism that maintains the E/I balance. The only significant change in NL 1-3 triple KO mice that we have observed was 30 % reduction in the number of GABA\textsubscript{A}R\textalpha{1} containing postsynaptic clusters. These findings are not in agreement with the effects of neuroligin RNAi knock-down in cultured neurons (Chih et al., 2005), where it is shown that the knock-down of even single neuroligin leads to the strong decrease in synapse numbers of cultured neurons in vitro, but are in line with observation obtained from \(\alpha\)-neurexin triple KO mice (Missler et al., 2003), which revealed that the deletion of all three \(\alpha\)-neurexins has little effect on synapse densities. We propose that such a discrepancy between our data on NL 1-3 triple KOs and the data obtained after RNAi knock-down of neuroligins in cultured neurons (Chih et al., 2005) can be explained by activity-dependent homeostatic effect on synaptogenesis (Turigiaano and Nelson, 2004) or it is possible that off-target effects of the RNAi constructs cause perturbations of synapse stability. An alternative explanation for this discrepancy
could be that differences between our data obtained from NL 1-3 triple KO mice and the published cell culture experiments on NLs are due to specific compensatory effects in the NL 1-3 triple KOs in vivo. Thus, the present study shows that even the absence of all three neuroligins is not essential for initial synapse formation and neuroligins determine functional parameters of synapses by regulating the recruitment of synaptic proteins (e.g., GABA$_A$ receptors).

4.1.2 The role of NLs 1-3 in synaptic function

4.1.2.1 Severe impairment of inhibitory synaptic transmission in neuroligin triple KO mice

The present study shows how compulsory are all three neuroligins together in synaptic function and the most obvious phenotypic characteristic of this is the early postnatal death of neuroligin triple KO mice. Because of the necessity to produce large numbers of NL triple knockout mice, we were forced to use NL 1 and NL 3 single and NL 1/3 double KO mice as littermate controls in electrophysiological analyses in NL 1-3 triple KO mice. Our investigations revealed dramatic impairment of inhibitory synaptic transmission. Spontaneous GABAergic/glycinergic sPSC amplitudes in the preBötzinger complex and evoked GABAergic/glycinergic ePSC amplitudes in the NH were both strongly reduced in NL 1-3 triple KO mice. The frequency of spontaneous inhibitory PSCs in PBC neurons was also significantly decreased in triple knockout mice. Together with finding that the numbers of postsynaptic clusters containing GABA$_A$R$_{\alpha 1}$ were reduced, and the numbers of inhibitory synapses were unchanged in the brainstem of NL 1-3 triple KO mice, these data indicate that initial formation of GABAergic synaptic contacts does not depend on neuroligins, but the absence of NLs leads to their dysfunction, possibly due to a deficiency of recruitment of receptors to GABAergic synapses. Interestingly, GABAergic/glycinergic mPSC frequencies in PBC were decreased, whereas mPSC amplitudes remained
unaltered in mice lacking NL 1-3, suggesting that this functional shut-down may be caused by postsynaptic changes and presynaptic dysfunction. These results are in line with recent electrophysiological data obtained from RNAi knock-down experiments performed in cultured hippocampal neurons, where a strong reduction of NLs 1-3 expression decreases the frequency and amplitude of mIPSCs (Chih et al., 2005).

