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Cooperative Action of Neuroligins and Brain-Derived Neurotrophic Factor Mediates Presynaptic Maturation

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Hiermit erkläre ich, die Dissertation mit dem Titel "Cooperative Action of Neuroligins and Brain-Derived Neurotrophic Factor Mediates Presynapic Maturation" eigenständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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List of Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
AU	Arbitrary units
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumine
Bsn	Bassoon
CASK	Calcium/calmodulin-dependent serine protein kinase
CAST	Cytomatrix-at-the-active-zone-associated structural protein
CMV	Cytomegalovirus
DABCO	1,4-Diazabicyclo(2,2,2)octane
DIV	Day *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELKS	Protein rich in the amino acids E, L, K, and S
FCS	Fetal calf serum
GABA	Gamma-aminobutyric acid
GAD65	Glutamate decarboxylase 2 (molecular weight 65 kDa)
GBSS	Gey's balanced salt solution
GFP	Green fluorescent protein
GFP-F, EGFP-F	(Enhanced) farnesylated green fluorescent protein
HBSS	Hanks' balanced salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IRES	Internal ribosomal entry site

LatA	Latrunculin A
LIMK1	LIM domain kinase 1
MAGUK	Membrane-associated guanylate kinases
MAP2	Microtubule-associated protein 2
MECP2	Methyl CpG binding protein 2
MEM	Minimum Essential Medium
mGluR	Metabotropic glutamate receptor
mOrange/mOr	Monomeric orange fluorescent protein
mTOR	Mechanistic target of rapamycin or mammalian target of rapamycin
NICHD	Eunice Kennedy Shriver National Institute of Child Health and Human Development
NIH	National Institutes of Health
NL	Neuroligin
NMDA	N-methyl-D-aspartate
NRXN	Neurexin
NT	Not treated (with buffer)
p75NTR	P75 neurotrophin receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PSD-95	Postsynaptic density protein 95
PVDF	Polyvinylidene fluoride
RIM	Rab3-interacting molecule
RIM-BP	RIM-binding protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNP	Single-nucleotide polymorphism

Syt1	Synaptotagmin-1
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine-5-isothiocyanate
TrkB	Tyrosine receptor kinase B
UBC	Ubiquitin C
VSV-G	Vesicular stomatitis virus G glycoprotein

Chapter One: Introduction

Everything we do, every thought we've ever had, is produced by the human brain. But exactly how it operates remains one of the biggest unresolved mysteries, and it seems the more we probe its secrets, the more surprises we find.

– Neil deGrasse Tyson

Our brain is the center of our nervous system – the hub where our conscious and subconscious and our dreams and memories are formed – the conductor of every moment of our lives. To generate a complex idea or perform a fine motor task, we need a high-functioning entity to define all processes involved. Therefore, it comes as no surprise that our brain has billions of interconnected neurons, intertwined in a sophisticated network, with each neuron communicating with up to a thousand other neurons in a highly specialized and precise way. Neurons are highly differentiated polarized cells that, albeit categorized into different subgroups, have a common structure. Each neuron has a cell body (or soma), an axon (a long projection of a neuron conveying information to other neurons), and one or more dendrites (shorter branched projections where input from other contacting neurons is received).

Certain aspects of brain function remain enigmatic, even fundamental ones such as why a certain neuron targets another neuron precisely and not the one next to it or how memories are stored and retrieved. However, we have been very successful at uncovering other processes, and one of the essential aspects that we do understand is that excitation and inhibition have to be balanced for the brain to function normally. This has been shown for diseases such as Alzheimer's disease, autism spectrum disorder (ASD), schizophrenia, epilepsy, etc., in which this balance is disrupted. The question is, what does this mean?

1.1 Neurons and brain connectivity

The brain is made up of billions of neurons. In other tissues and organs, the cells are usually connected with two, maybe four, or five other cells. The striking difference in the central nervous system is that one neuron connects to hundreds of others in a highly specific and precise way. The mechanisms behind this precise wiring are not yet understood and are one of the bigger questions that we need to answer in the long run. What is understood, however, is that neurons are polarized cells that have a receiving end for input from other neurons and a sending end to output signals to other neurons. The receiving processes of neurons are called dendrites and the sending processes are called axons. When an axon contacts a dendrite, a connection

between two neurons is formed called a synapse. Through the formation of many synapses is how the neurons in the brain communicate.

1.2. Synapses

Synapses are highly specialized asymmetric contact sites comprised of a presynaptic site and a postsynaptic site. Both of these specializations are at dedicated membrane regions of the sending (presynaptic) neuron and receiving (postsynaptic) neuron, respectively. In the most common type of synapses – the chemical synapses – electric currents in the presynaptic neuron lead to a series of intricately orchestrated events that prime, dock and release the synaptic vesicles located in the presynaptic bouton. The specialized region in the presynaptic bouton that contains the machinery involved in the priming and docking of synaptic vesicles and the subsequent neurotransmitter release is called the active zone. In electron microscope images, the presynaptic active zone and the corresponding postsynaptic site at excitatory synapses appear electron-dense. The reason for the high electron density is the large amount of pre- and postsynaptic scaffolding molecules. At the presynaptic site, the electron-dense accumulation of scaffolding proteins is termed the cytomatrix of the active zone; at the postsynaptic site, the complex meshwork of scaffolding proteins is called the postsynaptic density. In vertebrates, the cytomatrix of the active zone contains the Rab3-interacting molecules (RIMs), the RIM binding proteins (RIM-BPs), bassoon and piccolo/aczonin, the CAST/ELKS proteins (cytomatrix-at-the-active-zone-associated structural protein; protein rich in the amino acids E, L, K, and S), the liprins, and the Munc-13 (mammalian uncoordinated-13) proteins (Gundelfinger and Fejtova 2012). At the postsynaptic site, the major components of the postsynaptic density are proteins of the MAGUK family (membrane-associated guanylate kinases), such as PSD-95 (postsynaptic density protein 95), and proteins of the ProSAP/Shank family (proline-rich synapse-associated protein; SH3 domain and multiple ankyrin repeats (Verpelli et al. 2012). Figure 1 summarizes the mentioned synaptic proteins and structures depicted within a synapse.

Once the synaptic vesicles have been primed and docked, they can fuse and the neurotransmitters can be released. The binding of the neurotransmitters to the receptors results in the opening of ion channels and hence a propagated electric

current. Depending on the receptors, in this step the ion channels either open directly by the action of ionotropic receptors or indirectly involving the action of second messengers, as is the case with metabotropic receptors. Neurotransmitters can either excite or inhibit the postsynaptic neuron. Since one neuron receives input from hundreds of others, some exciting it and others inhibiting it, ultimately the ratio between those signals will determine what the output signal of that neuron will be. The overall balance of excitation and inhibition will then determine how the brain will function as a whole.

1.3 Impaired excitation/inhibition ratio

An impaired balance of the excitation/inhibition ratio could lead to a number of mental illnesses, with some of them categorized as autism spectrum disorder (ASD). ASD, with a great variability and heterogeneity behind the name, is a group of neurodevelopmental disorders that by latest statistical data are estimated to affect 1 in 54 children (Centers for Disease Control and Prevention, 2016) (Maenner et al. 2020). The number of children diagnosed with ASD nowadays is higher than ever. The reason for this, however, remains unknown. Curiously, because of popular culture, we tend to associate autism only with the possible positive features associated with the rare high-functioning variant of autism – Asperger's syndrome. However, the defining features for ASD are the triad of impaired social interactions, impaired communication, and stereotypical behavior (American Psychiatric Association, 2013).

In recent years, the emerging view is that the impaired balance of the excitation/inhibition ratio leading to ASD can be attributed to impaired synaptic function, with problems occurring mainly during the stages of formation and maturation of synapses (Zoghbi 2003; Dean and Dresbach 2006; Betancur et al. 2009; Bourgeron 2009).

1.4 Stages of synaptogenesis

Garner et al. (2006) proposed to summarize the process of synaptogenesis as a continuum of five steps. The first step is establishing an initial contact between the axon and the dendrite. In the second step, synaptic proteins and synaptic vesicles are

recruited to the nascent synapse. In the third step, a functional synapse is formed by precisely aligning the postsynaptic density to the cytomatrix of the active zone. In the fourth step, the synapse matures structurally and functionally. The fifth and final step involves the maintenance and stabilization of the mature synapse over long periods of time.

An astounding degree of coordination in time and space is required for the correct assembly of a synapse. In terms of space, the building blocks comprising the presynapse and the postsynapse have to be delivered to the exact locations at the cell membrane. For example, the presynaptic scaffolding molecules, the synaptic vesicles, and the presynaptic transmembrane cell adhesion molecules are delivered to the active zone, while the postsynaptic scaffolding molecules, the neurotransmitter receptors, and the postsynaptic cell adhesion molecules are delivered to the postsynaptic density. Both of these regions are exactly opposite each other and cover a stretch of the cell membrane that is only about 500 nm wide. In terms of time, it has been found that synaptic proteins can be localized at the nascent synapse as early as 30 min after the initial contact has been established (Ahmari et al. 2000; Friedman et al. 2000; Bresler et al. 2001; Okabe et al. 2001; Zhai et al. 2001; Bresler 2004). Within this short time frame, a presynapse can release neurotransmitters and the postsynapse can detect them, meaning that all the major components of the release machinery as well as postsynaptic receptors have already been recruited (Buchanan et al. 1989; Ziv and Garner 2001). This precise coordination between the presynapse and the postsynapse remains conserved during the next steps including further recruitment of synaptic vesicles, proteins, and receptors, as the proportions between the synaptic vesicle pool and the size of the postsynaptic density are maintained constant (Waites et al. 2005; Garner et al. 2006).

Three factors play a key role in the coordination of synapse formation and maturation (Garner et al. 2006):

1. Secreted signaling molecules
2. Cell adhesion molecules
3. Neuronal activity

In this thesis, I will focus on the proteins of the neuroligin (NL) family representing cell adhesion molecules and on brain-derived neurotrophic factor (BDNF), a secreted signaling molecule of the neurotrophin family.

1.5 Neuroligins and BDNF

Neuroligins are postsynaptic cell adhesion molecules that play a role in regulating the formation, stabilization, and maturation of synapses. Both increased and decreased levels of neuroligins have been linked to neurodevelopmental disorders such as autism spectrum disorders (Südhof 2008) and neurodegenerative disorders such as Alzheimer's disease (Sindi et al. 2014), which emphasizes their significance for physiological brain development and function. Rodents possess four isoforms (NL1, NL2, NL3, and NL4), while humans possess five isoforms (NL1, NL2, NL3, NL4X, and NL4Y). NL1 is predominantly present at excitatory synapses, while NL2 is predominantly present at inhibitory synapses. NL3 can be found at both excitatory and inhibitory synapses, while NL4 is specifically found at glycinergic synapses. (Varoqueaux et al. 2006; Hoon et al. 2011). Going hand in hand with this selective localization is the observation that the overexpression of NL1 primarily leads to the formation of excitatory synapses, while the overexpression of NL2 primarily leads to the formation of inhibitory synapses (Song et al. 1999; Graf et al. 2004; Prange et al. 2004; Varoqueaux et al. 2004; Chih 2005; Levinson et al. 2005).

The presynaptic ligands of neuroligins are the neurexins. There are three primary isoforms of neurexins in mammals: NRXN1, NRXN2, and NRXN3. However, thousands of variants are formed through alternative splicing (Ullrich et al. 1995). Interestingly, overexpressing neuroligins in fibroblasts leads to the formation of presynaptic specializations in axons that come into contact with these fibroblasts (Scheiffele et al. 2000), and the overexpression of neurexins in fibroblasts in this co-culture system leads to the formation of postsynaptic specializations in dendrites (Graf et al. 2004). In both cases, neuroligins or neurexins induce the formation of

only one side of a synapse in this co-culture system, and it is therefore called a hemisynapse.

In neurons, increasing the expression levels of neuroligins enhances synapse formation along the dendrites of the transfected neurons, while decreasing their expression levels has the opposite effect and leads to a reduction in the number of synaptic boutons (Dean et al. 2003; Prange et al. 2004; Dean and Dresbach 2006; Südhof 2008). The effects of neuroligins on synapse formation are only observed when the expression of neuroligins increased or decreased only in a subset of neurons, while the rest of the neurons remain unaltered (Chih 2005; Kwon et al. 2012; Shipman and Nicoll 2012; Futai et al. 2013; Schnell et al. 2014). This means that their action relies on competition between cells.

In contrast, synapse maturation is affected by altering neuroligin levels both globally and only in subsets of neurons, indicating that neuroligins play a fundamental and essential role in the induction of presynaptic maturation. For example, in global triple-knockout mice lacking neuroligins 1–3, the recruitment of GABA receptors to inhibitory synapses is reduced, perturbing synaptic maturation. In addition, evoked neurotransmission is impaired in GABAergic and glycinergic synapses in these mice (Varoqueaux et al. 2006). Global knockout of NL1 reduces the recruitment of NMDA receptors and thus the strength of glutamatergic synapses (Chubykin et al. 2007). Overexpression of NL1 by stereotaxic lentiviral injection in the hippocampus increases the recruitment of NMDA receptors, the amplitude of NMDA-mediated excitatory postsynaptic currents, and long-term potentiation in acute slices from these mice (Budreck et al. 2013). Similarly, overexpression of NL1 *in vivo* by generating transgenic NL1-knock-in mice increases the size of dendritic spines and enhances the recruitment of presynaptic and postsynaptic proteins (Dahlhaus et al. 2009; Hoy et al. 2013).

How do postsynaptic neuroligins affect presynaptic maturation? Wittenmayer et al. (2009) previously studied which aspects of presynaptic maturation are influenced by NL1 using primary hippocampal neuronal cultures. Overexpressing NL1 enhanced NMDA receptor recruitment in the postsynapse. In addition, it increased synaptic vesicle release probability, the number of presynaptic boutons, and the size of the recycling synaptic vesicle pool. Finally, it made active zones of immature neurons independent of F-actin (Wittenmayer et al. 2009). These are all features of mature synapses, and the results indicate an essential role of NL1 in synapse maturation.

Interestingly, BDNF has similar effects on presynaptic maturation. BDNF is a secreted transsynaptic signaling molecule of the neurotrophin family. It binds to the

neurotrophin receptors TrkB (tyrosine kinase receptor B) and p75NTR (Chao 2003). The most studied gene variation in the BDNF gene is the single-nucleotide polymorphism (SNP) Val66Met. The Val66Met SNP is associated with bipolar and depressive disorders, as well as anxiety, schizophrenia, obesity, and eating disorders (Egan et al. 2003; Hariri et al. 2003). Both increased and reduced serum levels of BDNF have been implicated in neurodevelopmental disorders including ASD (Nelson et al. 2001; Miyazaki et al. 2004; Correia et al. 2010). Moreover, in a mouse model for Rett syndrome (*Mecp2*-null mice), BDNF expression is reduced, leading to impaired BDNF-TrkB signaling (Chang et al. 2006). The best studied functions of BDNF are in enhancing neuronal survival and differentiation, dendritic and axonal differentiation, and axon guidance (Park and Poo 2013). Furthermore, BDNF aids synaptic plasticity during the neuronal wiring in the developing nervous system as well as in the developed nervous system (Park and Poo 2013; Zagrebelsky and Korte 2014). At the synaptic level, BDNF enhances synapse formation (Vicario-Abejón et al. 1998; Tyler and Pozzo-Miller 2001; Kellner et al. 2014) and the recruitment of synaptic vesicle proteins such as synaptotagmin, synaptobrevin, and synaptophysin (Tartaglia et al. 2001). BDNF also increases the size of the readily releasable synaptic vesicle pool and boosts synaptic vesicle recycling and release probability (Collin et al. 2001; Tyler et al. 2006; Shinoda et al. 2014). Considering all these properties of BDNF, I contemplated that BDNF may lead to early presynaptic maturation and play an essential role in neuroligin-induced events during early network development.

Taking into account the similarities between neuroligins and BDNF in terms of increasing synapse number and promoting presynaptic maturation, as well as being implicated in ASD, I proceeded, for the first time, to investigate the possibility that these proteins act in the same pathway. Here, employing specific techniques to examine the state of structural and functional presynaptic maturation, I found that applying recombinant BDNF to immature neuronal cultures and organotypic hippocampal slice cultures emulates the manner in which overexpressing NL1 or NL2 induces presynaptic formation and maturation. Moreover, applying recombinant BDNF reestablished the impaired presynaptic maturation in cultures from NL1 knock-out mice. On the other hand, overexpressing NL1 or NL2 did not reestablish the impaired presynaptic maturation in cultures from mice in which BDNF had been depleted. In fact, depleting BDNF in the cultures that I studied severely inhibited the action of NL1 and NL2 on presynaptic maturation, indicating that the joint action of neuroligins and BDNF is needed to precisely mediate presynaptic maturation.

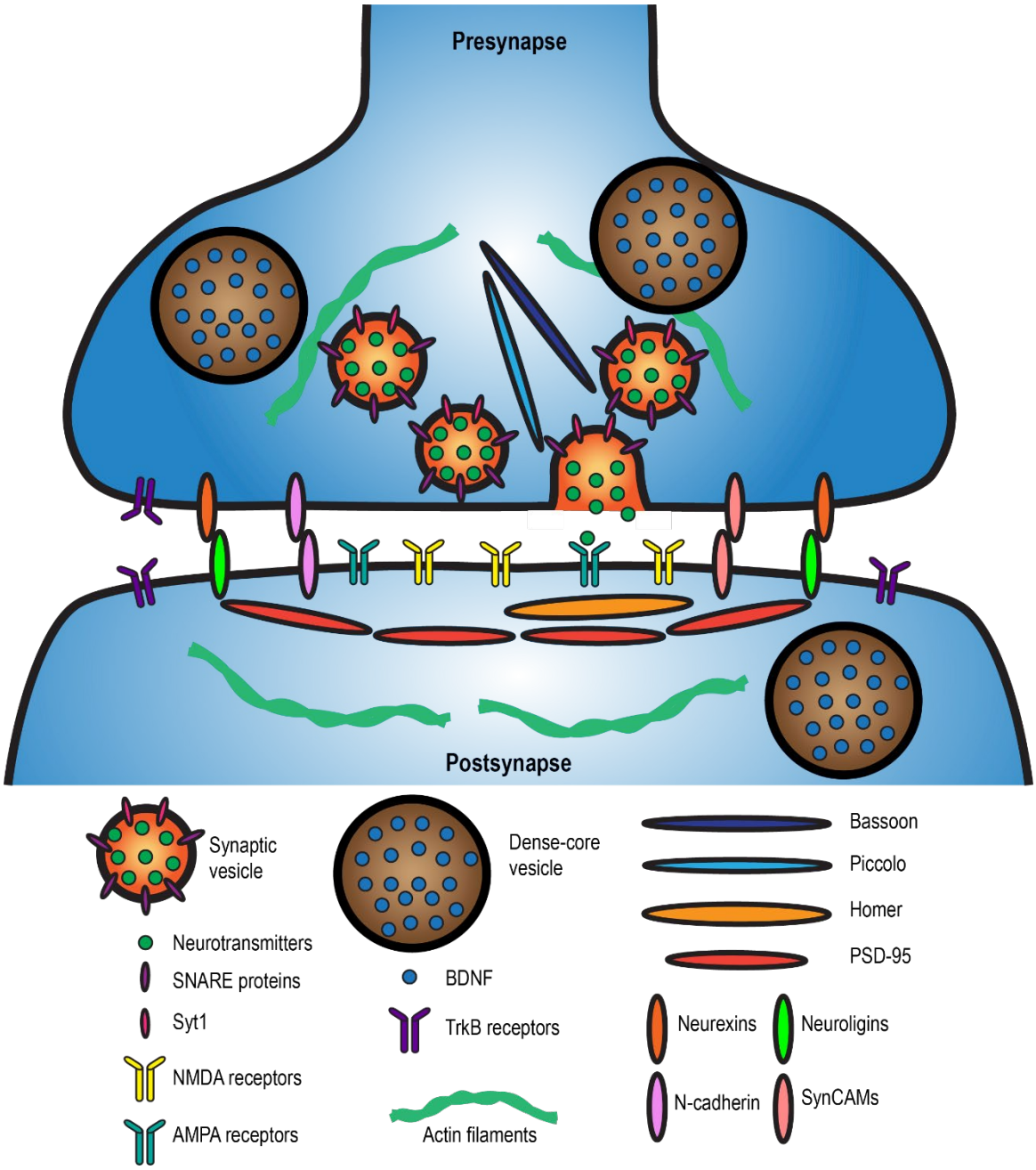

Figure 1: Schematic representation of a synapse. The presynaptic compartment is depicted on the top, and the postsynaptic compartment is depicted on the bottom. At the presynapse, we see some of the proteins at the cytomatrix of the active zone. At the postsynapse, we see some of the proteins at the postsynaptic density. A synaptic vesicle releasing neurotransmitters and a dense-core vesicle transporting BDNF are depicted. In the synaptic cleft, homologous and heterologous interactions of cell adhesion molecules are shown. (Image created using Adobe Illustrator.)

1.6 Motivations and aims of this study

The motivation for this study originates from the emerging view that faulty synaptic maturation may underlie certain psychiatric disorders such as ASD. Knowing how synapses mature to reach a fully functional state is both a fundamental cell biology question and a prerequisite to understanding how such disorders arise and whether they may become treatable.

Both neuroligins and BDNF have been implicated in the etiology of ASD. Here, I introduce BDNF as a potential mediator of NL-induced presynaptic maturation. Using neuronal rat and mouse cultures from either wild-type or genetically modified animals, I explore the link between these proteins.

The main aims of this thesis are as follows:

1. Establish assays for studying structural and functional presynaptic maturation.
2. Investigate the role of NL1, NL2, and BDNF in presynaptic maturation in cultured hippocampal neurons.
3. Investigate presynaptic maturation in a more conserved network by studying organotypic hippocampal slice cultures.
4. Investigate whether BDNF signaling is involved in NL-induced presynaptic maturation.
5. Investigate whether neuroligins binding to neurexins plays a role in inducing presynaptic maturation.
6. Investigate the effect of recombinant BDNF on neurons with impaired maturation from NL1-knockout mice.
7. Investigate the origin of BDNF involved in NL-induced presynaptic maturation.

Chapter Two: Materials and Methods

Table 1: Laboratory equipment

Item	Manufacturer
Bio photometer	Eppendorf
Centrifuge 5810R	Eppendorf
Dumont #3 forceps	Fine Science Tools
Dumont #5/45 forceps	Fine Science Tools
Dumont #7 forceps	Fine Science Tools
Electrophoresis chamber	Bio-Rad
Electrophoresis power supply	Gibco BRL, Life Technologies
Electrophoresis power supply CS	Biocom
Filling instrument 1054/160	Carl Martin, Solingen
Filling instrument LS1054/10A	Carl Martin, Solingen
Freezers	Liebherr
Fridges	Liebherr
Glass beakers	Schott
Glass bottles	Biochrom KG Berlin
Glass Erlenmeyer flasks	Schott
Glass measuring cylinders	Schott
Halogen light source KL 1500	Schott AG
Incubator Hera Cell 150	Thermo Electron Corporation
Laboratory balance Sartorius Extend	Sartorius
Laminar flow hood Hera Safe KS18	Thermo Electron Corporation
Magnetic stirrer MR 3000	Heidolph
Microcentrifuge Mikro200R	Hettich
Microscope Nikon Eclipse TS100	Nikon
Microscope Zeiss Axio Imager Z2, equipped with the following: – ApoTome 2.0	Carl Zeiss Carl Zeiss

Item	Manufacturer
– LED light source Lumencor Sola SE II	AHF Analysentechnik
– Hamamatsu Orca Flash 4.0 V2 Digital CMOS camera	Hamamatsu
– Objective Fluar 5×/0.25 M27	Carl Zeiss
– Objective Plan-Apochromat 10×/0.45 M27	Carl Zeiss
– Objective Plan-Apochromat 20×/0.8 M27	Carl Zeiss
– Objective Plan-Apochromat 40×/1.4 Oil DIC M27	Carl Zeiss
– Objective Plan-Apochromat 63×/1.4 Oil DIC M27	Carl Zeiss
– Filter set DAPI HC BP	AHF Analysentechnik
– Filter set EGFP HC	AHF Analysentechnik
– Filter set Cy3 HC	AHF Analysentechnik
– Filter set Cy5 HC	AHF Analysentechnik
Microscope Zeiss Axio Observer Z1, equipped with the following:	Carl Zeiss
– Definite Focus	Carl Zeiss
– CoolSnap HQ2 CCD camera	Photometrics
– Mercury short-arc reflective lamp	Lighting & Electronics Jena
– Objective Fluar 10×/0.25 Ph1 M27	Carl Zeiss
– Objective Fluar 20×/0.25 M27	Carl Zeiss
– Objective Plan-Apochromat 40×/1.4 Oil DIC M27	Carl Zeiss
– Objective Plan-Apochromat 63×/1.4 Oil DIC M27	Carl Zeiss
– Filter set DAPI HC BP	AHF Analysentechnik
– Filter set EGFP HC	AHF Analysentechnik
– Filter set Cy3 HC	AHF Analysentechnik

Item	Manufacturer
– Filter set Cy5 HC	AHF Analysentechnik
Mini centrifuge	Carl Roth
Neubauer chamber	Marienfeld Superior, Paul Marienfeld GmbH
pH meter pH523	WTW
Pipette Research plus 0.1–2.5 μL	Eppendorf
Pipette Research plus 0.5–10 μL	Eppendorf
Pipette Research plus 100–1000 μL	Eppendorf
Pipette Research plus 10–100 μL	Eppendorf
Pipetus red dot	Hirschmann Laborgeräte
Safe aspiration system Vacusafe	Integra
Semi-dry transfer cell Trans-Blot SD	Bio-Rad
Serological pipette controller Omega Plus	Argos Technologies
Shaker Vibramax 100	Heidolph
Shaker Vortex Genius 3	IKA
Sharp fine scissors	Fine Science Tools
Sharp–blunt surgical scissors	Fine Science Tools
Spectrophotometer NanoDrop 2000	Thermo Fisher Scientific
Standard pattern forceps	Fine Science Tools
Stereo microscope MZ75	Leica
Thermal cycler TProfessional	Biometra
Thermoblock Tb2	Biometra
Thermomixer 5436	Eppendorf
Thermoshaker TS-100	BIOSAN
Tissue slicer	Stoelting
TSX Ultra-Low freezer	Thermo Fisher Scientific
Water bath	Gesellschaft für Labortechnik

Item	Manufacturer
Water purification system Milli-Q	Millipore, Merck
Western blot imaging system Odyssey Fc	LI-COR

Table 2: Consumables

Item	Manufacturer
24-well tissue culture plates	Falcon, Corning Inc.
6-well tissue culture plates	Sarstedt
BD Discardit II syringe, 5 mL	Becton Dickinson
BD Microlance 3, Nr.1, 0.5 × 25 mm	Becton Dickinson
BD Microlance 3, Nr.1, 0.9 × 40 mm	Becton Dickinson
Cell culture dishes, PS, 100/20 mm	Greiner Bio-One
Cell culture dishes, PS, 35/10 mm	Greiner Bio-One
Cell culture dishes, PS, 60/15 mm	Greiner Bio-One
Cell scraper	Sarstedt
Cell strainer 100 μm Nylon	Falcon, Corning Inc.
Cellstar tubes 15 mL, PP, sterile	Greiner Bio-One
Cellstar tubes 50 mL, PP, sterile	Greiner Bio-One
Delicate task wipes	Kimtech Science
Disposable gloves, latex	Starlab
Disposable gloves, nitrile	Labsolute
Filter paper	Bio-Rad
Glass Pasteur pipettes	Brand
Immobilion-FL PVDF membrane	Millipore
Microscope cover glasses 12 mm Ø	Hecht Assistent
Microscope slides	Thermo Fisher Scientific

Item	Manufacturer
Millicell PVDF cell culture inserts	Millipore
Mini-PROTEAN TGX precast gels	Bio-Rad
Parafilm M	Bemis
PCR tubes 0.2 mL	Starlab
Pipette tips 10/20 µL XLTipOne	Starlab
Pipette tips 1000 µL Blue Graduated TipOne	Starlab
Pipette tips 200 µL Yellow TipOne	Starlab
Safe-lock tubes 0.5 mL	Eppendorf
Safe-lock tubes 1.5 mL	Eppendorf
Safe-lock tubes 2.0 mL	Eppendorf
Serological pipette 10 mL	Sarstedt
Serological pipette 25 mL	Sarstedt
Serological pipette 5 mL	Sarstedt
Transfer pipette	Sarstedt

Table 3: Cell culture media, solutions, and reagents

Item	Manufacturer or recipe
2 M $CaCl_2$	2 M $CaCl_2$ in distilled H_2O
2.5 % trypsin	Gibco, Thermo Fisher Scientific
Anisomycin	Tocris Bioscience
B27 supplement	Gibco, Thermo Fisher Scientific
Distilled water	Gibco, Thermo Fisher Scientific
DMEM	Gibco, Thermo Fisher Scientific
DMSO	Sigma-Aldrich, Merck
DPBS	Gibco, Thermo Fisher Scientific
FCS	Millipore, Merck
GBSS	Gibco, Thermo Fisher Scientific
Glucose solution	Gibco, Thermo Fisher Scientific
HBSS	Gibco, Thermo Fisher Scientific
Horse serum	Gibco, Thermo Fisher Scientific
Latrunculin A	Sigma-Aldrich, Merck
L-glutamine	PAN Biotech
MEM	Gibco, Thermo Fisher Scientific
Neurobasal	Gibco, Thermo Fisher Scientific
Nystatin	Sigma-Aldrich, Merck
Opti-MEM	Gibco, Thermo Fisher Scientific
PEI	Sigma-Aldrich, Merck
Penicillin–streptomycin	PAN Biotech
Poly-L-lysine	Sigma-Aldrich, Merck
Recombinant human BDNF	R&D Systems
Recombinant human TrkB-Fc	R&D Systems

Item	Manufacturer or recipe
Transfection buffer	274 mM NaCl, 9.5 mM KCl, 1.4 mM Na_2HPO_4, 15 mM glucose, 42 mM HEPES
Trypan Blue Solution 0.4 %	Thermo Fisher Scientific
Trypsin/EDTA	PAN Biotech
Tyrode's high-K^+ depolarization buffer	640 mM NaCl, 700 mM KCL, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 200 mM HEPES (pH 7.4), 300 mM glucose

Table 4: Other chemicals, reagents, and kits

Item	Manufacturer or recipe
1,4-Diazabicyclo(2,2,2)octane (DABCO)	Merck
10× phosphate-buffered saline (PBS)	Roche
4 % PFA in PBS without K^+	4 % PFA, 20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 9 g/L NaCl, 23.8 g/L glucose, pH 7.4
4-(2-Hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)	Sigma-Aldrich, Merck
4× Laemmli buffer	Bio-Rad
Blocking buffer for primary antibodies	45 mL PBS, 5 mL FCS, 2.5 g sucrose, 1 g BSA, 1.5 mL 10 % Triton X-100
Blocking buffer for secondary antibodies	45 mL PBS, 2.5 g sucrose, 1 g BSA, 1.5 mL 10 % Triton X-100
Bovine serum albumin (BSA)	Sigma-Aldrich, Merck
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Merck
D-glucose	Merck

Item	Manufacturer or recipe
Disodium hydrogen phosphate (Na_2HPO_4)	AppliChem
DNA extraction kit	Nexttec Biotechnologie, GmbH
Glycerin	Carl Roth
L-agarose	AppliChem
Magnesium chloride ($MgCl_2$)	Lab Honeywell
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Lab Honeywell
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma-Aldrich, Merck
Methanol (CH_3OH)	AppliChem
Midori Green Advance DNA Stain	NIPPON Genetics Europe
Mowiol + DABCO	0.2 M Tris HCl, 2.5 % DABCO, distilled H_2O, Mowiol, glycerin
Mowiol 4-88	Calbiochem
Nuclease-free H_2O	Thermo Fisher Scientific
Paraformaldehyde (PFA)	Sigma-Aldrich, Merck
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific
Potassium chloride (KCl)	Sigma-Aldrich, Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Santa Cruz Biotechnology
Protease inhibitors tablets	Roche
Qiagen EndoFree Plasmid Maxi Kit	Qiagen
Socium deoxyholate	AppliChem
Sodium chloride (NaCl)	neoFroxx
Sodium dihydrogen phosphate (NaH_2PO_4)	AppliChem
Sodium dodecyl sulfate (SDS)	AppliChem
Sodium fluoride (NaF)	Sigma-Aldrich, Merck

Item	Manufacturer or recipe
Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth
Sodium orthovanadate	AppliChem
Sucrose	neoFroxx
Taq Green Hot Start Master Mix	Thermo Fisher Scientific
Tris	Carl Roth
Tris HCl	neoFroxx
Tris/glycine buffer	Bio-Rad
Tris/glycine/SDS buffer	Bio-Rad
TRITC phalloidin	Sigma-Aldrich, Merck
Triton X-100	Merck
β-mercaptoethanol	Bio-Rad

2.1 Cell culture

Primary hippocampal neuronal cultures were prepared from E19 (embryonic day 19) Wistar rats, and primary cortical neuronal cultures were prepared from P0 (postnatal day 0) NL1-wild-type and NL1-knockout mice (kindly provided by the lab of Prof. Nils Brose), essentially as described previously (Wittenmayer et al. 2009). The hippocampi were excised from the brains of the rat embryos and neonatal mice. They were placed directly in Hanks' balanced salt solution (HBSS), washed three times with clean HBSS, and then 2.5 % trypsin was added to 2 µL total volume. After a 20 min trypsin digestion at 37 °C and three washes with HBSS, the hippocampi were homogenized using a syringe (Becton Dickinson) and needles of two diameters (Becton Dickinson) – first a needle with a diameter of 0.9 mm and then a needle with a diameter of 0.5 mm. The resulting cell suspension was passed through a cell strainer (Falcon, Corning Inc.) and diluted with Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific) containing 10 % FCS (PAN Biotech), 1 % penicillin-streptomycin (PAN Biotech), and 1 % L-glutamine (PAN Biotech) to a final volume of 10 mL. To count the cells, 10 µL of the cell suspension were mixed with 10 µL of Trypan Blue Solution 0.4 % (Thermo Fisher Scientific) and added to a Neubauer chamber (Marienfeld Superior, Paul Marienfeld GmbH). A density of 55 000 cells/cm^2 was plated on polyethylenimine (PEI)-coated (Sigma-Aldrich, Merck) coverslips in 24-well tissue culture plates (Falcon, Corning Inc.). Twelve hours after plating, the original DMEM solution was exchanged for Neurobasal medium (Gibco, Thermo Fisher Scientific) containing 1 % penicillin-streptomycin (PAN Biotech), 1 % L-glutamine (PAN Biotech), and 2 % B27 supplement (Gibco, Thermo Fisher Scientific).

Primary hippocampal mouse cultures were prepared from C57BL/6J-SV129 mice on E16.5 (kindly provided by the lab of Prof. Martin Korte). Immediately after being taken out, the hippocampi were placed in ice-cold GBSS (Gey's balanced salt solution: 137 mM NaCl, 5.55 mM D-glucose, 4.89 mM KCl, 0.33 mM KH_2PO_4, 1.033 mM $MgCl_2 \cdot 6H_2O$, 0.284 mM $MgSO_4 \cdot 7H_2O$, 2.7 mM $NaHCO_3$, 0.845 mM Na_2HPO_4, and 1.98 mM $CaCl_2$) (Gibco, Thermo Fisher Scientific). After a 30 min digestion in Trypsin/EDTA (Gibco, Thermo Fisher Scientific) and three to four washes with serum medium comprised of DMEM (Gibco, Thermo Fisher Scientific) and 2 % FCS (PAN Biotech), the hippocampi were homogenized and then plated on poly-L-lysine-coated (Sigma-Aldrich, Merck) coverslips at a 70 000 cells/cm^2 density and kept in Neurobasal medium (Gibco, Thermo Fisher Scientific) containing 0.25 % L-glutamine (PAN Biotech), 2 % B27 supplement (Gibco, Thermo Fisher Scientific), and 10 % 10× N2 supplement (Gibco, Thermo Fisher Scientific).

The cultures were incubated at 37 °C and 5 % CO_2.

2.2 Genotyping

To determine the genotype of the P0 mice from the heterozygous crossing of NL1-wild-type and NL1-knockout mice, a small cut of the tail was used. The DNA was extracted using a DNA (deoxyribonucleic acid) extraction kit (nexttec Biotechnologie GmbH), containing Buffer G, Proteinase K, Prep Solution, and nexttec cleanColumns. At first the tail cuts were incubated in a solution containing Buffer G and Proteinase K for 30 min at 56 °C. During that time, the clean columns were equilibrated – they were incubated for 5 min with the Prep Solution. Then they were spun at 350 × *g* for 1 min. After the lysis of the tail cuts was complete, the lysates were transferred to the equilibrated nexttec cleanColumns and eventually spun at 700 × *g* for 1 min.

A list of the primers used for the DNA amplification can be found in Table 5.

The components for the Master mix can be found in Table 6.

The PCR (polymerase chain reaction) program that was used is outlined in Table 7.

In a final step, the amplified DNA fragments were separated using gel electrophoresis. A 2 % L-agarose (AppliChem) gel was prepared and 4 mL of Midori Green Advance DNA Stain (NIPPON Genetics Europe) was added to stain the DNA fragments. The gel was run for 1.5 h at 90 V. An image was acquired using the Odyssey Fc imaging system (LI-COR).

Table 5: Primer set for NL1-knockout/wild-type genotyping

Sequence	Detects
5'–CGGTCAACAAACCTACTCAGAATCAGG–3'	NL1-wild-type mouse allele
5'–CTGAACTCACATGGAGGCAGGATATAA–3'	NL1-wild-type and NL1-knockout mouse alleles
5'–GAGCGCGCGCGGCGGAGTTGTTGAC–3'	NL1-knockout mouse allele

Table 6: Master mix for NL1-knockout/wild-type genotyping

Component	Volume
Taq Green Hot Start Master Mix (Thermo Fisher Scientific)	5 µL
Nuclease-free H_2O (Thermo Fisher Scientific)	10 µL
Primer set (see Table 5)	4 µL (1 pmol/µL of each primer)
Extracted DNA	1 µL

Table 7: PCR program for NL1-knockout/wild-type genotyping

Step	Description
1.	94 °C for 5 min
2.	94 °C for 30 s
3.	64 °C for 30 s
4.	72 °C for 1 min
5.	Goto 2. for 31 cycles
6.	72 °C for 7 min
7.	10 °C forever

2.3 Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared from P3 Wistar rats. The hippocampi were extracted from the brains and placed in a cell culture dish (100/20 mm, Greiner Bio-One) containing slicing medium (HBSS (Gibco, Thermo Fisher Scientific) containing 25 mM HEPES). They were then individually cut into 300 µm slices using a tissue slicer (Stoelting). The intact slices were selected and placed on PVDF (polyvinylidene fluoride) cell culture inserts (Millipore, Merck) and cultivated in culture medium containing 50 % MEM (Minimum Essential Medium, Gibco, Thermo Fisher Scientific) with Glutamax-1 (Gibco, Thermo Fisher Scientific), 24 % HBSS (Gibco, Thermo Fisher Scientific), 36 mM D-glucose (Merck), 25 % heat-inactivated horse serum (Gibco, Thermo Fisher Scientific), 1 % penicillin-streptomycin (PAN Biotech), and 0.06 % nystatin (Sigma-Aldrich, Merck).

2.4 Transfection

Neurons were transfected on DIV2 (day *in vitro* 2) or DIV4 using the calcium phosphate transfection method as described by Thomas Dresbach (Dresbach et al. 2003). In short, the original medium was first substituted with Opti-MEM medium (Gibco, Thermo Fisher Scientific); a mixture containing the plasmid, sterile distilled water, 2 M $CaCl_2$, and the transfection buffer (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM glucose, 42 mM HEPES, pH 7.06) was prepared and incubated for 20 min before being added to the cell medium. The cells were incubated for 60 min and then washed with Neurobasal medium (Gibco, Thermo Fisher Scientific). In the end, the original medium was restored. The following constructs were overexpressed: a farnesylated GFP (green fluorescent protein); an mOrange (monomeric orange fluorescent protein) construct; a NL1 construct containing a GFP tag inserted into the intracellular domain (Dresbach et al. 2004); a newly generated construct containing the NL1 sequence, an internal ribosomal entry site (IRES), and mOrange; a NL1 construct with the L399A/N400A/D402N/K306A mutations (Ko et al. 2009), kindly provided by the lab of Thomas Südhof; a newly generated NL2 construct with a GFP tag attached to the C-terminus, i.e., to the intracellular domain; and a newly generated construct containing the NL2 sequence, an IRES, and mOrange. For the vectors, see Table 8. In addition, a Cre-IRES-GFP construct was co-transfected in cultures form $BDNF^{flox/lox}$ mice. This construct was inserted in a pUBC (ubiquitin C) vector.

Table 8: DNA constructs used

Name	Description
Cre–IRES–GFP	pUBC vector, Cre, IRES, GFP
GFP-F	pCMV (cytomegalovirus) vector, farnesylated GFP
mOrange	pcDNA3 vector, monomeric Orange
Mutated NL1-GFP	pCMV vector, L399A/N400A/D402N/K306A mutations (Ko et al. 2009)
NL1-GFP	pCMV vector, GFP tag inserted in the intracellular domain (C-terminus) of NL1 (Dresbach et al. 2004)
NL1–IRES–mOrange	pCMV vector, NL1, IRES, mOrange
NL2-GFP	pCMV vector, C-terminal GFP-tag
NL2–IRES–mOrange	pCMV vector, NL2, IRES, mOrange

2.5 BDNF application, TrkB-Fc scavenger, F-actin depolymerization, and anisomycin treatment

Recombinant human BDNF (R&D Systems) was added at a concentration of 0.1 μg/mL to non-transfected neurons 22 h before fixation, and 1 % BSA dissolved in sterile PBS (used to reconstitute BDNF) was added to the cell medium in the control condition.

Recombinant human TrkB-Fc chimera protein (R&D Systems) dissolved in sterile PBS was added at a final concentration of 4 μg/mL to inhibit the TrkB receptor on DIV3 (12 h after transfection).

Latrunculin A (Sigma-Aldrich, Merck) dissolved in DMSO was applied to the medium 6 h prior to fixation at a concentration of 2.5 μM (Zhang and Benson 2001). To visualize F-actin, TRITC-labeled phalloidin (Sigma-Aldrich, Merck) was used (1:6000).

Anisomycin (Tocris Bioscience) dissolved in DMSO was applied at a final concentration of 10 μM to the medium 6 h prior to fixation.

2.6 Synaptotagmin-1 antibody uptake

A monoclonal antibody directed against the luminal domain of synaptotagmin-1 (Synaptic Systems) was diluted (1:200) in depolarization buffer (64 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES at pH 7.4, 30 mM glucose) to reach a final dilution of 1:600 in the cell medium. The neurons were then incubated for 5 min and subsequently washed three times with Neurobasal medium (Gibco, Thermo Fisher Scientific).

2.7 Recombinant lentivirus production and infection

The production of the lentivirus was performed in HEK293T (human embryonic kidney) cells (5×10^6 cells in a 10 cm cell culture dish). The cells were co-transfected in serum-free DMEM with 10 μg of the lentiviral expression vector (pUBC-Cre–IRES–GFP) in conjunction with the 7.5 μg of delta 8.9 vector and 5 μg of VSV-G vesicular stomatitis virus G glycoprotein) using PEI (Sigma-Aldrich). The transfection solution was incubated for 30 min at room temperature and then added dropwise to the cells. Between 4 and 6 h after the transfection, the culture medium was replaced by fresh DMEM containing 10 % FCS. Lentivirus was harvested from the cell culture supernatant 48–72 h after transfection and immediately stored at −70 °C. To excise the *Bdnf* gene, primary hippocampal neurons from mice carrying two floxed *Bdnf* alleles were transduced with the Cre-recombinase-expressing lentivirus. The lentivirus-containing medium was added dropwise to the primary neurons in a 1:5 dilution. The transduction was performed 4 h after preparation of the primary hippocampal cultures.

2.8 Western blot

All steps during the sample preparation were performed on ice or at 4 °C. Hippocampal neurons were cultured in a six-well tissue culture plate (Sarstedt). Prior to lysis, the cells were washed three times with cold PBS. Lysates from cultured hippocampal neurons were prepared using a lysis buffer containing the following: 50 mM Tris HCl (neoFroxx), 150 mM NaCl (neoFroxx), 0.1 % Triton X-100 (Merck), 0.5 % sodium deoxycholate (AppliChem), 0.1 % sodium dodecyl sulfate (SDS, AppliChem), 1 mM sodium orthovanadate (AppliChem), 1 mM NaF (Sigma-Aldrich, Merck), and 1 protease inhibitors tablet (Roche). The cells were scraped using a plastic cell scraper (Sarstedt), and the lysate was transferred to a microcentrifuge tube. The

tubes were placed on a shaker at 4 °C for 30 min. After that they were centrifuged at 16000 × *g* in a precooled centrifuge. The supernatant was transferred to a fresh precooled tube, and the pellet was discarded. A small amount of the sample was used to perform a BCA assay to determine the protein content (see Section 2.8). For each sample, 20 µg was mixed with an equal volume of 2× Laemmli buffer (4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol, 0.004 % bromophenol blue, and 0.125 M Tris HCl; Bio-Rad) and boiled at 95 °C for 5 min. After that the samples were centrifuged at 16000 × *g* for 1 min. The samples as well as a molecular weight marker were loaded into the wells of a 4–20 % gradient SDS-PAGE (polyacrylamide gel electrophoresis) gel (Bio-Rad). The gel was run at 100 V for 1 h. The running buffer contained 25 mM Tris, 190 mM glycine, and 0.1 % SDS.