4.1.2.2 Lack of NLs alters excitatory synaptic activity

As known NL 1 (Song et al., 1999, Chih et al., 2006), NL 3 are preferentially localized to glutamatergic synapses and NL 2 is localized to inhibitory synapses (Varoqueaux et al., 2004), but interestingly the loss of neuroligins seem to affect inhibitory synaptic transmission more strongly than excitatory transmission. What could be the explanation of this? A biological reason for a preferential effect of the NL 1-3 triple KO on inhibitory synapses could be explained by the fact that glutamatergic synapses, in contrast to GABAergic/glycinergic synapses, contain multiple transsynaptic cell adhesion and signaling systems that interact with intracellular proteins (Garner et al., 2002; Kim and Sheng, 2004; Li and Sheng, 2003; Montgomery et al., 2004; Scheiffelle et al., 2003; Yamagata et al., 2003). The data obtained from excitatory synaptic transmission in brainstem revealed significant functional changes in mice that had deletion of NLs 1-3. Our analyses of spontaneous excitatory synaptic transmission showed reduction of frequency of sEPSCs in the preBötzinger complex of NL 1-3 triple KOs, while sEPSC amplitudes were similar in all NL KO combinations. The slight decrease of evoked excitatory ePSC amplitudes in hypoglossal neurons of NL 1-3 triple KOs was not statistically significant. Analyzing the miniature glutamatergic PSCs we found strongly reduced mPSC frequency in PBC neurons of neuroligin 1-3 triple KO mice and almost identical mPSC amplitudes in all NL KO combinations. By contrast, electrophysiological data obtained in cultured neocortical neurons showed no differences in AMPA mEPSCs between control and neuroligin triple KO neurons. In addition, the electrophysiological studies in triple-neuroligin
knock-down hippocampal neurons showed that the strong reduction of NLs 1-3 expression has little effect on mEPSC frequency and amplitude (Chih et al., 2005). Such a discrepancy between cultured neurons and acute slices indicate that these changes induced by a lack of neuroligins can be obscured in cultured neurons. These data, together with findings on brainstem morphology and glutamatergic transmission demonstrate that the loss of NLs 1-3 causes the functional shut-down of a large number of glutamatergic synapses, which develops significant decrease in sPSC and mPSC frequency, but does not influence on sPSC and mPSC amplitudes. The reason of these results obtained from respiratory brainstem of neuroligin 1-3 triple KOs could be both postsynaptic changes and presynaptic dysfunction.

4.1.2.3 Na⁺-, K⁺-, Ca²⁺-channels function is not affected in NL 1-3 KO mice

Testing the function of voltage-gated Na⁺-, K⁺-, Ca²⁺-channels in PBC neurons of NL 1-3 triple KO mice, we observed that neither Na⁺- nor K⁺-or Ca²⁺-currents or current densities were significantly altered. These data suggest that the impairment of synaptic transmission in respiratory brainstem of NL 1-3 triple KOs is not due to dysfunction of these voltage-gated channels. Similar to NL 1-3 triple KO mice, voltage-gated Na⁺-, K⁺-currents exhibited not significant changes in PBC neurons of α-neurexin triple KO mice, in contrast to the voltage-dependent Ca²⁺-current, which was strongly reduced in α-neurexin triple KO mice (Missler et al., 2003).

Thus, our data obtained from respiratory brainstem of NL 1-3 triple KO mice demonstrate that the early postnatal death of NL 1-3 triple KO mice could be a result of combination of different subtle synaptic phenotypes of the all three NL single KOs.
4.2 The effects of NL 2 deletion in vivo

After examination the effects of NLs 1-3 in respiratory rhythm generating brainstem network, and finding that the loss of NLs 1-3 results more reduction of inhibitory synaptic transmission than excitatory, next we turned to analyze the inhibitory synaptic activity and synaptogenesis only in the absence of NL 2, using NL 2 KO mice. In this study first time we show the role of NL 2 in synaptic activity in vivo. Our results demonstrate that the lack of NL 2 leads to a very strong impairment of inhibitory synaptic function and does not change the initial synapse formation in the respiratory brainstem. In contrast to a GABAergic/glycinergic synaptic activity glutamatergic synaptic transmission is not altered in absence of neuroligin 2. NL 2 KO mice exhibit an increased ratio of glutamatergic versus GABAergic/glycinergic synapses in preBötzinger complex.