To transfer the proteins from the gel onto a PVDF membrane (Millipore, Merck), the semi-dry blotting technique was used. First, the membrane was washed for several seconds in pure methanol, and then the gel, the filter paper, and the membrane were equilibrated in the blotting buffer for 15 min. The blotting buffer contained 25 mM Tris, 190 mM glycine, 20 % methanol, and 0.1 % SDS. The sandwich was assembled so that the gel is on the anode side and the membrane on the cathode side. The transfer was done at 150 mA for 1 h. After the transfer, the blot was washed three times for 5 min with washing buffer (neoFroxx); the antibody solution for immunodetection containing both primary and secondary antibodies was prepared in Western Froxx pure (neoFroxx). The membrane was incubated in the antibody solution for 1 h on a shaker in the dark. After the incubation, the membrane was washed three times for 5 min with the washing buffer. The primary antibodies used were the following: mouse anti-NL1 (Synaptic Systems) 1:1000, rabbit anti-NL2 (Synaptic Systems) 1:1000, mouse anti-β-III-tubulin 1:1000 (Promega), and chicken anti-β-III-tubulin 1:1000 (Abcam). The secondary antibodies used were the following: goat anti-mouse IRDye 680RD (LI-COR), goat anti-rabbit IRDye 680RD (LI-COR), goat anti-mouse IRDye 800CW (LI-COR), and donkey anti-chicken IRDye 800CW (LI-COR). The membrane was imaged using the Odyssey Fc imaging system (LI-COR).

2.9 BCA assay

The BCA (bicinchoninic acid) assay colorimetrically determines the protein concentration in samples. A pre-formulated kit from Thermo Scientific was used to perform the assay. First, standard samples of fixed concentrations were prepared; then the samples to be measured and the standards were mixed with the working reagent in

a ratio of 1:20. All samples were incubated at room temperature for 2 h. The measurements were taken using the NanoDrop 2000 spectrophotometer (Thermo Scientific). In a first step, the standards were measured as predefined by the software; in the next step, the absorption of the samples was measured against the absorption of the standards. Thus, the protein concentration could be determined.

2.10 Immunocytochemistry and microscopy

Neurons were fixed with 4 % PFA in K^+-free PBS for 20 min. Afterwards, they were permeabilized using PBS with 0.3 % Triton X-100 (Merck), 2 % BSA (Sigma-Aldrich, Merck), 10 % FCS (PAN Biotech), and 5 % glucose (Merck).

The primary antibodies were the following: mouse anti-bassoon (Enzo Life Sciences) 1:1000, guinea pig anti-synaptophysin (Synaptic Systems) 1:500, mouse anti-synaptotagmin-1 (Synaptic Systems) 1:200, chicken anti-MAP2 (microtubule-associated protein 2, Synaptic Systems) 1:1000, and mouse anti-BDNF (monoclonal antibody developed by Y.-A. Barde, obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242, USA) 1:100. They were diluted in PBS with 0.3 % Triton X-100, 2 % BSA, 10 % FCS, and 5 % glucose.

The secondary antibodies were the following: donkey anti-mouse Alexa 647 (Jackson ImmunoResearch) 1:1000, donkey anti-mouse Cy3 (Jackson ImmunoResearch) 1:500, goat anti-guinea pig Alexa 647 (Jackson ImmunoResearch) 1:1000, and goat anti-chicken Alexa 488 (Jackson ImmunoResearch) 1:1000. They were diluted in PBS with 0.3 % Triton X-100, 2 % BSA, and 5 % glucose.

Epifluorescence microscopy images were acquired using a CoolSNAP HQ2 CCD camera (Photometrics) attached to a Zeiss AxioObserver Z1 (Carl Zeiss) with a 40× magnification or a Hamamatsu Orca Flash V2 (Hamamatsu) attached to a Zeiss AxioImager Z2 (Carl Zeiss) with a 40× magnification. Exposure time for the images with active zone and vesicle labeling was kept consistent for each set of experiments.

Table 9: Primary antibodies used

Item	Manufacturer
Chicken anti-MAP2	Synaptic Systems
Chicken anti-β-III-tubulin	Abcam
Guinea pig anti-synaptophysin	Synaptic Systems
Mouse anti-bassoon	Enzo Life Sciences
Mouse anti-BDNF	Developmental Studies Hybridoma Bank
Mouse anti-NL1	Synaptic Systems
Mouse anti-synaptotagmin-1	Synaptic Systems
Mouse anti-β-III-tubulin	Promega
Rabbit anti-NL2	Synaptic Systems

Table 10: Secondary antibodies used

Item	Manufacturer
Donkey anti-chicken IRDye 800CW	LI-COR
Donkey anti-mouse Alexa 647	Jackson ImmunoResearch
Donkey anti-mouse Cy3	Jackson ImmunoResearch
Goat anti-chicken Alexa 488	Jackson Immuno Research
Goat anti-guinea pig Alexa 647	Jackson ImmunoResearch
Goat anti-mouse IRDye 680RD	LI-COR
Goat anti-rabbit Alexa 647	Jackson ImmunoResearch
Goat anti-rabbit IRDye 680RD	LI-COR
Goat-anti mouse IRDye 800CW	LI-COR

2.11 Analysis

900 × 900 pixel selections of each image were made with Adobe Photoshop. Synaptic puncta were counted using OpenView (created by Noam E Ziv, Technion, Haifa). Synaptic puncta were selected automatically after setting a threshold and keeping it constant for all images. Dendritic length was measured in MetaMorph (Molecular Devices) and Fiji software (Schindelin et al. 2012). Synaptotagmin-1 intensity was measured using OpenView and Fiji software. The threshold was set in a way that all visible puncta in a neuron were selected, and it was kept constant throughout a set. Statistical analysis involved a two-tailed Student's *t* test and a two-way ANOVA that was performed using Prism (GraphPad Software).

Table 11: Software used

Name	Creator
Adobe Illustrator	Adobe
Adobe Photoshop	Adobe
Blender	The Blender Foundation
Fiji	Schindelin et al. (2012)
MetaMorph	Molecular Devices
MS Office Excel	Microsoft
MS Office Word	Microsoft
OpenView	Noam E Ziv
Prism	GraphPad Software
VisiView	Visitron
ZEN Blue Pro	Carl Zeiss

Chapter Three: Results

The results from this study have partially been published in Petkova-Tuffy et al. 2021:

Petkova-Tuffy A, Gödecke N, Viotti J, Korte M, Dresbach T (2021): Neuroligin-1 mediates presynaptic maturation through brain-derived neurotrophic factor signaling. BMC Biol 19, 215

3.1 Assays for testing structural and functional presynaptic maturation

In neuronal cultures, we can use two time windows to distinguish between immature and mature presynaptic terminals, namely, before DIV7, when presynaptic terminals are still predominantly immature, and after DIV14, when most presynaptic terminals have become mature. It has been observed that presynaptic terminals gain independence from F-actin in the process of becoming structurally mature (Zhang and Benson 2001; Wittenmayer et al. 2009). The mechanism of this process remains unknown, but the hypothesis is that the molecules at the presynapse form increasingly tight molecular structures. If we apply latrunculin A (LatA, an F-actin-disrupting drug) to cultured neurons before DIV7, the synaptic proteins disperse from the presynaptic terminals (Figure 2). In contrast, if we apply LatA to cultured neurons after DIV14, this does not occur (Figure 3). Observing the functional maturation of synapses, it has been established that the total size of the synaptic vesicle recycling pool at presynaptic terminals increases. Therefore, we can use assays measuring synaptic vesicle recycling in order to determine the functional maturation stage of presynaptic boutons (Mozhayeva et al. 2002; Wittenmayer et al. 2009) (Figure 4). In this study, I used gain-of-function experiments to examine what promotes and boosts presynaptic maturation before DIV7 and loss-of-function experiments to examine what inhibits presynaptic maturation after DIV14.

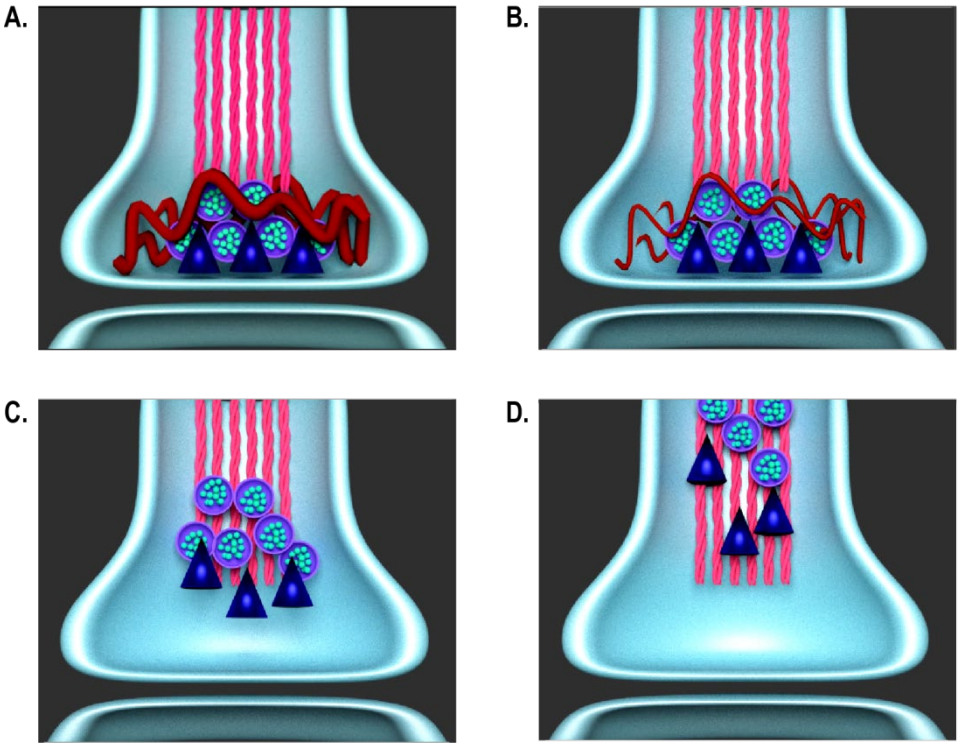

Figure 2: F-actin depolymerization in immature synapses. When latrunculin A depolymerizes F-actin in immature synapses, they disassemble. This is a schematic representation of an immature synapse. (A) F-actin filaments are still intact (red) and stabilize the active zone. (B) Latrunculin A depolymerizes the F-actin filaments. (C) and (D) The presynaptic active zone of the immature synapse is disassembled (3D animation created using Blender software).

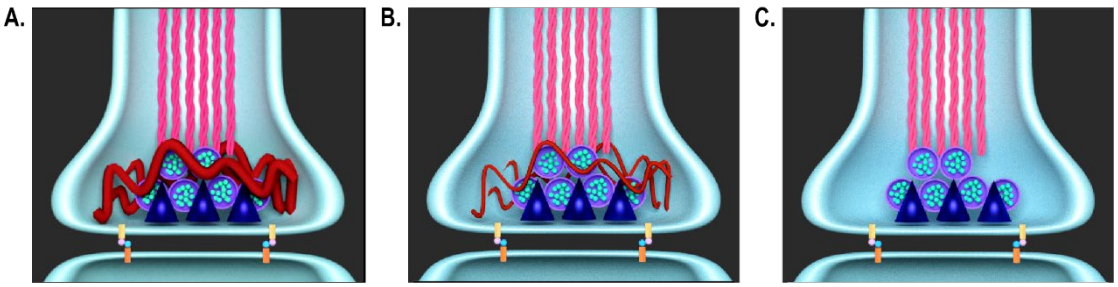

Figure 3: F-actin depolymerization in mature synapses. When latrunculin A depolymerizes F-actin in mature synapses, they do not disassemble. This is a schematic representation of a mature synapse. (A) F-actin filaments are still intact (red). (B) Latrunculin A depolymerizes the F-actin filaments. (C) The presynaptic active zone of the mature synapse remains intact even though F-actin is depolymerized (3D animation created using Blender software).

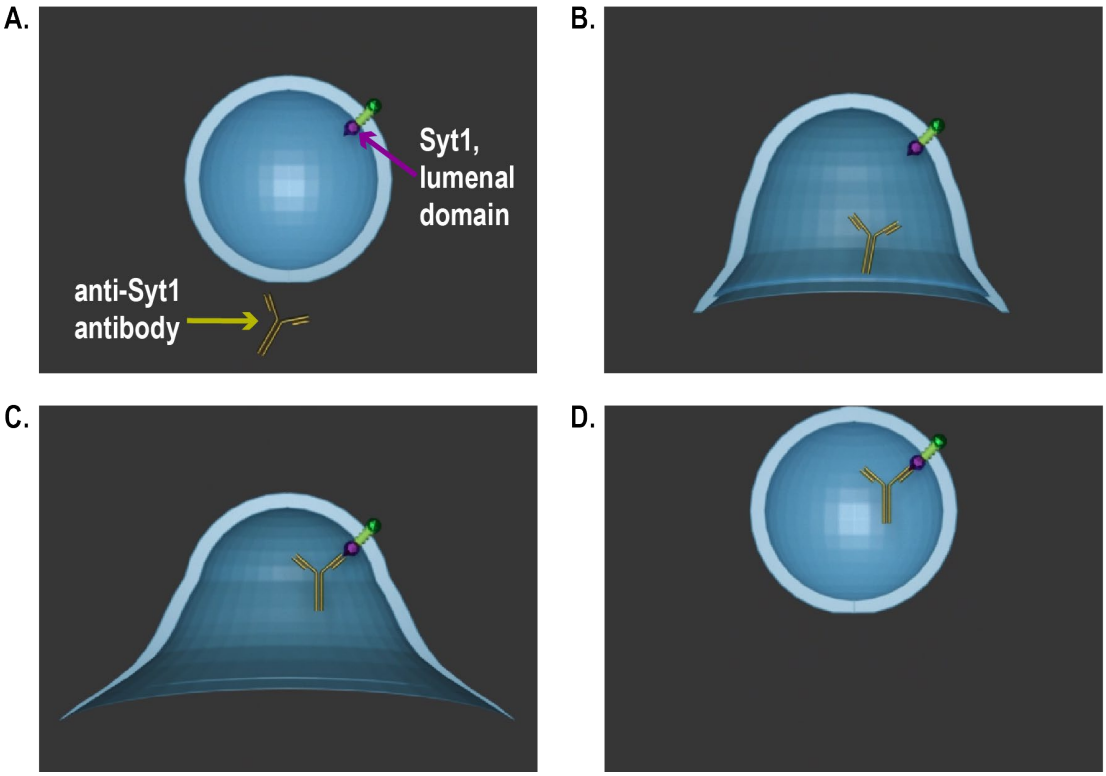

Figure 4: Synaptotagmin-1 uptake in a schematic representation of a recycling synaptic vesicle. (A) Neurons are stimulated with a high-K^+ buffer in the presence of antibodies directed against the lumenal domain of synaptotagmin-1 (Syt1). (B) Once the synaptic vesicle has fused, the antibodies can enter. (C) The antibodies bind to the now exposed lumenal domain of Syt1. (D) The vesicle together with the bound antibody is recycled, and the antibody is thus taken up (3D animation created using Blender software).

3.2 NL1, NL2, and BDNF promote structural and functional presynaptic maturation

Taking advantage of these assays, it has previously been shown that NL1 promotes structural and functional maturation of presynaptic terminals (Wittenmayer et al. 2009). To assess if NL2 has an analogous function, I transfected the constructs NL1-GFP, NL2-GFP, or farnesylated EGFP (EGFP-F) into cultured hippocampal neurons from E19 rats on DIV2. On DIV5, I fixed the neuronal cultures that had previously been treated with LatA or DMSO as a control, and I immunostained them for bassoon to identify how many active zones there are per 10 µm dendritic length (Wittenmayer et al. 2009; Petkova-Tuffy et al. 2021). The number of active zones per 10 µm dendritic length was increased in neurons overexpressing NL1 or NL2, indicating that both isoforms enhance synaptogenesis (Figure 5 A). Depolymerization

of F-actin using LatA had no effect on the number of active zones formed on the dendrites of NL1- or NL2-expressing cells, rendering them resistant to the LatA treatment (Figure 5 B). Consequently, both NL isoforms induce the formation of presynaptic terminals and their structural maturation. To investigate whether BDNF enhances synaptogenesis and maturation of the presynaptic terminals, I applied exogenous BDNF to hippocampal neuronal cultures on DIV4 and fixed these neuronal cultures on DIV5 following treatment with LatA or DMSO. I immunostained them for bassoon to detect if the active zones in these young BDNF-treated neurons become LatA resistant. Following exogenous BDNF application, the number of active zones was increased, indicating that BDNF promoted synaptogenesis (Figure 6). In addition, depolymerization of F-actin using LatA had no effect on the number of active zones on BDNF-treated dendrites, indicating that BDNF advances structural presynaptic maturation. From these results, we can conclude that BDNF application as well as the overexpression of the neuroligin isoforms NL1 or NL2 has comparable properties in promoting the structural maturation of active zones (Figures 5 and 6).

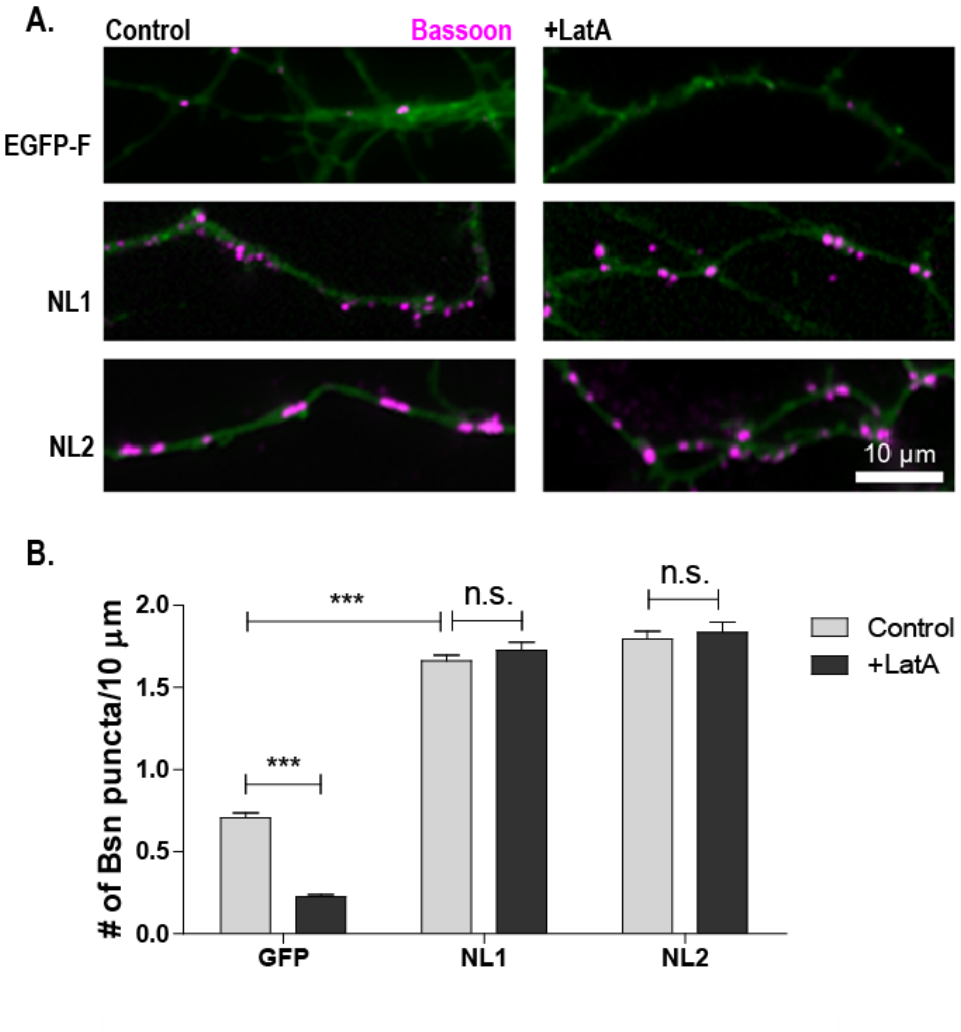

Figure 5: Induction of structural presynaptic maturation by NL1 and NL2. (A) DIV5 cultured hippocampal neurons transfected with membrane-targeted EGFP (farnesylated EGFP, EGFP-F), GFP-tagged NL1, or GFP-tagged NL2 (green); treated with LatA or DMSO; and immunostained for bassoon (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. Virtually all bassoon puncta formed on NL1- or NL2-expressing cells are LatA resistant. (Mean + SEM is shown; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 174) = 31.51$; $^{***}p < 0.0001$ for EGFP-F control vs. EGFP-F + LatA, $p > 0.05$ for NL1 control vs. NL1 + LatA and NL2 control vs NL2 + LatA). Scale bar is 10 µm.

This figure has partially been published in Petkova-Tuffy et al. (2021).

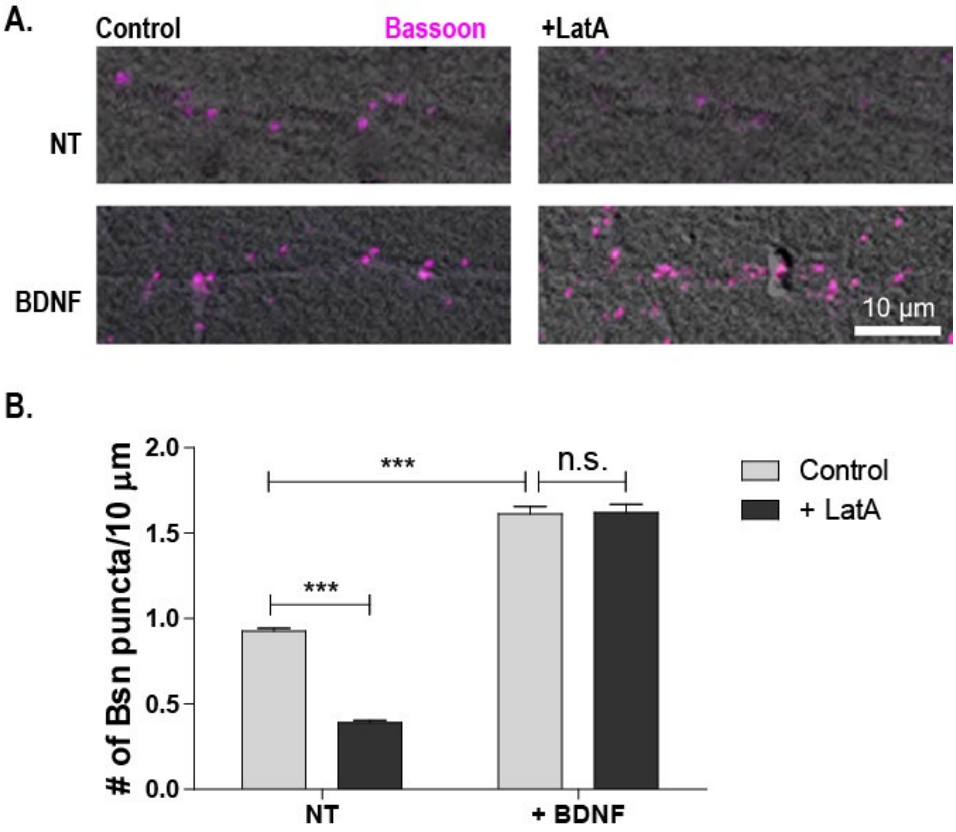

Figure 6: Induction of structural active zone maturation by BDNF. (A) DIV5 cultured hippocampal neurons treated with BDNF or buffer (NT, not treated) on DIV4, treated with LatA or DMSO 6 h prior to fixation, fixed on DIV 5, and immunostained for bassoon (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. (Mean + SEM is shown; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 67.49$; $^{***}p < 0.0001$ for NT control vs. NT + LatA, $p > 0.05$ for BDNF control vs. BDNF + LatA).

This figure has been published in Petkova-Tuffy et al. (2021).

It remains unknown what LatA resistance represents on a molecular or cellular level. I hypothesized that it might represent the increased lifetime of presynaptic terminals and therefore a reduced turnover rate of active zones. To test this hypothesis, I used anisomycin as an inhibitor of protein synthesis, a drug that was utilized by Bednarek and Caroni (2011) to establish the lifetime of the scaffolding protein bassoon at active zones. In immature neurons, treated with buffer or transfected with the control construct GFP, anisomycin treatment led to a decrease in the number of active zones per 10 µm dendritic length. In contrast, anisomycin treatment did not lead to a decrease in the number of active zones in DIV5 cultured neurons treated with BDNF

or overexpressing NL1 (Figures 7 and 8). From these results, we can conclude that the application of exogenous BDNF and the overexpression of NL1 lead to an increased lifetime of bassoon at the active zone. I also studied the effect of BDNF application in organotypic hippocampal slices as a model system with better preserved connectivity. I applied BDNF to immature organotypic hippocampal slices and treated them with LatA, anisomycin, or DMSO as a control. The results reveal that BDNF application in these slices leads to LatA resistance of bassoon as well as to an increased lifetime of bassoon at the active zones (Figure 9).

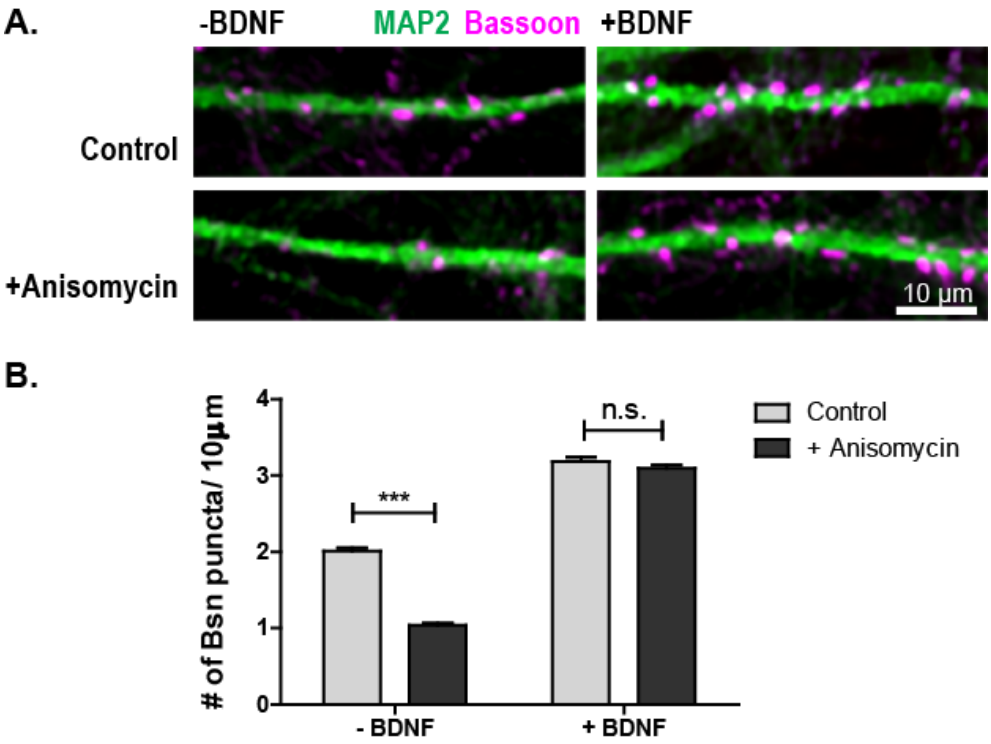

Figure 7: BDNF increases the lifetime of active zones. (A) Cultured hippocampal neurons treated with buffer (−BDNF) or BDNF (+BDNF) on DIV4, treated with DMSO (control) or anisomycin (+Anisomycin) on DIV5, fixed 6 h later, and immunostained for bassoon (magenta) and MAP2 (green). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. (Mean + SEM is shown; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 99.82$; $^{***}p < 0.0001$ for NT control vs. NT + anisomycin, $p > 0.05$ for BDNF control vs. BDNF + anisomycin). Scale bar is 10 µm.

This figure has been published in Petkova-Tuffy et al. (2021).

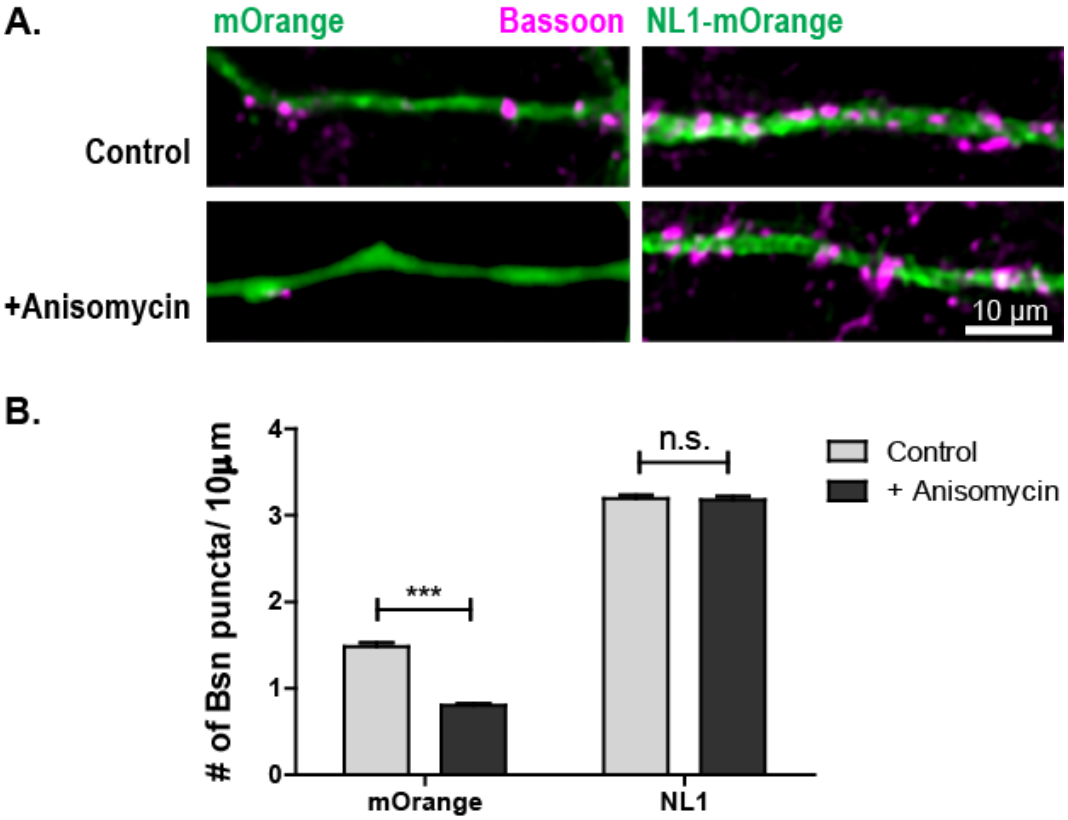

Figure 8: NL1 increases the lifetime of active zones. (A) DIV5 cultured hippocampal neurons transfected with mOrange or mOrange-tagged NL1 (green), treated with anisomicin or DMSO for 6 h, fixed, and immunostained for bassoon (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. (Mean + SEM is shown; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 82.33$; $^{***}p < 0.0001$ for mOrange control vs. mOrange + anisomycin, $p > 0.05$ for NL1 control vs. NL1 + anisomycin). Scale bar is 10 µm.

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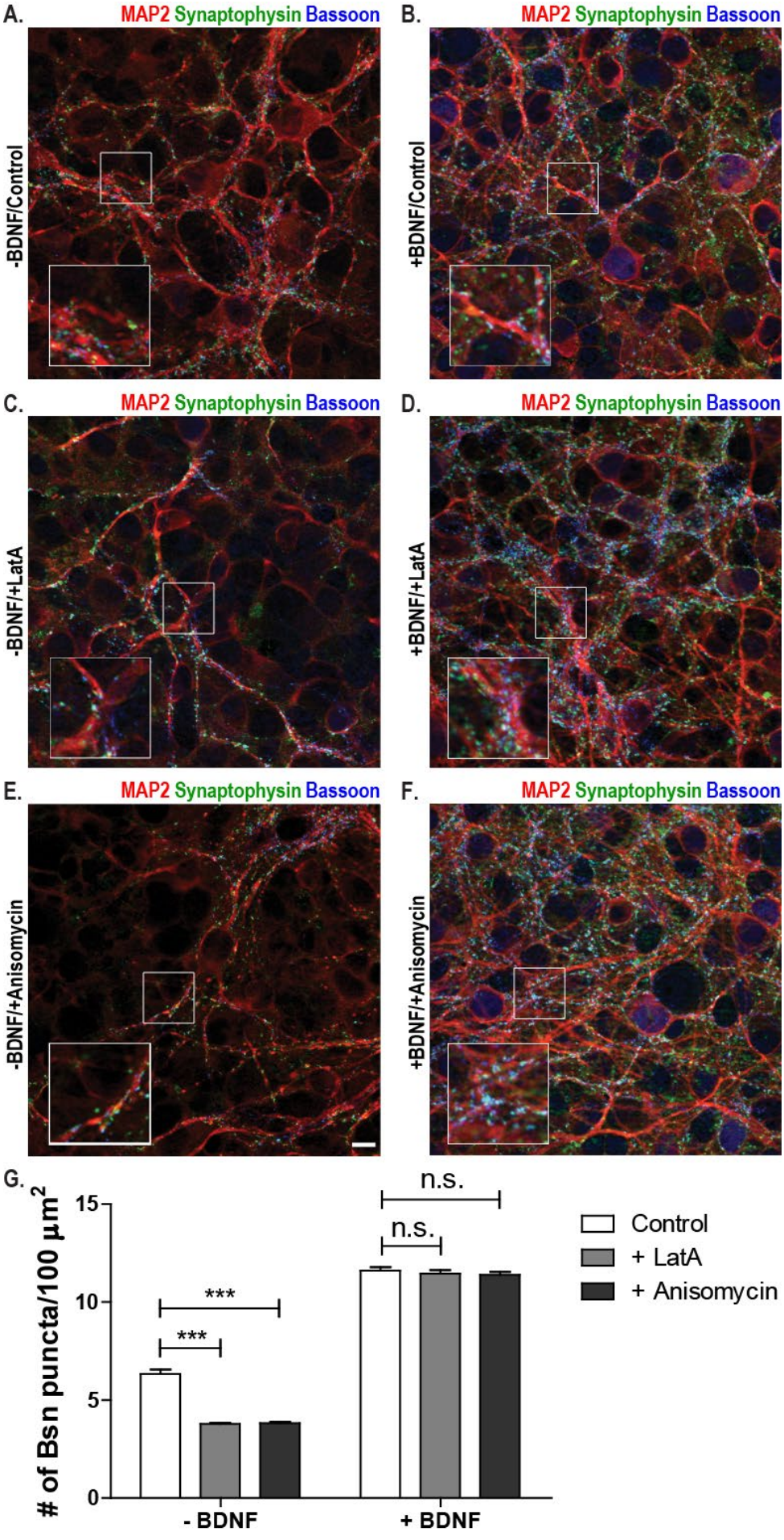
A.
MAP2 Synaptophysin Bassoon
-BDNF/Control
B.
MAP2 Synaptophysin Bassoon
+BDNF/Control
C.
MAP2 Synaptophysin Bassoon
-BDNF/+LatA
D.
MAP2 Synaptophysin Bassoon
+BDNF/+LatA
E.
MAP2 Synaptophysin Bassoon
-BDNF/+Anisomycin
F.
MAP2 Synaptophysin Bassoon
+BDNF/+Anisomycin
G.
# of Bsn puncta/100 µm2
15
10
5
0
***
***
n.s.
n.s.
- BDNF
+ BDNF
Control
+ LatA
+ Anisomycin

Figure 9: BDNF increases the structural presynaptic maturation and the lifetime of active zones in organotypic cultures. (A) Hippocampal organotypic cultures treated with buffer (−BDNF) on DIV5; treated with DMSO (control) on DIV6; fixed 6 h later; and immunostained for MAP2 (red), synaptophysin (green), and bassoon (blue). (B) Hippocampal organotypic cultures treated with BDNF (+BDNF) on DIV5; treated with DMSO (control) on DIV6; fixed 6 h later; and immunostained for MAP2, synaptophysin, and bassoon. (C) Hippocampal organotypic cultures treated with buffer (−BDNF) on DIV5; treated with latrunculin A (+LatA) on DIV6; fixed 6 h later; and immunostained for MAP2, synaptophysin, and bassoon. (D) Hippocampal organotypic cultures treated with BDNF (+BDNF) on DIV5; treated with latrunculin A (+LatA) on DIV6; fixed 6 h later; and immunostained for MAP2, synaptophysin, and bassoon. (E) Hippocampal organotypic cultures treated with buffer (−BDNF) on DIV5; treated with anisomycin (+Anisomycin) on DIV6; fixed 6 h later; and immunostained for MAP2, synaptophysin, and bassoon. (F) Hippocampal organotypic cultures treated with BDNF (+BDNF) on DIV5; treated with anisomycin (+Anisomycin) on DIV6; fixed 6 h later; and immunostained for MAP2, synaptophysin, and bassoon. (G) Quantification of the number of bassoon puncta per 100 μm² for the conditions indicated in panels A–F. Mean + SEM are shown; $N = 3$ experiments, $n = 10$ images per condition and experiment; two-way ANOVA with post hoc Sidak tests: interaction is significant (***$p < 0.0001$, $F(2, 174) = 38.43$; ***$p < 0.0001$ for NT control vs. NT + LatA and for NT control vs. NT + Anisomycin; $p > 0.05$ for BDNF control vs. BDNF + LatA and for BDNF control vs. BDNF + Anisomycin). Scale bar is 10 μm.

This figure has been published in Petkova-Tuffy et al. (2021).

3.3 NL1 and NL2 require BDNF signaling to promote presynaptic maturation

To study whether BDNF signaling is required for the maturation-promoting effect of neuroligins on the stability of the active zone, I overexpressed both neuroligin isoforms in hippocampal neurons from E19 rats. On DIV2, a soluble form of the receptor for BDNF TrkB, i.e., TrkB-Fc, was introduced into the growth medium with the goal of reducing BDNF concentration in the medium (Dean et al. 2009). Interestingly, the overexpression of NL1 and NL2 with bath application of TrkB-Fc was affected differently for the two isoforms. In the control neurons expressing GFP, bath application of TrkB-Fc led to a decreased number of active zones per 10 μm dentritic length of those neurons, but while the synaptogenic effect of NL2 overexpression was prevented by TrkB-Fc application, the synaptogenic effect of NL1 overexpression remained unaffected (Figure 10). I also examined the LatA resistance of the remaining bassoon puncta in the presence of the BDNF scavenger, in order to determine whether the active zones have reached a structurally mature state or not. With bath application of TrkB-Fc the LatA resistance of the active zones on the dendrites of NL1-overexressing neurons was reduced while the number of active zones on the dentrites of NL2-overexpressing neurons was unaffected (Figure 11). These results suggest that when BDNF signaling is blocked, NL1 overexpression leads to increased synaptogenesis, but the presynaptic terminals that are formed remain structurally immature, while NL2 overexpression no longer leads to increased synaptogenesis, but the few presynaptic terminals that are formed become structurally mature. Therefore, we can conclude that BDNF plays a role in the synaptogenic effect of NL2 but not NL1, while it appears to be required for the maturation-inducing effect of NL1 but not NL2. The latter observation should, however, be only accepted provisionally, because only a low number of bassoon puncta can be observed on the dendrites of NL2-overexpressing neurons in the presence of the BDNF scavenger, so the LatA-resistant bassoon puncta could be an artifact or caused by unscavenged BDNF in the medium.

To study whether neuroligins require BDNF signaling for the maturation-promoting effect on synaptic vesicle recycling, I overexpressed the two neuroligin isoforms in cultured neurons from E19 rats and used the uptake of synaptotagmin-1 (Syt1) antibodies upon depolarization as a readout for the amount of synaptic vesicle recycling at the presynaptic terminals. For these experiments, I used a specific Syt1 antibody that binds to the lumenal domain of the transmembrane synaptic vesicle

protein synaptitagmin-1. Depolarization triggers the release of synaptic vesicles, which undergo exocytosis. During exocytosis, the lumenal domain of Syt1 becomes exposed in the synaptic cleft. The antibodies that have been presented in the medium can now bind to the lumenal domain of Syt1 and are then taken up during the endocytotic phase of the exo- and endocytotic cycle of synaptic vesicle release. Afterwards, the antibodies that have been taken up can be imaged using secondary antibodies coupled to fluorophores. The resulting fluorescence signals can be used as a readout for the amount of synaptic vesicle recycling and the number of recycling vesicles, because they correspond to the number of primary antibodies directed against Syt1 that have been taken up. On DIV5, I stimulated transfected live neuronal cultures while Syt1 antibodies against the lumenal domain were presented in the medium to bind to Syt1 in the recycling synaptic vesicles. The cells were washed with neuronal medium without any additives and then fixed and immunostained with secondary antibodies against the primary Syt1 antibodies. In comparison to the GFP-transfected cells, the NL1- and NL2-overexpressing neurons showed an increase in the fluorescence intensity of the Syt1 signal per presynaptic terminal, meaning that NL1 and NL2 lead to increased synaptic vesicle recycling (Figure 12). In the presence of TrkB-Fc to perturb BDNF signaling, the fluorescence intensity of the Syt1 signals was reduced in GFP-transfected neurons, and the enhancing effect of NL1 and NL2 on synaptic vesicle recycling was blocked, indicating that NL1 and NL2 required BDNF signaling for their enhancing effect on synaptic vesicle recycling in terms of functional maturation.

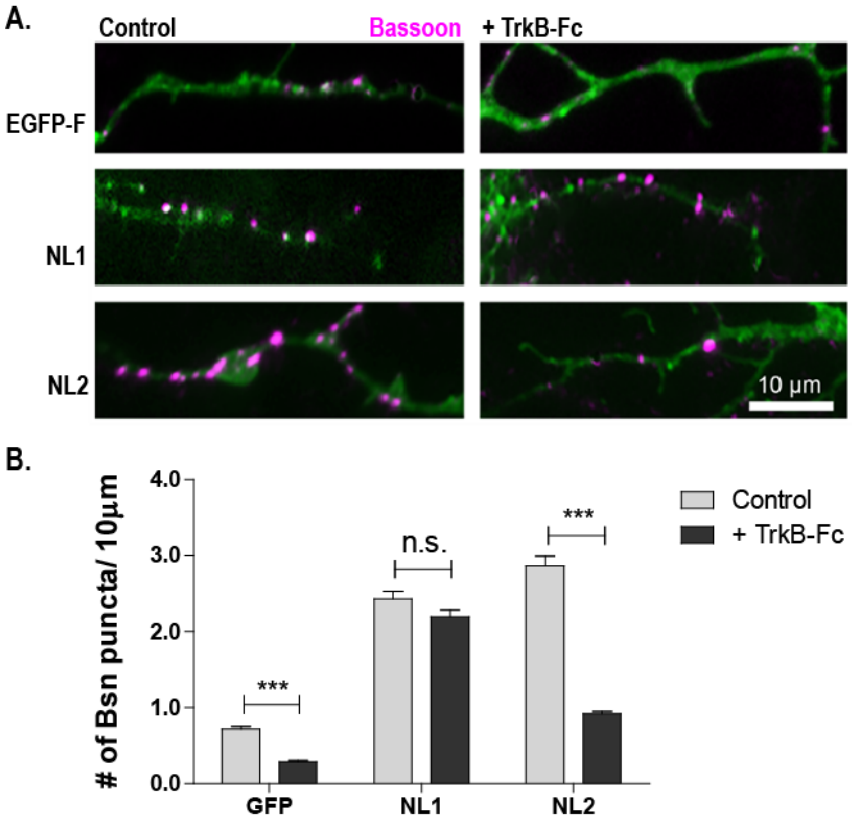

Figure 10: BDNF signaling is required for NL2-induced synapse formation. (A) Representative images of DIV5 cultured hippocampal neurons transfected with farnesylated EGFP (EGFP-F), NL1-GFP (NL1), or NL2-GFP (NL2) (green) grown in the absence or presence of TrkB-Fc and immunostained for bassoon (magenta) to determine the number of active zones. (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant (***$p < 0.0001$, $F(2, 174) = 74.86$); ***$p < 0.0001$ for GFP control vs. GFP + TrkB-Fc; and for NL2 control vs. NL2 + TrkB-Fc; $p > 0.05$ for NL1 control vs. NL1 + TrkB-Fc). Scale bar is 10 µm.