4.2.1 Loss of NL 2 does not alter synaptogenesis in PBC

Despite the high homology with NL 1, which is specifically localized to glutamatergic postsynaptic specializations, NL 2 is localized to inhibitory synapses and is not recruited to glutamatergic synapses, when NL 1 is absent. Some studies show that despite NL 2 is concentrated at inhibitory synapses, it can also associate with postsynaptic proteins from excitatory synapses, including PSD 95 (Graf et al., 2004, Chih et al., 2005). It has been shown that postsynaptic mechanisms and not interactions with presynaptic cell adhesion molecules determine the localization of NL 2 (Graf et al., 2004; Varoqueaux et al., 2004). The studies done in cultured neurons (Chih et al., 2005) revealed that overexpression and knock-down of NL 2 result substantial reduction of both excitatory and inhibitory synapse numbers, demonstrating that NL 2, similar to other members of neuroligin family, is important regulator of synatogenesis. Our present data obtained from NL 2 KO mice are not entirely agree with these results. Our immunofluorescence double labeling experiments in preBötzinger
complex revealed that the number of glutamatergic and GABAergic/glycinergic pre- and postsynaptic structures was not significantly different between control and NL 2 KO mice, as we have also seen in double labeled experiments obtained from NL 1-3 triple KO mice. Likewise, we did not observe statistically significant difference in the number of pre- and postsynaptically double labeled glutamatergic and GABAergic/glycinergic synapses in all tested genotypes. Moreover, the staining intensity was similar in control and NL KO mice. The small change that we observed was the insignificantly increased ratio between the number of glutamatergic synapses and GABAergic/glycinergic synapses in NL 2 KO mice, whereas other studies performed in neuronal cultures (Graf et al., 2004, Chih et al., 2005, Prange et al., 2004) demonstrate that NL 2 affects the E/I ratio. Thus, our data demonstrate that NL 2 is dispensable not only for the initial formation of glutamatergic, but also for GABAergic/glycinergic synaptic contacts in vivo.

4.2.2 Excitatory synaptic activity remains unaltered in NL 2 KO mice

Our analyses of glutamatergic synaptic transmission did not reveal any functional changes in glutamatergic activity in PBC and NH of NL 2 KO mice. The frequencies and amplitudes of spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) measured in preBötzinger neurons were almost identical in control and NL 2 KO mice. Likewise, evoked glutamatergic ePSC amplitudes and failure rates were unaltered in NL 2 KOs. In addition, the responses elicited by direct application of glutamate in presence of tetrodotoxin were not significantly different between control and NL 2 KO mice, and it shows that properties of excitatory postsynaptic receptors are not altered in absence of NL 2. These results together with our morphological data indicate that loss of NL 2 does not alter glutamatergic synapse numbers and glutamatergic synaptic transmission, which supports the hypothesis that other splice variants of neuroligins that are preferentially localized to glutamatergic synapses, are not recruited to inhibitory synapses, when NL 2 is absent.
4.2.3 Deletion of NL 2 strongly impairs GABAergic/glycinergic transmission

As shown in our results obtained from NL 1-3 KO mice, the loss of all three neuroligins has a preferential effect on inhibitory synaptic activity. Investigating the consequences of only NL 2 deletion, we observed strong reduction of total and pharmacologically separated inhibitory synaptic activity in respiratory brainstem. The total spontaneous PSC and spontaneous inhibitory IPSC frequencies and amplitudes were strongly decreased in PBC neurons. Electrically evoked PSC and IPSC amplitudes in hyposglossal neurons were less affected in NL 2 KO mice as compared to controls, indicating that in absence of NL 2 the synaptic activity in PBC neurons is affected stronger than in NH. Our recordings of pharmacologically isolated GABAergic synaptic transmission and glycinergic synaptic transmission revealed that both are significantly reduced in respiratory brainstem of mice lacking NL 2. Comparison between them revealed that GABAergic sPSC frequencies and amplitudes were decreased more severe, than glycinergic sPSC frequencies and amplitudes in NL 2 KO mice. Likewise, the deletion of NL 2 resulted stronger decrease of miniature GABAergic postsynaptic currents compared to miniature glycinergic postsynaptic currents. The large decrease in the frequency of GABA_A and glycine-receptor mediated minis in NL 2 KO mice without a change in numbers of GABAergic/glycinergic synapses could be caused by a general defect in the function of presynaptic terminals, which can support our observation obtained in experiments involving hypertonic sucrose application. The frequency of miniature inhibitory postsynaptic currents elicited by pressure application of hypertonic sucrose was significantly decreased in PBC neurons of NL 2 KO mice as compared to controls. The reduction of GABAergic mPSC and glycinergic mPSC amplitudes in NL 2 KO mice suggests that the decrease in mini amplitude is caused by alterations of properties of postsynaptic receptors. The direct application of GABA_A-agonist muscimol during recording of miniature GABAergic postsynaptic currents elicited decreased response in NL
2 KO mice compared to controls. Likewise, the response evoked by pressure applied glycine was significantly reduced in mice lacking NL 2, but less than response evoked by muscimol.