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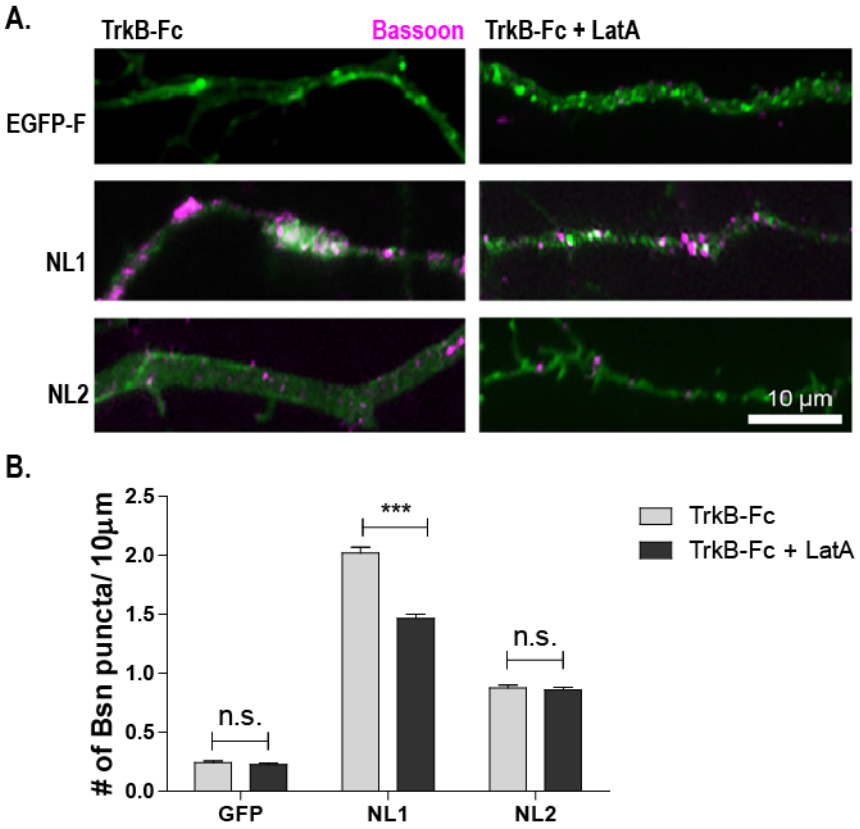

Figure 11: BDNF signaling is required for NL1-induced structural presynaptic maturation. (A) Representative images of DIV5 cultured hippocampal neurons transfected with farnesylated EGFP (EGFP-F), NL1-GFP (NL1), or NL2-GFP (NL2) (green); grown in the presence of TrkB-Fc; treated with LatA or DMSO; and immunostained for bassoon to determine the number of LatA-resistant active zones. (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant (***$p < 0.0001$, $F(2, 174) = 54.63$); ***$p < 0.0001$ for NL1 + TrkB-Fc vs. NL1 + TrkB-Fc + LatA; $p > 0.05$ for GFP + TrkB-Fc vs. EGFP-F + TrkB-Fc + LatA and for NL2 + TrkB-Fc vs. NL2 + TrkB-Fc + LatA). Scale bar is 10 µm.

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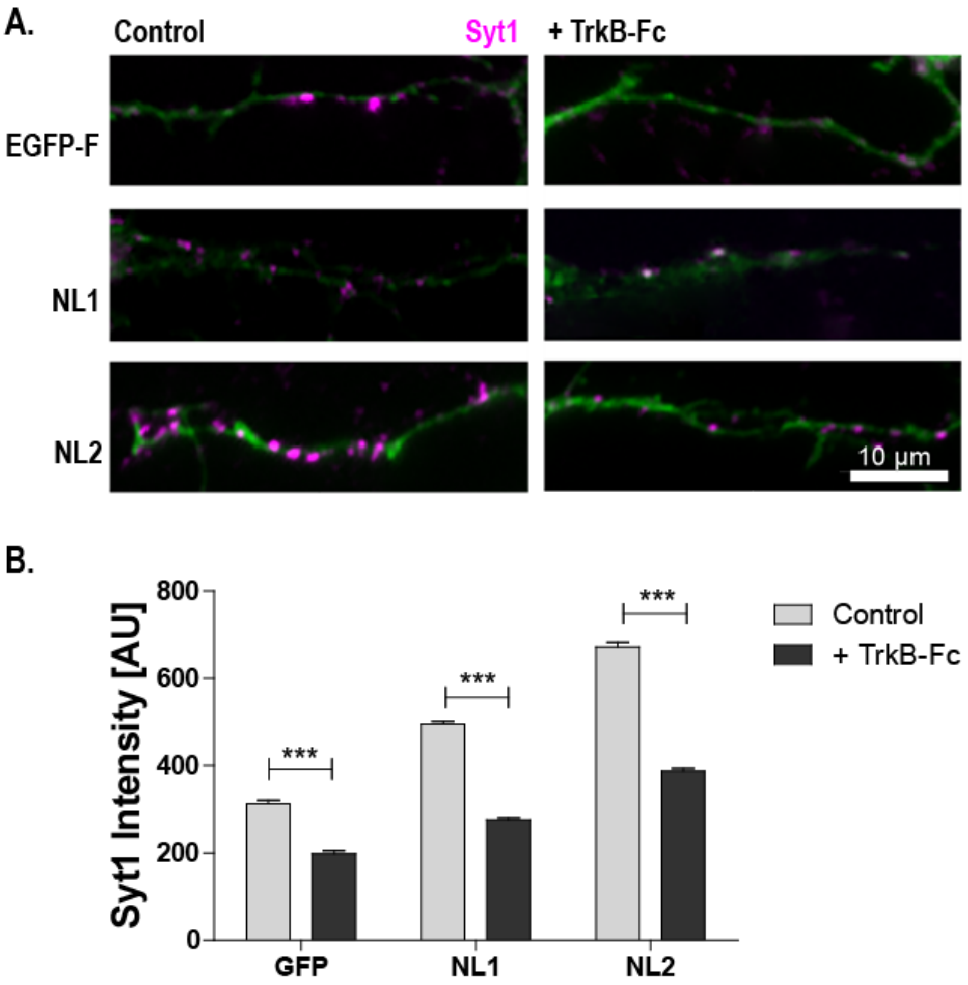

Figure 12: BDNF signaling is required for NL-mediated functional presynaptic maturation. (A) Representative images of cultured hippocampal neurons transfected on DIV2 with EGFP-F, NL1-EGFP (NL1), or NL2-EGFP (NL2) (green); grown in the absence or presence of TrkB-Fc; and stimulated in the presence of antibodies directed against the lumenal domain of Syt1 to label recycling synaptic vesicles. After washing, the cells were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (magenta) (B) Quantification of the fluorescence intensity of the Syt1 label (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant (***$p < 0.0001$, $F(2, 174) = 74.26$); ***$p < 0.0001$ for GFP control vs. GFP + TrkB-Fc; for NL1 control vs. NL1 + TrkB-Fc, and for NL2 control vs. NL2 + TrkB-Fc). Scale bar is 10 µm.

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To further investigate the involvement of BDNF signaling in NL-mediated presynaptic maturation, I used hippocampal neuronal cultures from $BDNF^{flox/lox}$ conditional knockout mice. Transducing these cultures with a Cre-recombinase-expressing lentivirus (pUBC-Cre–IRES–GFP) on DIV0 excises *Bdnf*. Considering that neuronal cultures from NL1-knockout mice do not reach structural and

functional maturation (Wittenmayer et al. 2009), BDNF-depleted cultures should also fail to do so if BDNF is involved in NL-mediated presynaptic maturation. To test this assumption, I studied the LatA resistance of bassoon puncta on DIV15 in neurons in BDNF-depleted cultures. Depolymerizing F-actin with LatA significantly reduced the number of active zones in these neurons compared to the neurons in non-transduced cultures (Figure 13).

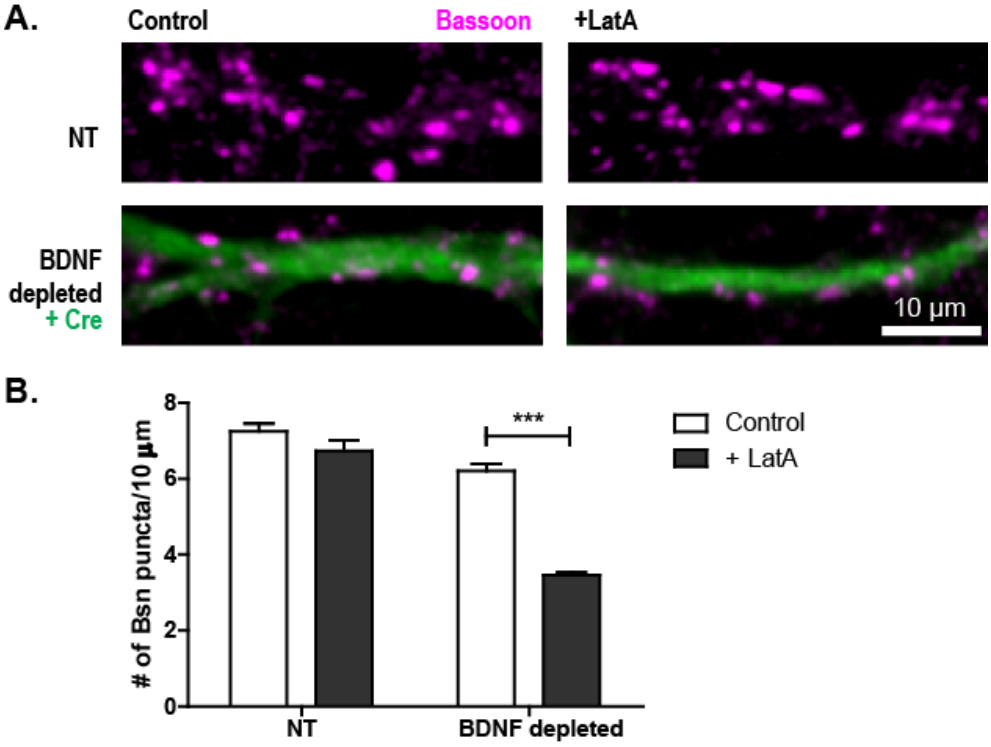

Figure 13: BDNF-depleted cultures fail to mature structurally. (A) Cultures of $BDNF^{flox/lox}$ hippocampal neurons with and without Cre-recombinase lentiviral transduction (green) to deplete BDNF levels. On DIV15 these cultures were fixed and immunostained for bassoon (magenta) to determine the number of active zones following an 18 h LatA treatment to depolymerize F-actin. (B) Quantification of the number of bassoon puncta for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 30.82$; $^{***}p < 0.0001$ for BDNF depleted vs. BDNF depleted + LatA, $p > 0.05$ for NT vs. NT + LatA). Scale bar is 10 µm.

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I also performed the Syt1 antibody uptake assay in these cultures to study their presynaptic functional maturation. In Cre–IRES–GFP-transduced cultures on DIV15, the fluorescence intensity of the Syt1 signals was significantly decreased compared to non-transduced cultures (Figure 14), suggesting that functional maturation is impaired upon BDNF depletion. Therefore, we can conclude that

BDNF-depleted cultures, similarly to NL1-knockout cultures, fail to reach structural and functional maturation.

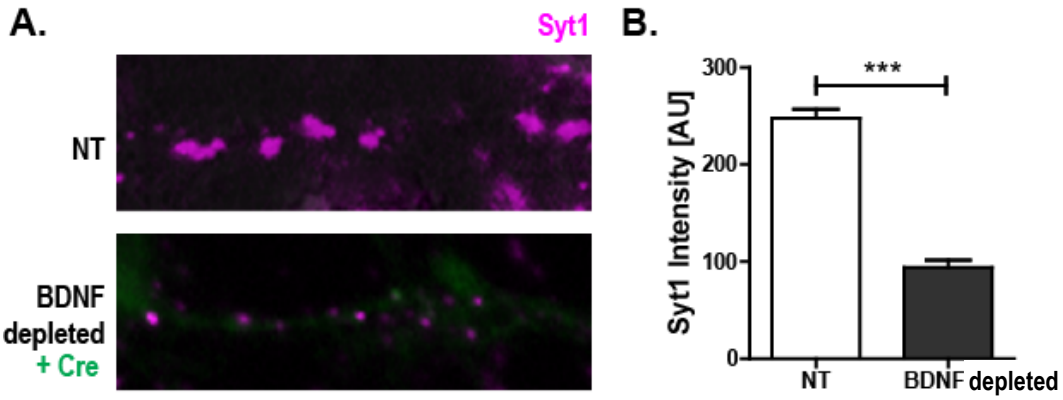

Figure 14: BDNF-depleted cultures fail to mature functionally. (A) Representative images of hippocampal neurons from BDNF$^{flox/lox}$ mice transduced with Cre–IRES–GFP (green) compared to non-transduced cultures from the same mice, which were stimulated in the presence of antibodies directed against the lumenal domain of Syt1 to label recycling synaptic vesicles. After washing, the cells were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (magenta). (B) Quantification of the intensity of the Syt1 label (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; ***$p < 0.0001$, Student's t test). Scale bar is 10 μm.

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The TrkB-Fc experiments indicated that neuroligins likely require BDNF signaling for their function in inducing presynaptic maturation. To verify these findings, I used the BDNF-depleted cultures, in which I overexpressed NL1–IRES–mOrange and NL2–IRES–mOrange on DIV4. As a control I overexpressed mOrange. After performing the LatA assay on DIV6 and immunostaining for bassoon, I found that in non-transduced cultures where BDNF levels remained unaffected, the number of active zones was significantly decreased in the control neurons expressing mOrange, while the number of active zones on the dendrites of neurons overexpressing NL1–IRES–mOrange and NL2–IRES–mOrange was unaffected (Figure 15), similarly to my findings in rat neuronal cultures on DIV5. On the other hand, BDNF depletion affected NL1 and NL2 function distinctly. While the total number of active zones was unaffected in NL1-overexpressing neurons upon BDNF depletion, it was significantly reduced in NL2-overexpressing neurons, meaning that synapse formation was only affected in NL2-overexpressing neurons. After treating the cultures with LatA, I found that F-actin depolymerization significantly decreased the number of active zones per 10 μm dendritic length in both NL1- and NL2-

overexpressing neurons, meaning that the function of both NL isoforms was affected by BDNF depletion in terms of their ability to induce structural presynaptic maturation. I also performed the Syt1 antibody uptake assay in the same setting and found that BDNF depletion led to a significant decrease in the fluorescence intensity of the Syt1 signals in mOrange-expressing neurons compared to mOrange-expressing neurons in non-transduced cultures. Both NL1 and NL2 failed to increase the fluorescence intensity of the Syt1 signals upon BDNF-depletion, meaning that their function was also affected by BDNF depletion in terms of their ability to induce functional presynaptic maturation (Figure 16).

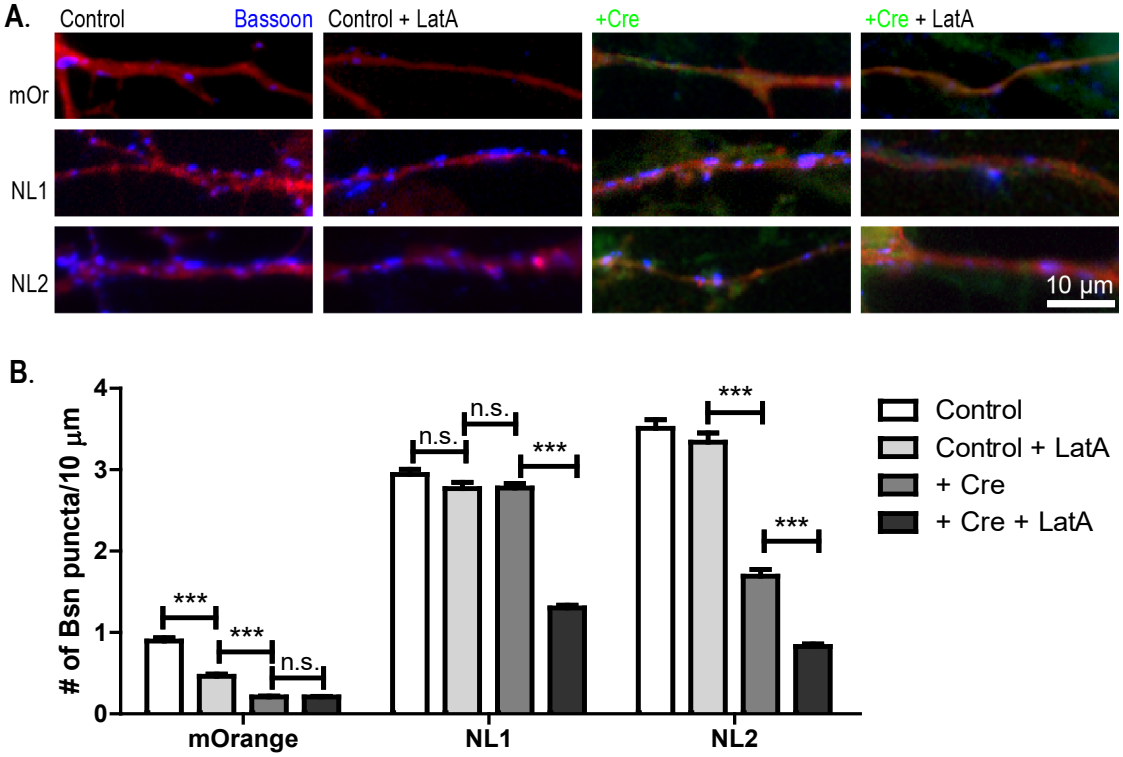

Figure 15: NL1 and NL2 fail to induce structural presynaptic maturation in BDNF-depleted cultures. (A) Representative images of DIV6 $BDNF^{flox/lox}$ cultures and Cre-recombinase-transduced cultures transfected with mOrange, NL1-mOrange (NL1), or NL2-mOrange (NL2) (red) and immunostained for bassoon (blue). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(6, 348) = 86.78$; $^{***}p < 0.0001$ for mOr control vs. mOr + LatA, NL1 + Cre vs. NL1 + Cre + LatA, NL2 + LatA vs. NL2 + Cre, and NL2 + Cre vs. NL2 + Cre + LatA; $^{*}p < 0.01$ for mOr + LatA vs. mOr + Cre; $p > 0.05$ for mOr + Cre vs. mOr + Cre + LatA, NL1 control vs. NL1 + LatA, NL1 + LatA vs. NL1 + Cre, and NL2 control vs. NL2 + LatA). Scale bar is 10 µm.

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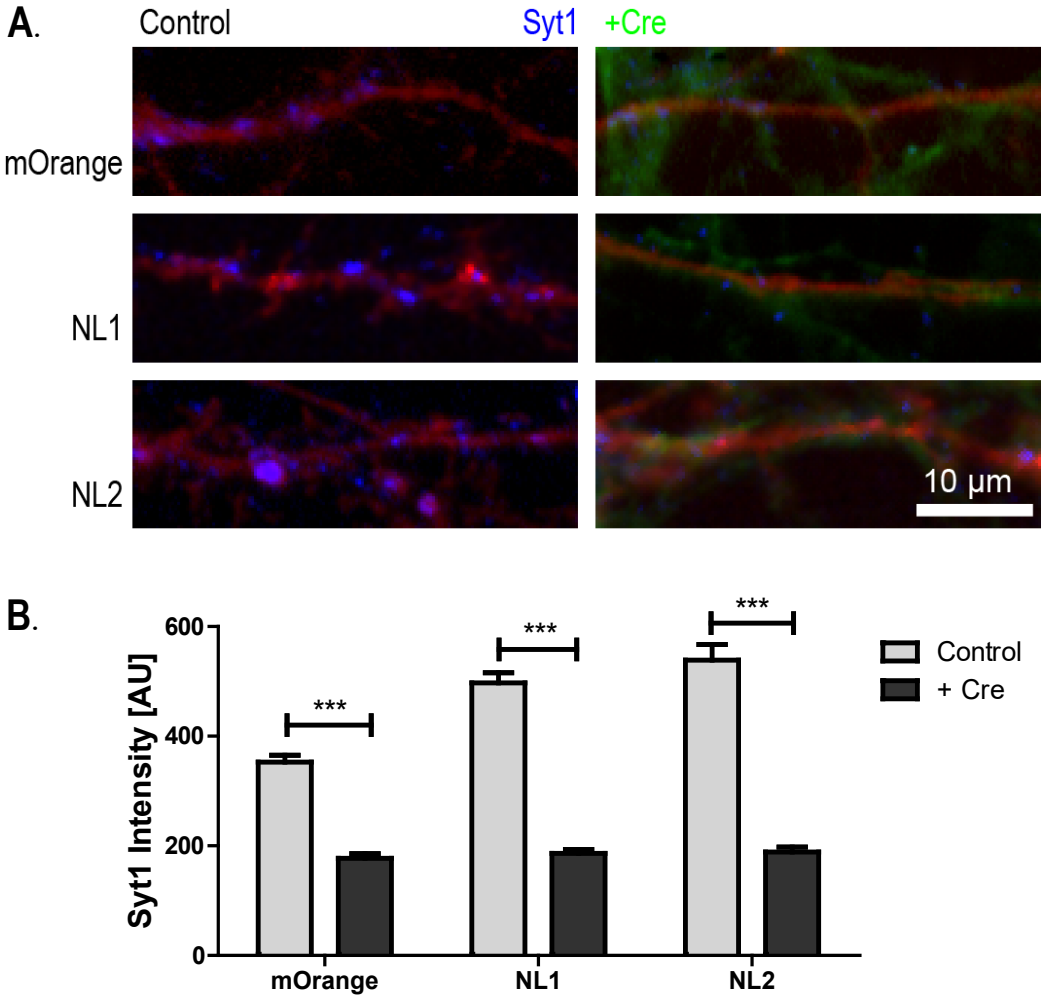

Figure 16: NL1 and NL2 fail to induce functional presynaptic maturation in BDNF-depleted cultures. (A) Representative images of cultured hippocampal neurons from $BDNF^{flox/lox}$ mice with or without Cre-recombinase transduction (green) transfected with mOrange, NL1-mOrange (NL1), or NL2-mOrange (NL2) (red) on DIV4. On DIV6, cultures were stimulated in the presence of antibodies against the lumenal domain of Syt1 to label recycling synaptic vesicles. After washing, the cells were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (blue). (B) Quantification of the fluorescence intensity of the Syt1 label for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(2, 174) = 16.44$; $^{***}p < 0.0001$ for mOr control vs. mOr + Cre, NL1 control vs. NL1 + Cre, and NL2 control vs. NL2 + Cre). Scale bar is 10 µm.

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3.4 NL1- and NL2-induced presynaptic maturation is likely independent of neurexin binding

So far, the assumption has been that most properties of neuroligins are dependent on their binding to their presynaptic partners, the neurexins. However, not all functions are dependent on neurexin-binding (Ko et al. 2009). Until now, it has remained unknown whether neurexin binding is required for the maturation-promoting effects of neuroligins. To test whether this is the case, I overexpressed a mutated variant of NL1 that lacks the ability to bind to neurexins (Ko et al. 2009) and compared its properties to overexpressing a non-mutated NL1 on DIV5. The effects of both NL1 variants on LatA resistance of active zones and on increasing the uptake of Syt1 antibodies were the same in neuronal cultures from E19 rats (Figure 17). I also compared the effects of the wild-type NL1 variant and the mutated NL1 variant in DIV5 neuronal cultures from NL1-knockout mice. Here, their function in inducing LatA resistance was also similar (Figure 18).

To exclude a possible interaction of neurexins with endogenous NL1 or NL2, I performed Western blot experiments to determine whether the NL1 and NL2 isoforms are expressed on DIV6 (Figure 19). As expected, endogenous NL1 and NL2 are below the detection limit – or not expressed – on DIV6. Therefore, we conclude that neurexin binding is not likely to be involved in NL1- and NL2-induced presynaptic maturation.

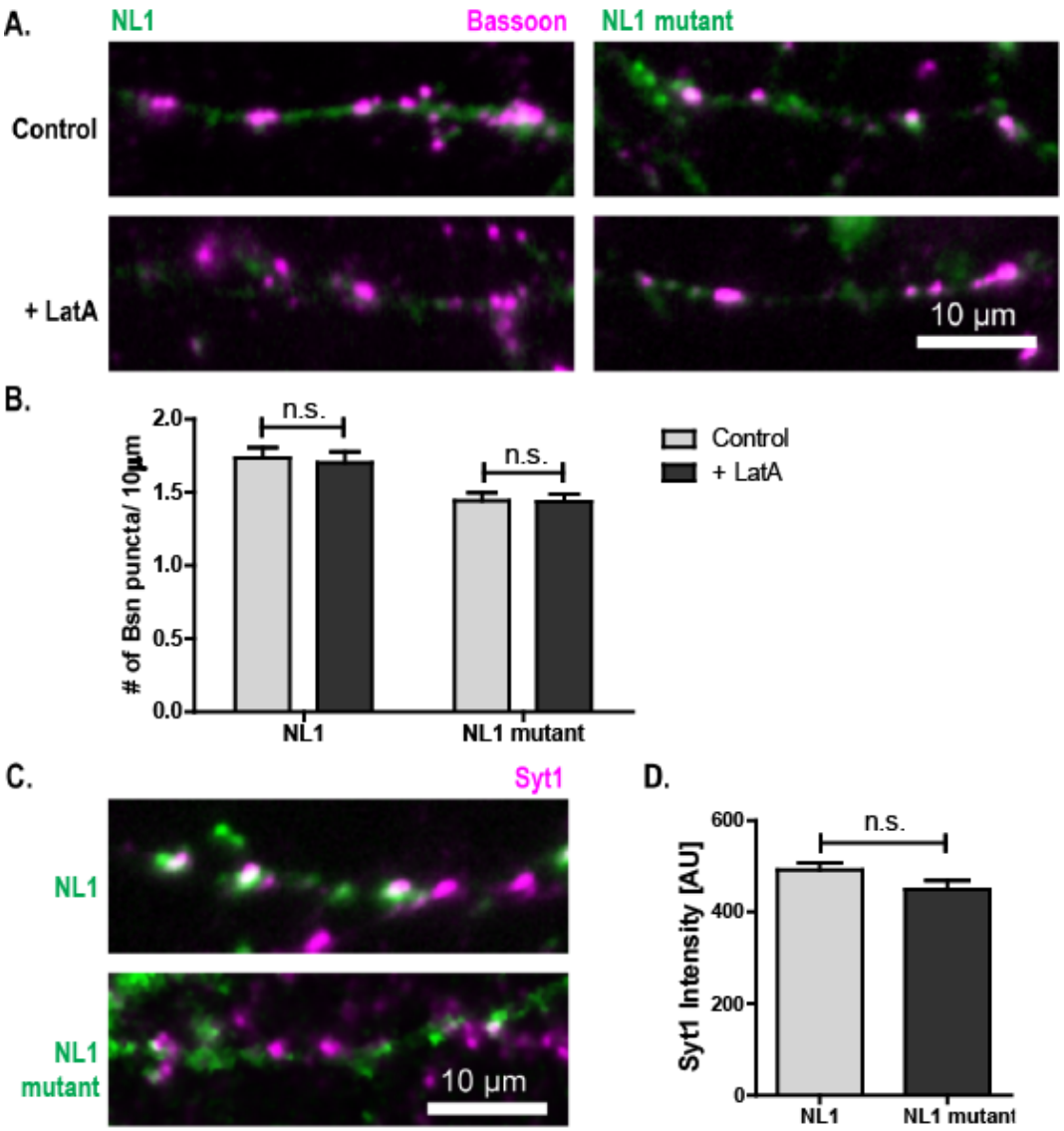

Figure 17: Similar effects of NL1 and a neurexin-binding-deficient NL1 mutant on structural and functional presynaptic maturation. (A) DIV5 cultured hippocampal neurons transfected with NL1–IRES–mOrange (NL1) or mutated NL1–IRES–mOrange (NL1 mutant), treated with LatA or DMSO, and immunostained for bassoon (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. Mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment; two-way ANOVA with post-hoc Sidak tests: $p>0.05$ for NL1 control vs. NL1 + LatA and for NL1 mutant control vs. NL1 mutant + LatA (C) The cultures were stimulated in the presence of antibodies directed against the lumenal domain of Syt1 to label recycling synaptic vesicles (magenta). (D) Quantification of the fluorescence intensity of the Syt1 label for the conditions indicated in panel C. Mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; Student's t test: $p > 0.05$. Scale bar is 10 µm.

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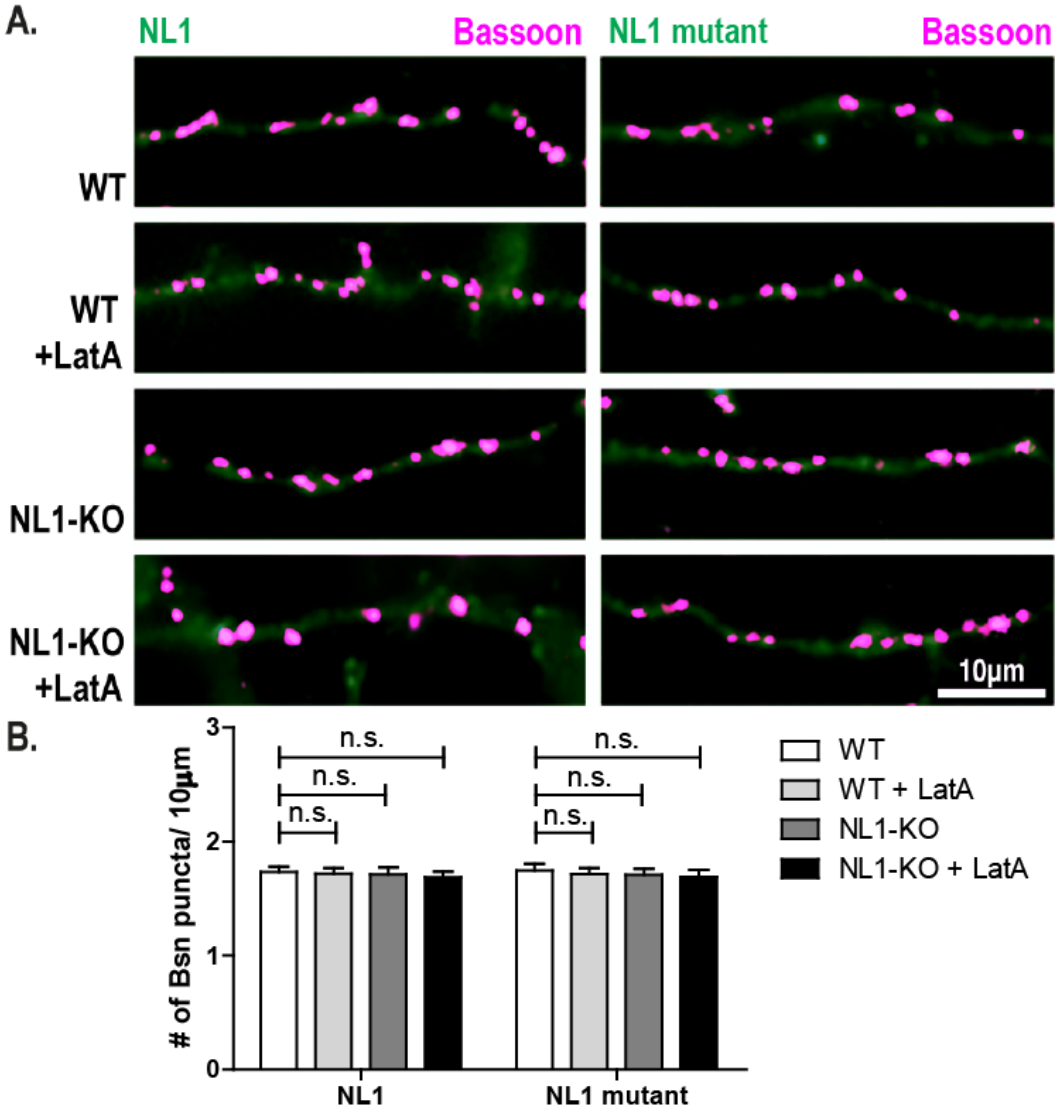

Figure 18: Similar effects of NL1 and a neurexin-binding-deficient NL1 mutant on structural presynaptic maturation in NL1-knockout cultures. (A) DIV5 cultured cortical wild-type and NL1-knockout neurons transfected with NL1–IRES–mOrange (NL1) or mutated NL1–IRES–mOrange (NL1 mutant), treated with LatA or DMSO, and immunostained for bassoon (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A. Mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post-hoc Sidak tests: $p > 0.05$ for all comparisons. Scale bar is 10 µm.

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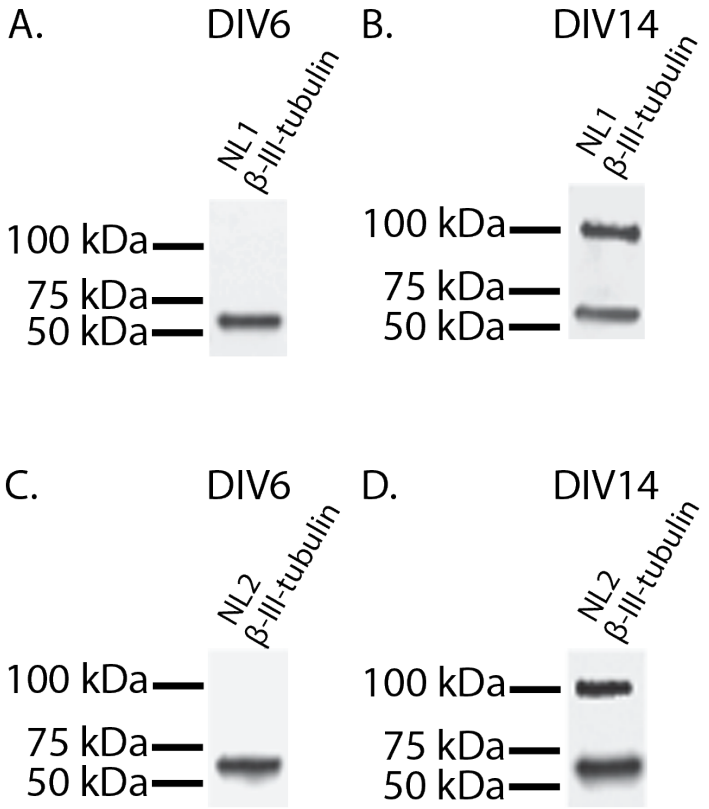

Figure 19: Endogenous expression of NL1 and NL2. Lysates were prepared from DIV6 (panels A and C) and DIV14 (panels B and D) cortical neurons. (A) Western blot showing the expression of β-III-tubulin. The lack of an NL1 band indicates that the protein is not expressed on DIV6. (B) Western blot showing the expression of β-III-tubulin and NL1 on DIV14. (C) Western blot showing the expression of β-III-tubulin. The lack of an NL2 band indicates that the protein is not expressed on DIV6. (D) Western blot showing the expression of β-III-tubulin and NL2 on DIV14.

3.5 BDNF application reestablishes impaired presynaptic maturation in cultured neurons from NL1-knockout mice

It has previously been observed that cultured neurons from NL1-knockout mice do not reach structural and functional maturation (Wittenmayer et al. 2009). Seeing that NL-induced presynaptic maturation is impaired when BDNF levels have been reduced, the question arises whether neuroligins trigger or boost BDNF signaling in order to induce presynaptic maturation. If this is indeed the case, then the application of exogenous BDNF should be able to rescue the failed maturation in cultured neurons from NL1-knockout mice. To explore this possibility, I applied exogenous BDNF to cultured cortical neurons from wild-type and NL1-knockout mice and performed the LatA assay and Syt1 uptake assay to determine the state of structural and functional maturation. In immature cultures on DIV6, BDNF application promoted structural and functional presynaptic maturation in NL1-knockout cultures (Figures 20 and 21). Interestingly, in DIV15 neurons, failed structural and functional maturation was in fact reestablished after BDNF application (Figures 22 and 23).

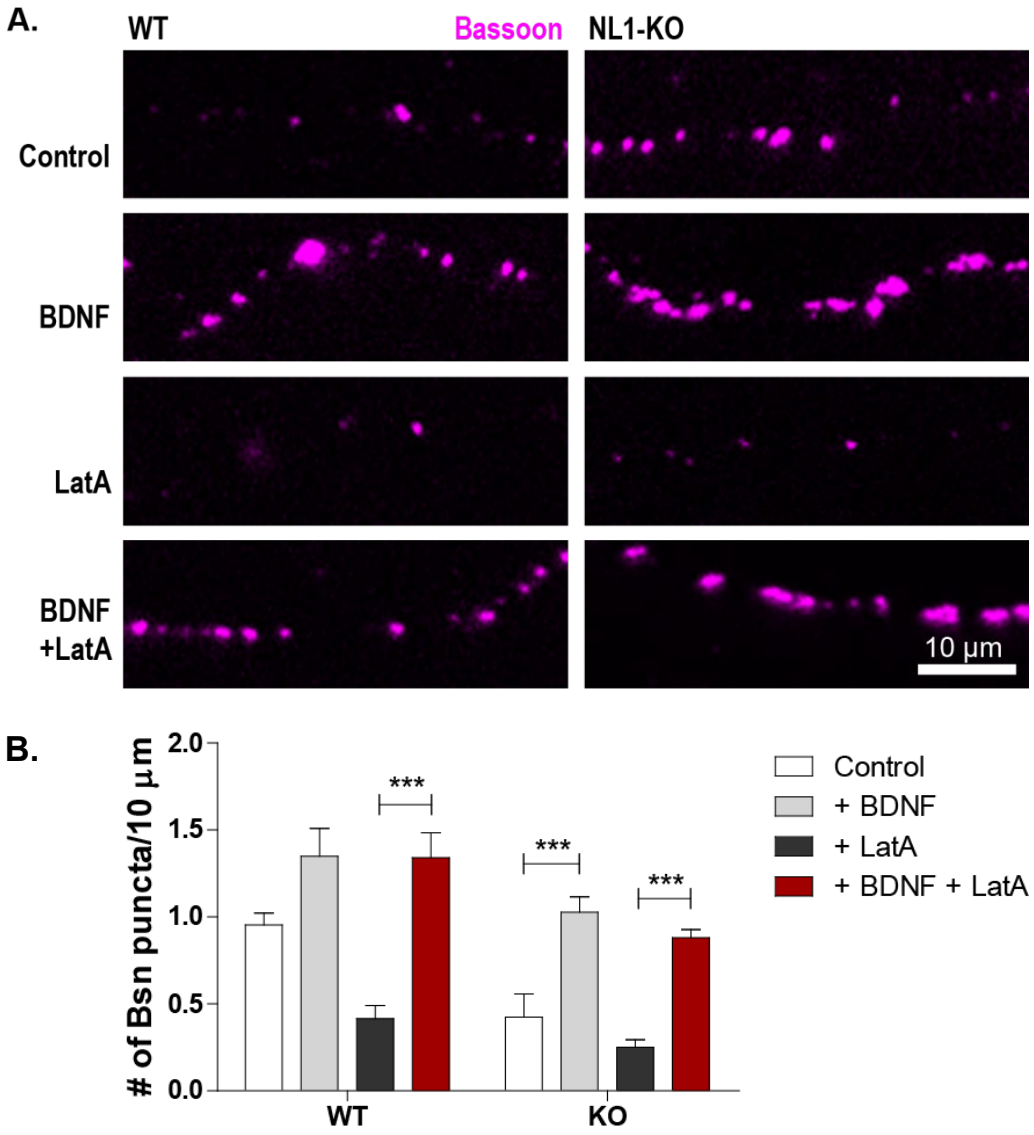

Figure 20: BDNF enhances structural presynaptic maturation in the absence of NL1. (A) Representative images of cultured cortical neurons from wild-type and NL1-knockout mice fixed on DIV6. Prior to fixation, the neurons were treated with BDNF for 22 h, LatA for 6 h, or both (see labeling on the left). Following fixation, the neurons were immunolabeled with a bassoon antibody (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post-hoc Sidak tests: effects of treatment (***$p < 0.0001$; $F(3, 232) = 29.52$) and genotype (***$p < 0.0001$; $F(3, 232) = 25.46$) are significant ***$p < 0.0001$ for wild type + LatA vs. wild type + BDNF + LatA, knockout control vs. knockout + BDNF, and knockout + LatA vs. knockout + BDNF + LatA). Scale bar is 10 µm.

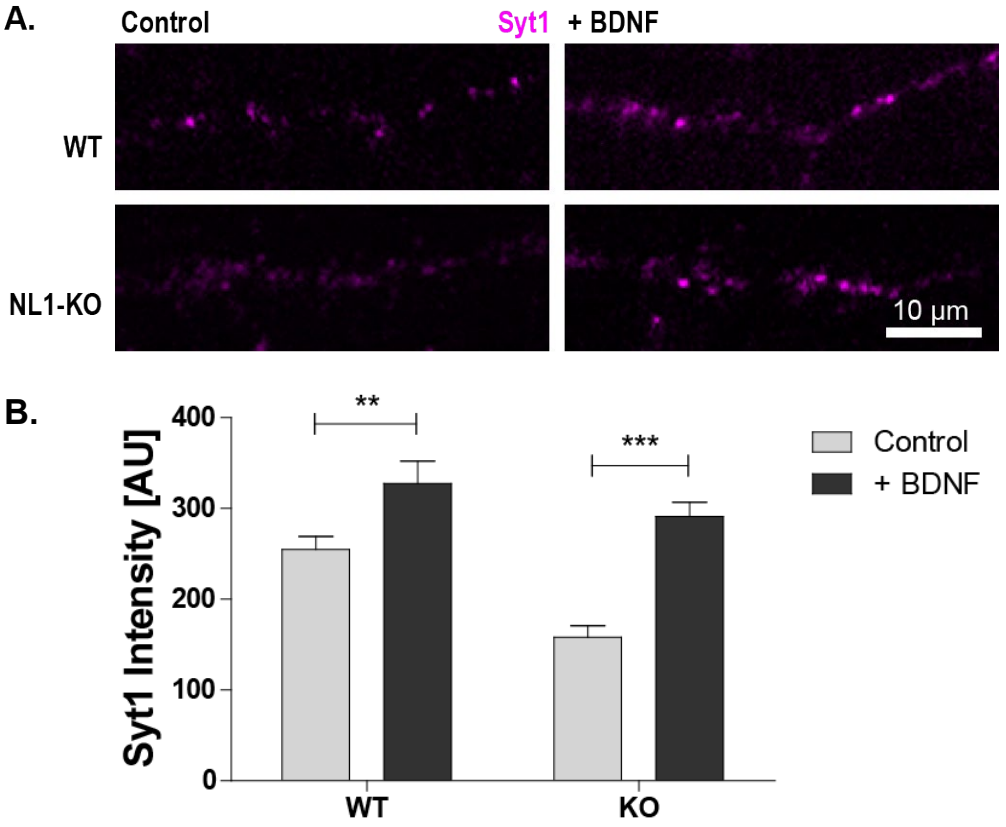

Figure 21: BDNF enhances functional presynaptic maturation in the absence of NL1. (A) Representative images of cultured cortical neurons from wild-type and NL1-knockout mice fixed on DIV6. On DIV6 cultures were stimulated in the presence of antibodies against the lumenal domain of Syt1 to label the recycling of synaptic vesicles. After washing, the neurons were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (magenta). (B) Quantification of the fluorescence intensity of the Syt1 label for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post-hoc Sidak tests: effects of BDNF treatment ($^{***}p < 0.0001$; $F(1, 116) = 28.42$) and genotype ($^{***}p = 0.0008$; $F(1, 116) = 11.83$) are significant; $^{**}p < 0.001$ for wild type control vs. wild type + BDNF, $^{***}p < 0.0001$ for knockout control vs. knockout + BDNF). Scale bar is 10 µm.

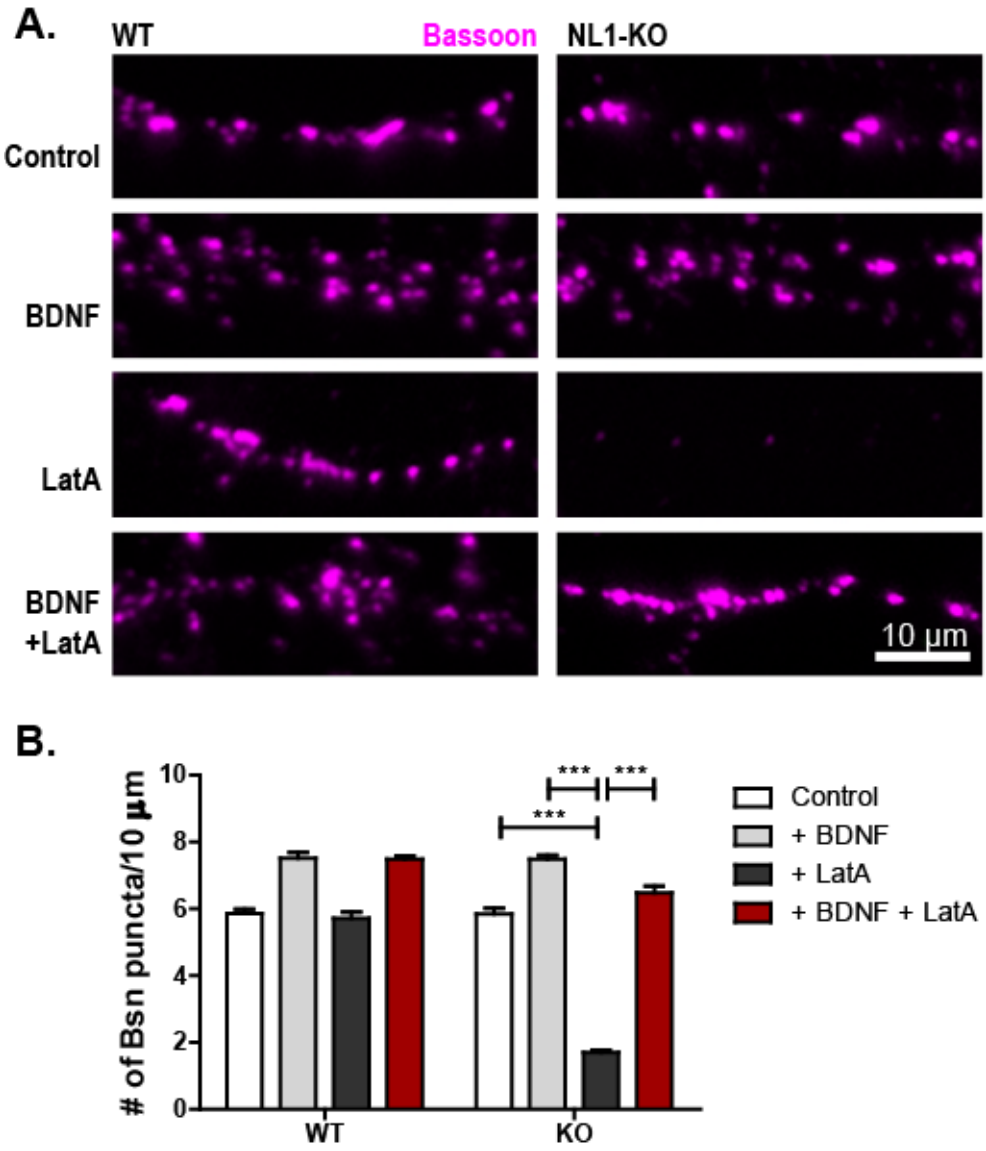

Figure 22: BDNF reestablishes impaired structural presynaptic maturation in the absence of NL1. (A) Representative images of cultured cortical neurons from wild-type and NL1-knockout mice fixed on DIV15. Prior to fixation, the neurons were treated with BDNF for 22 h, LatA for 18 h, or both (see labeling on the left). Following fixation, the neurons were immunolabeled with a bassoon antibody (magenta). (B) Quantification of the number of bassoon puncta per 10 µm dendrite for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post-hoc Sidak tests: interaction is significant: $^{***}p < 0.0001$; $F(3, 232) = 95.61$; $^{***}p < 0.0001$ for knockout control vs. knockout + LatA, for knockout + BDNF vs. knockout + LatA, and for knockout control vs. knockout + BDNF + LatA). Scale bar is 10 µm.