Taken together our morphological and electrophysiological data demonstrate that NL 2 is not important for initial formation of glutamatergic and GABAergic/glycinergic synaptic contacts in vivo, but it has an essential role for normal inhibitory synaptic function in intact brain, and the deletion of NL 2 impairs the GABAergic synaptic transmission stronger, than glycinergetic transmission in the respiratory rhythm generating network.
5. Summary

This project investigated the role of neuroligins in brainstem respiratory network of NL 1-3 and NL 2 conventional knockout mice. The deletion of all three neuroligins led to a lethal phenotype most likely due to respiratory failure, as we saw in our whole-body plethysmography recordings, whereas mice lacking only NL 2 are viable, fertile and behaviorally normal in the cage conditions. Whole-cell voltage-clamp studies on acute brainstem slices of newborn NL 1-3 KO mice, containing preBötzinger complex (PBC) and hypoglossal nucleus (NH), showed that early postnatal death of these animals is a consequence of impaired synaptic activity in these brainstem centers that control respiration. Our detailed analyses of pharmacologically isolated glutamatergic and GABAergic/glycinergic synaptic transmission revealed dramatic decrease in GABAergic/glycinergic activity and moderately reduced glutamatergic synaptic activity in neuroligin 1-3 deficient respiratory brainstem neurons, which is not due to dysfunction of voltage-dependent Ca\(^{2+}\) channels, while our analyses of Ca\(^{2+}\) current in PBC neurons showed that these channels are functioning properly in NL 1-3 KO mice. In contrast to some recent works performed in neuronal cultures, where authors found that suppression of neuroligins in neuronal cultures does result in a substantial reduction of excitatory and inhibitory synapse numbers, and this change consequently alters synaptic transmission in these neurons, we observed unaltered symmetric and asymmetric synapse numbers in mice lacking all three neuroligins. Interestingly, NL 1-3 triple KOs exhibit an increased E/I ratio and as known, several neurobiological disorders, such as autism is thought to be caused by an increase in the E/I ratio, and considering the potential role of neuroligins in dictating the E/I ratio, neuroligin knockout mice could serve as a useful animal model of neurological diseases.

Regarding the fact that the loss of neuroligins affected inhibitory synaptic transmission more strongly than excitatory, we next investigated in detail the role
NL 2, which is preferentially localized at inhibitory synapses. As it was expected, similar to data obtained from NL 1-3 KO mice neither the excitatory nor inhibitory synapse number was changed in NL 2 KO PBC neurons. Electrophysiological analyses revealed unaltered glutamatergic and dramatically reduced GABAergic/glycinergic transmission. Pharmacological separation of the GABAergic and glycinergic postsynaptic currents showed that both were significantly decreased in NL 2 deficient mice, but the deletion of NL 2 caused a stronger impairment of GABAergic synaptic transmission, than glycinergic synaptic activity.

Taken together, our results indicate that neuroligins are not required for initial synapse formation, but are essential for proper synapse maturation and brain function, and the loss of single NL 2 impairs GABAergic/glycinergic synaptic function, which is not compensated in the presence of other members of neuroligin family.
6. References


References


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Sargsyan V, Stettner GM, **Aramuni G**, Bettler B, Dutschmann M, Zhang W (2007) GABA\textsubscript{B} receptors are not involved in breathing, but are involved in gasping. *Manuscript in preparation*

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