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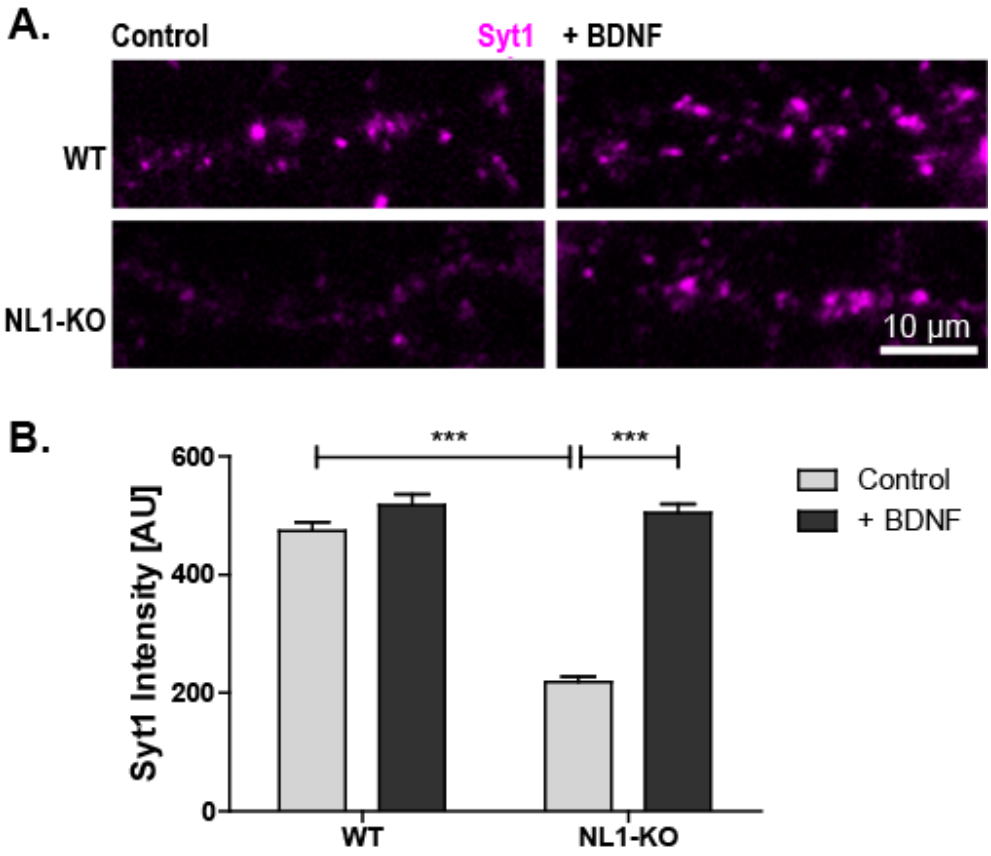

Figure 23: BDNF reestablishes impaired functional presynaptic maturation in the absence of NL1. (A) Representative images of cultured cortical neurons from wild-type and NL1-knockout mice fixed on DIV15. On DIV15, cultures were stimulated in the presence of antibodies against the lumenal domain of Synaptitagmin-1 (Syt1) to label the recycling of synaptic vesicles. After washing, the neurons were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (magenta). (B) Quantification of the fluorescence intensity of the Syt1 label for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post-hoc Sidak tests: interaction is significant: $^{***}p < 0.0001$; $F(1, 116) = 68.32$; $^{***}p < 0.0001$ for wild type control vs. knockout control and for knockout control vs. knockout + BDNF). Scale bar is 10 µm.

This figure has been published in Petkova-Tuffy et al. (2021).

3.6 NL1-induced presynaptic maturation relies on postsynaptic BDNF

It is known that BDNF acts presynaptically and postsynaptically. Interestingly, both pre- and postsynaptic BDNF release has been reported. To examine the origin of BDNF, I once again used hippocampal neuronal cultures from $BDNF^{flox/lox}$ mice. This time, however, they were not transduced with pUBC-Cre–IRES–GFP. Instead, on DIV4, I co-transfected a Cre–IRES–GFP construct together with either NL1–IRES–mOrange or NL2–IRES–mOrange. As a control, I transfected the neurons with GFP together with either NL1–IRES–mOrange or NL2–IRES–mOrange. On DIV6 I treated the cultures with LatA or performed Syt1 antibody uptake to evaluate the state of structural and functional presynaptic maturation (Figures 24 and 25). Interestingly, the results for NL1 and NL2 were very distinct. In the neurons co-transfected with NL1–IRES–mOrange and Cre–IRES–GFP, the number of active zones and the fluorescence intensity of the Syt1 signals were significantly reduced in comparison to the control neurons co-transfected with NL1–IRES–mOrange and GFP. This suggests that in this case NL1 requires postsynaptic BDNF to promote presynaptic maturation. In contrast, in the neurons co-transfected with NL2–IRES–mOrange and Cre–IRES–GFP, there was no significant reduction in the number of active zones or the fluorescence intensity of the Syt1 signals when compared to the control neurons. This suggests that in this case NL2 requires presynaptic BDNF to mediate presynaptic maturation.

Taking into account that NL-induced presynaptic maturation is impaired when BDNF levels have been reduced and that exogenous BDNF reestablished impaired maturation in NL1-knockout cultures, I wanted to test whether NL1 and NL2 overexpression will increase BDNF levels. For this experiment, I transfected rat hippocampal neuronal cultures on DIV3 with NL1–IRES–mOrange or NL2–IRES–mOrange, as well as with mOrange as a control. On DIV 5, I fixed the cultures and immunostained them for BDNF. The fluorescence intensity of BDNF was significantly increased in the neurons overexpressing NL1 and NL2 in comparison to the control neurons (Figure 26).

Taken together, the data compiled in this study indicate that BDNF plays a significant role in NL1- and NL2-induced presynaptic maturation.

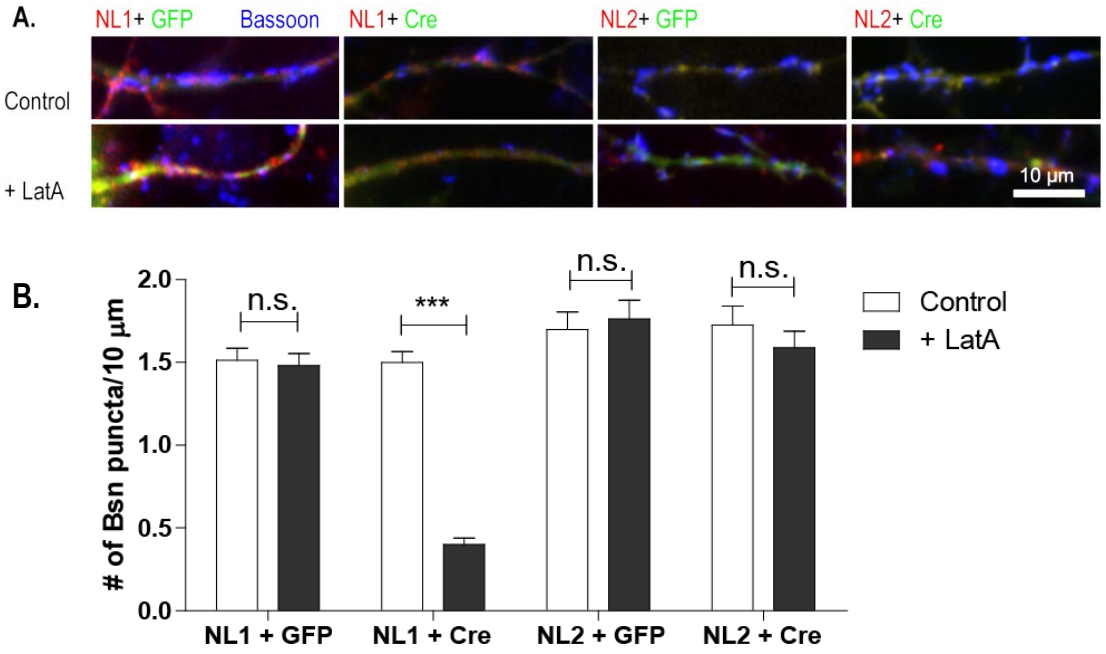

Figure 24: Postsynaptic BDNF is required for NL1-induced structural presynaptic maturation. (A) Representative images of cultured hippocampal neurons from BDNF$^{flox/lox}$ mice co-transfected with NL1–IRES–mOrange (red) + Cre–IRES–GFP (green), NL1–IRES–mOrange (red) + GFP (green), NL2–IRES–mOrange (red) + Cre–IRES–GFP (green), or NL2–IRES–mOrange (red) + GFP (green) on DIV4. Neurons were fixed on DIV6 and immunostained for bassoon (blue) following a 6 h LatA treatment to depolymerize F-actin. (B) Quantification of the number of bassoon puncta for the conditions indicated in panel A (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 18.14$; $^{***}p < 0.0001$ for NL1 + Cre vs. NL1 + Cre + LatA, $p > 0.05$ for NL1 + GFP vs. NL1 + GFP + LatA, NL2 + GFP vs. NL2 + GFP + LatA, and NL2 + Cre vs. NL2 + Cre + LatA). Scale bar is 10 µm.

This figure has been partially published in Petkova-Tuffy et al. (2021).

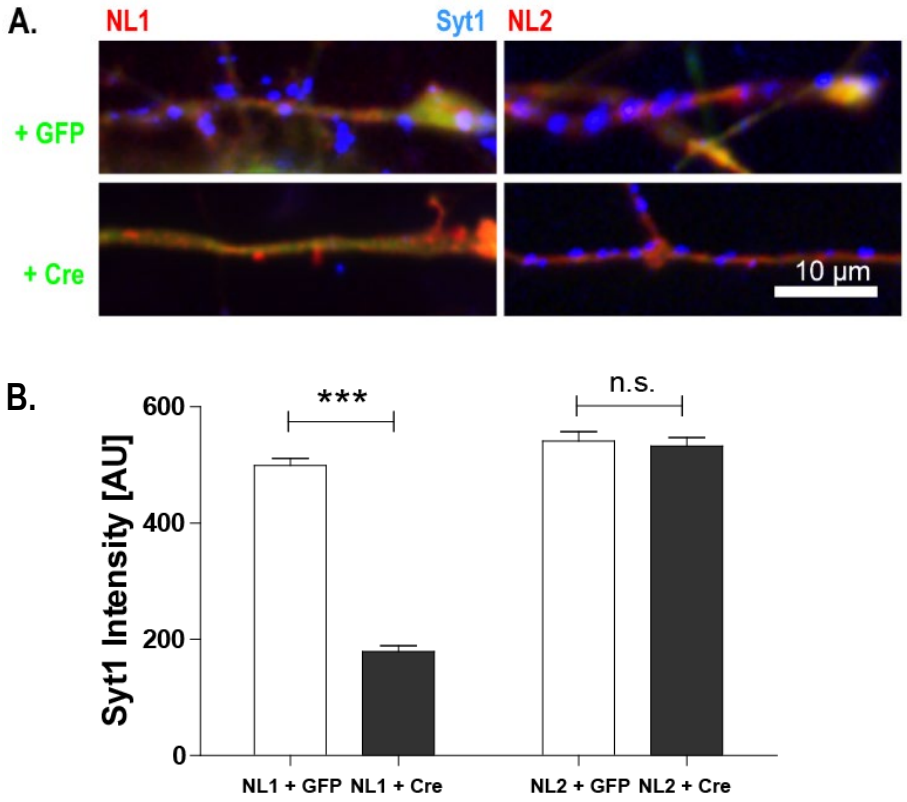

Figure 25: Postsynaptic BDNF is required for NL1-induced functional presynaptic maturation. (A) Representative images of cultured hippocampal neurons from $BDNF^{lox/lox}$ mice co-transfected with NL1–IRES–mOrange (red) + Cre–IRES–GFP (green), NL1–IRES–mOrange (red) + GFP (green), NL2–IRES–mOrange (red) + Cre–IRES–GFP (green), or NL2–IRES–mOrange (red) + GFP (green) on DIV4. On DIV6, cultures were stimulated in the presence of antibodies against the lumenal domain of Syt1 to label recycling synaptic vesicles. After washing, the cells were fixed and immunolabeled with secondary antibodies against the Syt1 antibody (blue). (B) Quantification of the fluorescence intensity of the Syt1 label for the conditions indicated in panel A (mean + SEM; $N = 3$ exp., $n = 10$ cells per experiment and condition; two-way ANOVA with post hoc Sidak tests: interaction is significant, $^{***}p < 0.001$, $F(1, 116) = 139.2$; $^{***}p < 0.0001$ for NL1 + GFP vs. NL1 + Cre, $p > 0.05$ for NL2 + GFP vs. NL2 + Cre). Scale bar is 10 µm.

This figure has been partially published in Petkova-Tuffy et al. (2021).

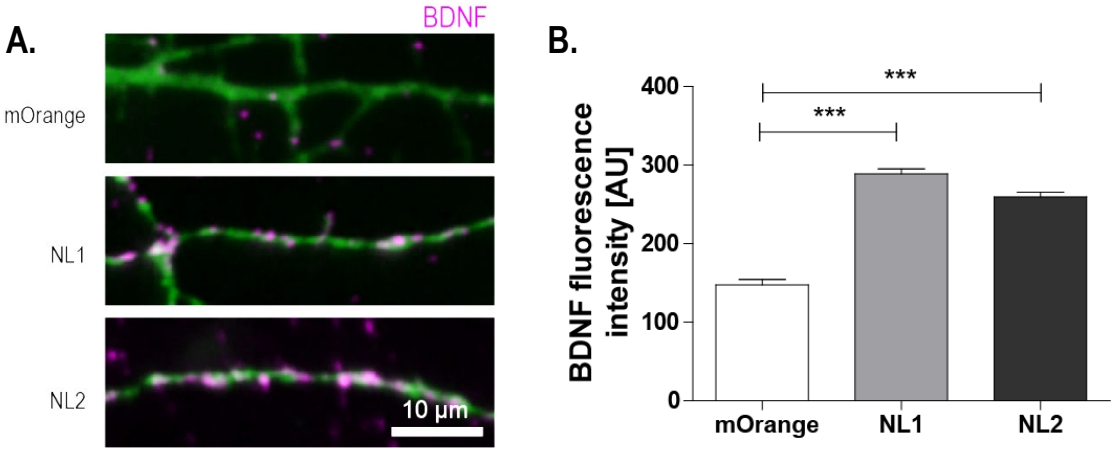

Figure 26: BDNF fluorescence is increased on the dendrites of neurons overexpressing NL1 and NL2. (A) Representative images of BDNF fluorescence on the dendrites of hippocampal neurons transfected with mOrange, NL1–IRES–mOrange, or NL2–IRES–mOrange (green) on DIV3. On DIV5 the neurons were fixed and immunostained for BDNF (magenta). (B) Quantification of the intensity of fluorescent BDNF puncta (mean + SEM; $N = 3$ experiments, $n = 10$ cells per experiment and condition; $^{***}p < 0.001$, one-way ANOVA and post hoc Bonferroni tests). Scale bar is 10 µm.

This figure has been partially published in Petkova-Tuffy et al. (2021).

Chapter Four: Discussion

BDNF is a transsynaptic signaling molecule that is involved in synapse development and has been implicated to play a role in neurodevelopmental diseases, in particular ASD (Ricci et al. 2013; Gilman et al. 2015; Meng et al. 2016) and anxiety (Lang et al. 2005; Moreira et al. 2015). Separately, neuroligins, which are postsynaptic cell adhesion molecules and also play an essential role in synapse formation and maturation, have also been linked to the etiology of ASD (Jamain et al. 2003; Zoghbi 2003; Südhof 2008; Betancur et al. 2009; Ju et al. 2014) and anxiety (Blundell et al. 2009; Babaev et al. 2016; Parente et al. 2017; Chen et al. 2017; Ali et al. 2020).

In this study, I tested the hypothesis that BDNF and neuroligins act in the same pathway in developing cultured neurons to promote synapse formation and presynaptic maturation. I initially found that applying recombinant BDNF and overexpressing NL1 and NL2 have a very similar effect on developing neurons; both induce structural active zone maturation at an immature stage (DIV5). Blocking BDNF signaling using the TrkB-Fc scavenger impaired NL1-induced maturation and NL2-induced active zone formation as well as NL1- and NL2-induced functional maturation, suggesting a link between neuroligins and BDNF. In addition, I found that BDNF-depleted cultures (where *Bdnf* was excised by Cre-recombinase lentiviral transduction in cultures from $BDNF^{lox/lox}$ mice) remain structurally and functionally immature on DIV15. Furthermore, overexpressing NL1 and NL2 no longer induced active zone maturation at DIV6 in BDNF-depleted cultures. These results further confirm previous findings that NL1 regulates presynaptic maturation and BDNF modulates synapse formation and structural and functional maturation (Dean and Dresbach 2006; Krueger et al. 2012; Park and Poo 2013). Interestingly, overexpressing neuroligins increased BDNF fluorescence in cultured neurons, and applying BDNF to cultures from NL1-knockout mice reestablished their otherwise compromised ability to mature. This strongly suggests that NL and BDNF act in the same signaling pathway, where BDNF mediates NL-induced events.

4.1 Methodological considerations

4.1.1 Immunofluorescence

To visualize endogenous proteins (e.g., bassoon, BDNF, synaptotagmin-1), I used an indirect immunofluorescence method. Hereby, a primary antibody binds to an epitope in the protein, and a secondary antibody recognizes the primary antibody, thereby binding indirectly to the protein. The detection of the protein is possible because a fluorescent dye is conjugated to the secondary antibody. The fluorophore is excited by filtered light of a specific wavelength, and the emitted light is recorded on the detector chip of the camera attached to the epifluorescence microscope. Several advantages make immunofluorescence a widely used standard technique: it is a relatively easy and cost-efficient method to label proteins, a wide range of primary antibodies are available for many proteins of interest, and a variety of secondary antibodies with conjugated dyes enable their visualization. This makes the method easily adjustable to specific needs. There are, however, a few disadvantages that should be considered. While there might be many primary antibodies binding to a particular protein, their specificity and binding affinity require verification, and this entails comprehensive testing. In this study, I needed a reliable antibody to label the presynapse; therefore, I resorted to a monoclonal antibody raised in mouse against the Sap7f epitope of bassoon, which is an active zone protein. This antibody shows consistency in its high affinity and specificity. It was also verified in a bassoon knock-out mouse in which the staining was absent (Altrock et al. 2003). This antibody has been used frequently to label active zones (tom Dieck et al. 2005; Fairless et al. 2008; Wittenmayer et al. 2009; Bär et al. 2016; Banerjee et al. 2020; Siddig et al. 2020).

4.1.2 Syt1 uptake assay

I used the synaptotagmin-1 antibody uptake assay to determine the vesicle recycling and thus the functional maturation state of synapses. The method first entails a stimulation of neurons using a Tyrode's solution buffer with a 70 mM K^+ concentration in the presence of a primary antibody against the lumenal domain of synaptotagmin-1. If the vesicle fuses, this lumenal domain is exposed to the medium now containing the primary antibodies, and the antibody will bind. After fixation, the neurons can be stained with secondary antibody using conventional protocols. This assay is a well-established method, as published by us (Riemann et al. 2018) and others (Willig et al. 2006; Fuchs et al. 2013; Shinoda et al. 2014), to ensure that most of the primary antibody in the medium is taken up and that the neurons do not show any

sign of damage. We should note that the neurons were stimulated for 5 min, and this stimulation may not correspond to physiological conditions. However, to what extent these conditions reflect physiological stimulation was inconsequential, because I used the assay to compare control synapses with manipulated synapses. Importantly, I used stimulation conditions employed before by our lab to label active synapses in control neurons and neurons overexpressing NL1 (Wittenmayer et al. 2009). Thus, my new data are directly comparable to the study that prompted my hypothesis.

4.1.3 Overexpression

I achieved overexpression of proteins by using the $CaPO_4$ transfection technique. In this experiment, the neurons are exposed to a sterile mixture of distilled water, $CaCl_2$, a plasmid, and transfection buffer containing Na_2HPO_4. During the incubation time of the mixture, the plasmid together with $CaCl_2$ and the transfection buffer forms crystals that later on, during the incubation time in the neuronal medium, make pores in the membrane of the neurons and allow the delivery of the plasmid into the cell. The neuron will then overexpress the protein encoded in the plasmid. To ensure that only neurons will overexpress the protein of interest, I used a protocol optimized for the transfection of neurons (Dresbach et al. 2003). Admittedly, transfection can potentially harm the neurons, which is why I was very meticulous in selecting only the healthiest-looking neurons showing no disintegration or rupture of dendrites and no large aggregates. Strong overexpression could lead to artifacts that would not be representative of the function of the protein of interest. For this reason, I excluded neurons that showed known signs of strong overexpression such as unnatural extremely high fluorescence and large localized clusters. However, it was necessary to use overexpression of neuroligins in my experimental paradigm, because neuroligins are not integrated into the plasma membrane at the early stage of development that I studied (Wittenmayer et al. 2009). In addition, my Western blot experiments (Figure 19) confirm that the expression of NL1 and NL2 is below the detection limit on DIV6. I took advantage of this, as it allowed me to specifically test if neuroligins would induce presynaptic maturation at a very early stage of development. Overexpressing neuroligins and applying BDNF before DIV7 were gain-of-function assays that enabled me to study the effects of neuroligins and BDNF early on.

4.1.4 Pharmacological treatments

I employed several pharmacological treatments to test for induced presynaptic maturation and to find out whether neuroligins and BDNF could act in the same

pathway during the process of presynaptic maturation. First, I applied recombinant BDNF once to check if BDNF has the same effect as neuroligins on establishing structural maturation and later in the course of my study to check if BDNF would help reestablish presynaptic maturation in neuronal cultures from NL1-knockout mice. Generally, BDNF expression levels are particularly low in the brain (Barde et al. 1982; Park and Poo 2013), but even minor changes can have a high impact on the function of synapses. The expression levels are tightly regulated by neuronal activity. Here, I used an established protocol for applying recombinant BDNF, which exceeds normal levels yet would clearly show what the effect of the neurotrophin on presynaptic maturation is. BDNF could bind to p75NTR and hence lead to apoptosis (Lee 2001). In my experiments, BDNF did not reduce but in fact increased the number of synapses and enhanced the maturation state of synapses, indicating that apoptosis was not occurring.

I also treated the neurons with latrunculin A to depolymerize F-actin. No notable damage to neurons has been reported using the standardized protocol which I adopted, and the neurons in our cultures show no visible sign of damage. Admittedly, latrunculin A has no effect on small populations of F-actin clusters. This usually occurs in very mature neurons and represents highly stabilized F-actin, which does not become depolymerized even after extremely long (48 h) drug treatment. It is believed that increased activity leads to the formation of these clusters (Zhang and Benson 2001).

It has been established that once active zones of cultured neurons have become independent of F-actin and therefore resistant to the application of latrunculin A, the presynapse is rendered mature (Zhang and Benson 2001; Bozdagi et al. 2004; Wittenmayer et al. 2009). However, it remains unknown what synaptic property this represents. In neurons from NL1-knockout mice, the presynaptic terminals do not become independent of F-actin but their release probability is not altered, indicating that latrunculin-A resistance and synaptic release probability do not correlate (Chubykin et al. 2007; Wittenmayer et al. 2009; Jiang et al. 2017). Here, I hypothesized that specific features of active zone stability are represented by active zones becoming resistant to F-actin depolymerization. Interestingly, after applying anisomycin to block protein synthesis, I detected an increase in the lifetime of bassoon at the active zones of immature synapses brought about by BDNF and NL1, signifying one mechanism of how these proteins can make active zones stable. In a previous study using anisomycin, the authors found that bassoon at active zones had a higher turnover rate in mice placed in an enriched environment (Bednarek and Caroni 2011).

Therefore, one explanation for the NL1 and BDNF interaction leading to latrunculin-A resistance of active zones could be that they stabilize synapses with increased activity following remodeling. If this signaling pathway is impaired, this would lead to continuous remodeling. In four mouse models for autism (patDp/+, NLG3 R451C, BTBR T+tf/J, and Fmr1-knockout mice) the lifetimes of synaptic proteins at dendritic spines are decreased. In addition, the neurons show an increase in spine formation and elimination (Tabuchi et al. 2007; McFarlane et al. 2008; Nakatani et al. 2009; Cruz-Martín et al. 2010; Pan et al. 2010; Okabe 2017). Therefore, NL1 and BDNF may be acting jointly to prevent a continuous formation and elimination of synapses by stabilizing active zones and promoting presynaptic maturation.

Applying TrkB-Fc to scavenge BDNF from the medium has proven very effective (Cheng and Yeh 2003; Gottmann et al. 2009; Matsuda et al. 2009; Henry et al. 2012; Kellner et al. 2014). However, I could not exclude the possibility that there might be residual BDNF affecting presynaptic maturation. This is why I corroborated the results of the experiments using TrkB-Fc with the data from the experiments using Cre recombinase to excise *Bdnf* in the neuronal cultures from $BDNF^{lox/lox}$ mice. The experiments using TrkB-Fc prove that extracellular BDNF is required, while the experiments using BDNF depletion with Cre recombinase prove that BDNF expression is required for NL-induced presynaptic maturation.

4.2 Induction of presynaptic maturation and involvement of BDNF signaling

In this study I showed that BDNF application induces structural presynaptic maturation similarly to NL1 and NL2 overexpression. A previous study has shown that BDNF application also enhances functional presynaptic maturation in old cultures (Shinoda et al. 2014). To study this, the authors of the publication performed a synaptotagmin-1 antibody uptake at a later DIV stage (DIV13–DIV15). I implemented the same experiment to test if NL1 and NL2 overexpression enhances functional presynaptic maturation, and I found that this was indeed the case. These similarities led me to believe that BDNF signaling could be necessary for neuroligins to induce presynaptic maturation. Therefore, my next experimental steps were to perturb BDNF signaling and find out how it would affect NL action. I hypothesized that BDNF signaling is involved in NL-mediated induction of presynaptic maturation; hence, impairing BDNF signaling would interfere with this process. My three main findings are the following. Firstly, scavenging BDNF from the culture medium using

TrkB-Fc significantly impaired functional presynaptic maturation in NL1- and NL2-overexpressing neurons. Secondly, it led to a significant decrease in the number of active zones formed on the dendrites of NL2-overexpressing neurons. Keeping in mind that NL2 is found primarily in inhibitory synapses, this result is in line with studies showing that BDNF is necessary especially for inhibitory synapse formation (Rutherford et al. 1997; Vicario-Abejón et al. 1998; Paul et al. 2001; Yamada et al. 2002). Thirdly, scavenging BDNF from the culture medium impaired structural presynaptic maturation in NL1-overexpressing neurons. As NL1 is excitatory synapse specific, this result is in agreement with studies proving that BDNF is necessary for inducing maturation at excitatory synapses (Tyler and Pozzo-Miller 2001; Gottmann et al. 2009; Yoshii and Constantine-Paton 2010).

At this stage, my results concerning the induction of structural presynaptic maturation in neurons overexpressing NL2 were inconclusive, as my analysis did not reveal a significant reduction in the number of bassoon puncta following latrunculin-A treatment after scavenging BDNF. However, the number of active zones formed was significantly reduced following only TrkB-Fc treatment, and I could not exclude the effect that any residual amount of BDNF could aid the few synapses formed to reach a structurally mature state. For this reason, I also investigated whether NL1- and NL2-overexpressing neurons would reach a mature state early on in BDNF-depleted neuronal cultures where *Bdnf* is excised using Cre recombinase in neurons from $BDNF^{flox/lox}$ mice. Initially, I tested whether these cultures reached a mature state naturally, and my results show that both structural maturation and functional maturation are impaired on DIV15 when these events should already have occurred (Zhang and Benson 2001). When I overexpressed NL1 and NL2 in these neuronal cultures, both proteins failed to induce structural and functional maturation in the absence of BDNF expression. Interestingly, in neurons overexpressing the isoform NL2, there was a significant initial reduction in the number of bassoon puncta, which then further decreased upon F-actin depolymerization. This result hints towards the importance of BDNF for inhibitory synapses in two ways – on the one hand, for their formation and, on the other hand, for their functional maturation. This observation is once again in agreement with studies showing that BDNF is necessary for the formation and maturation of inhibitory synapses (Rutherford et al. 1997; Vicario-Abejón et al. 1998; Paul et al. 2001; Yamada et al. 2002).

This study introduces, for the first time, a previously unknown connection between BDNF function and NL-induced presynaptic maturation. Therefore, it is fitting that my results are corroborated by previous findings for BDNF function.

4.3 Presynaptic and postsynaptic BDNF

One of the most challenging questions concerning BDNF function is whether BDNF is secreted presynaptically or postsynaptically. Somatic (Brigadski 2005 et al. 2005; Kolarow et al. 2007; de Wit et al. 2009; Adachi et al. 2013); dendritic, i.e., postsynaptic (Hartmann et al. 2001; Brigadski et al. 2005; Kolarow et al. 2007; de Wit 2009; Dean 2009: Matsuda 2009; Adachi et al. 2013); and axonal, i.e., presynaptic (Kohara et al. 2001; Dean et al. 2009; Matsuda 2009; Shinoda et al. 2011) release of BDNF has been described so far in *in vitro* studies of hippocampal and cortical neuronal cultures. Presynaptic BDNF secretion has also been reported in acute slices (Jia et al. 2010), while postsynaptic BDNF secretion has been reported in acute slices (Korte et al. 1996) and organotypic slices (Kohara et al. 2007). *In vivo* studies find both presynaptic (Smith et al. 1997; Dieni et al. 2012) and postsynaptic (Arancibia et al. 2007) localization and release of BDNF. To address this, I co-transfected neurons with fluorescently tagged versions of either NL1 and Cre-IRES-GFP or NL2 and Cre-IRES-GFP – the Cre recombinase to excise *Bdnf*. In this experimental setting, my aim was to only suppress BDNF expression locally as opposed to a global suppression in the case of lentiviral infection. This way, I was able to determine whether the BDNF necessary for NL action on presynaptic maturation is coming from the neuron expressing the NL construct, i.e., the postsynaptic neuron, or other presynaptic neurons. Having this sparse inhibition of BDNF expression practically in only the neurons that are also overexpressing the neuroligin constructs, I could show that since NL1 was no longer able to induce presynaptic maturation. Thus, postsynaptic expression and by extension postsynaptic release of BDNF are definitely required for the NL1-induced presynaptic maturation. While the experiment shows this clearly, it does not rule out the possibility that presynaptic release may be required in addition. To test this, the opposite experiment needs to be conducted: presynaptic knockout of BDNF combined with postsynaptic expression of NL1. This is a technically challenging experiment, because this way only a subset of synapses can be analyzed where the axon of a presynaptic BDNF-knockout neuron contacts a postsynaptic NL1-overexpressing neuron. Interestingly, NL2 still induced both structural and functional maturation in these experiments. This result would argue that exclusively presynaptic BDNF is required in the case of NL2-induced presynaptic maturation. This could mean that there are two distinct mechanisms by which BDNF mediates NL-induced events.

4.4 Possible mechanism and BDNF rescue

More than 4500 genes have been implicated in ASD; however, each one accounts for a maximum of 1–2 % of cases (Basu et al. 2009). Interestingly, the proteins as products of these genes fall into convergent pathways, with one example being the mTOR pathway (Bourgeron 2009; Sawicka and Zukin 2012; Wang and Doering 2013; Takei and Nawa 2014; Ebrahimi-Fakhari and Sahin 2015; Sato 2016). This is why recent attempts to look for a therapeutic target have focused on finding the pathways where the proteins implicated in ASD act (Ebert and Greenberg 2013). Here, I propose that neuroligins and BDNF act in the same pathway to induce presynaptic maturation.

While BDNF does act in the mTOR pathway, neuroligins have thus far not been shown to act directly in this pathway (Henry et al. 2012). Nevertheless, neuroligins establish a physical link between the postsynaptic group-I mGluR/Homer/Shank/PSD-95 complex and presynaptic elements through interactions with neurexins (O'Connor et al. 2014). Activation of postsynaptic mGluRs triggers several signaling pathways, including the mTOR pathway (Ebert and Greenberg 2013). Keeping my findings in mind, I propose an alternative pathway in which BDNF and neuroligins could act together to induce presynaptic maturation. Namely, NL overexpression or BDNF application would increase highly stable clusters of F-actin via the LIMK1/cofilin pathway. BDNF is regulated in an activity-dependent manner and appears to be acting downstream of neuroligins. Therefore, I suggest that neuroligins in an initial step will increase BDNF secretion and expression. Then BDNF via TrkB dimerization will lead to LIMK1 dimerization and transphosphorylation, which in turn phosphorylates cofilin. This inactive form of cofilin results in the stabilization of F-actin (Figure 27). The LIMK1/cofilin pathway has already been explored in the context of mouse models of autism: in SHANK3-deficient mice, autism-related symptoms were rescued by inhibiting the non-phosphorylated form of cofilin (Duffney et al. 2015). We should not, however, exclude the possibility that BDNF can activate the mTOR signaling pathway, which in turn can then activate LIMK1 via rac1 (Huber et al. 2015).

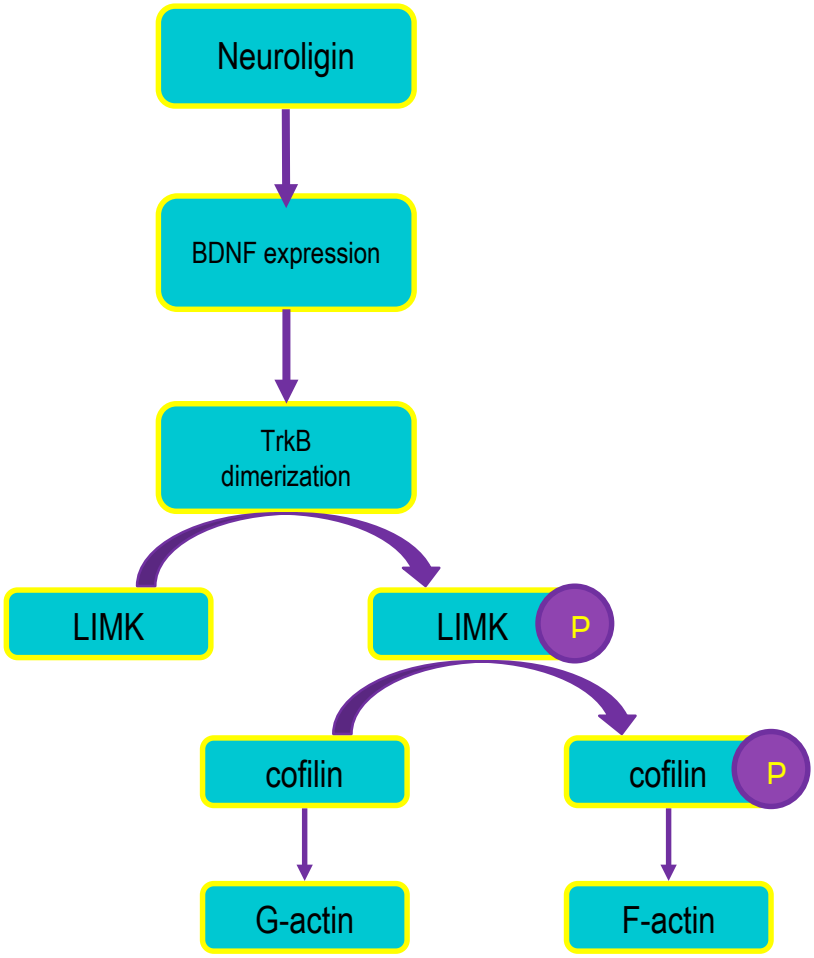

Figure 27: A chart outlining one possible pathway in which neuroligins and BDNF act together to influence F-actin and thus structural presynaptic maturation.

Some actions of neuroligins depend on neurexins, while others do not. For example, binding specifically to alpha-neurexins is essential for the function of NL1 in inducing synapse formation onto non-neuronal cells. However, neurexin binding is not required for enhanced synapse formation upon NL1 overexpression in neurons (Ko et al. 2009). Interestingly, in mice lacking all NL isoforms, overexpression of NL1 induces NMDA-dependent long-term potentiation but only if it can bind to neurexins, indicating that neurexin binding is essential in this scenario. In contrast, overexpressing an NL1 variant lacking its intracellular domain cannot rescue the decreased NMDA/AMPA ratio in quadruple NL-knockout mice, indicating that only the intracellular domain but not neurexin binding is essential in this scenario (Wu et al. 2019). This would mean that the intracellular domain of NL1 is necessary for the recruitment of NMDA receptors. This recruitment of NMDA receptors and their subsequent activation could be necessary for triggering BDNF expression (Zafra et al.

1991; Ghosh et al. 1994). Interestingly, a variant of NL1 lacking the intracellular domain fails to induce early structural presynaptic maturation, indicating that the intracellular domain is required for this action of NL1 (Wittenmayer et al. 2009). So far, my results regarding the induction of presynaptic maturation suggest that neurexin binding is not essential for these processes, as overexpressing a mutated NL1 construct that does not bind to neurexins showed no loss of the ability to induce presynaptic maturation. However, neuroligins have been shown to form homo- and heterodimers (Poulopoulos et al. 2012). And while neuroligins do not get integrated into the cell membrane early on, we cannot exclude the possibility that the overexpressed mutated isoform of NL1 could dimerize with endogenous NL1 and in this way induce presynaptic maturation. A previous study by T. Biederer and T. Südhof shows that CASK and protein 4.1 interact with the cytosolic tail of neurexin to induce local assembly of actin/spectrin filaments (Biederer and Südhof 2001). Therefore, an interaction between neuroligins and neurexins, additionally supported by BDNF action, could be linked to localized cytoskeleton modifications, circumventing the LIMK1/cofilin pathway.

Another possible mechanism could be local recruitment and phosphorylation of receptors. It has been shown that NL1 recruits NMDA receptors, and BDNF recruits them and increases their activity. However, BDNF is expressed and secreted in an activity-dependent manner. Therefore, it is possible that in a first step neuroligins need to recruit NMDA receptors, thus increasing the activity of the neuron, and in this way lead to BDNF release. Then BDNF would lead to the phosphorylation of NMDA receptors and increase their activity. My two proposed mechanisms are not necessarily separate. It has been shown that increased activity induces the formation of highly stable F-actin clusters. This would mean that F-actin polymerization could be the result of LIMK1/cofilin activation by BDNF as a result of increased neuronal activity following NMDA receptor recruitment by NL1.

While there is no direct link between neuroligins and the mTOR pathway so far, it should not be excluded as a possible target pathway. NL1 has been shown to recruit NMDA receptors whose activation results in Ca^{2+} influx (Barrow et al. 2009). This in turn increases neuronal activity and activates CaMKII. Increased neuronal activity triggers BDNF secretion, and secreted BDNF binds to TrkB which activates, among others, the mTOR signaling pathway (Ebert and Greenberg 2013; Park and Poo 2013). Interestingly, Gephyrin, a postsynaptic scaffolding protein that binds to NL2, has been shown to directly bind to mTOR (Sabatini et al. 1999). This link, however, remains to be further explored.

One more reason to explore alternative pathways linked to ASD is that one of the most promising therapeutic targets – mGluR5 – has had no success in clinical trials to alleviate ASD symptoms (Mullard 2015).

Since I found that BDNF acts downstream of neuroligins and that its application can reestablish failed synaptic maturation in cultures from NL1-knockout mice, increasing BDNF levels locally or globally in the brain of subjects with ASD could represent a novel therapeutic solution. It would therefore be extremely interesting to study at first whether BDNF and neuroligins would also cooperate *in vivo* to induce presynaptic maturation. Remarkably, one study has already shown that a local BDNF infusion in the rat brain is possible; furthermore, if the infusion is performed in the nucleus accumbens of aged rats with impaired cognition, it improves their symptoms (Li et al. 2012). To what degree the BDNF–NL interaction is present *in vivo* and whether compensatory mechanisms exist remains to be tested. One intriguing possibility could be to breed mice with ASD-like symptoms with synaptotagmin-IV knock-out mice, which show increased BDNF secretion (Dean et al. 2009) to find out whether the symptoms will be relieved.

4.5 The big picture and excitation/inhibition ratio

Epilepsy is a symptom that is very commonly associated with autism and occurs in up to one-third of individuals with ASD (Muhle et al. 2004). In 2001, J. Hussman proposed that autism may be the result of an altered excitation/inhibition balance in neurotransmitter release in the brain (Hussman 2001). He suggested that impaired GABAergic transmission emerges as a common factor in several autism etiologies and that both compromised GABAergic transmission and excessive stimulation of non-NMDA glutamate receptors generate autism-like pathology. This hypothesis was further explored and explained in detail by J. Rubenstein and M. Merzenich (Rubenstein and Merzenich 2003). Since then, several studies have confirmed that an increased excitation/inhibition ratio leads to hyper-excitability in cortical circuits in the brains of autistics (Bear et al. 2004; Bateup et al. 2013; Lozano et al. 2014; Nelson and Valakh 2015). Interestingly, some recent studies have found the opposite instead – a decreased excitation/inhibition ratio (Carlsson 1998; Dani et al. 2005; Dani and Nelson 2009; Etherton et al. 2009; Etherton et al. 2011; Rabaneda et al. 2014; Nelson and Valakh 2015). It remains to be shown what the underlying causes for a shift in the excitation/inhibition ratio are and whether neuroligins and BDNF play a role in the events involved, particularly in the early stages of circuit development.

Interestingly, neuroligins 1–4, which have been implicated in a number of autism cases, show neuron subtype specificity. While NL1 is primarily found at excitatory synapses, NL2 is predominantly found at inhibitory synapses (Song et al. 1999; Varoqueaux et al. 2004). NL3 is found at both excitatory and inhibitory synapses (Budreck and Scheiffele 2007), and NL4 in mice is located at glycinergic synapses (Hoon et al. 2011). Thus, impairing one neuroligin isoform has the potential for shifting the excitation/inhibition balance. As an example, global knockout of NL1 in mice reduces the strength of glutamatergic synapses by reducing NMDA receptor recruitment (Chubykin et al. 2007), while overexpression of NL1 in acute slices increases NMDA receptor recruitment, synaptic strength, and the stability of active excitatory synapses (Budreck et al. 2013). Mice lacking neuroligins 1–3 show impaired evoked and spontaneous GABAergic/glycinergic transmission in the brainstem (Varoqueaux et al. 2006). Knocking out NL1 in mice decreases the NMDA/AMPA receptor ratio, while knocking out NL2 in mice decreases inhibitory synaptic responses (Chubykin et al. 2007). In the NL3 R451C knock-in mutant mouse, the NMDA/AMPA receptor ratio was increased (Tabuchi et al. 2007). Therefore, a shift in the expression of some neuroligin isoforms could lead to an altered excitation/inhibition ratio.

BDNF, on the other hand, has effects on both excitatory and inhibitory synapses. In cultured hippocampal neurons, BDNF promotes the formation of both excitatory and inhibitory synapses and the maturation of GABAergic synapses (Vicario-Abejón et al. 1998; Huang et al. 1999). Chronic BDNF application has been shown to increase the frequency of AMPA receptor-mediated excitatory postsynaptic currents, while reducing BDNF/TrkB signaling decreases the amplitudes of excitatory postsynaptic currents and the number of presynaptic release sites (Vicario-Abejón et al. 1998; Collin et al. 2001; Klau et al. 2001; Paul et al. 2001; Tyler and Pozzo-Miller 2001). Interestingly, *in vivo* studies on BDNF knock-out mice showed no difference in the number of glutamatergic synapses, whereas synapse formation was largely impaired in cultured neurons from these mice, hinting that there are strong compensatory mechanisms *in vivo* (Korte et al. 1995; Itami et al. 2003; Singh et al. 2006; Gottmann et al. 2009). BDNF application in neuronal cultures enhances the formation and maturation of GABAergic synapses by increasing the expression of GAD65 and recruiting presynaptic Ca^{2+} channels and $GABA_A$ receptors (Rutherford et al. 1997; Vicario-Abejón et al. 1998; Marty et al. 2000; Yamada et al. 2002; Henneberger et al. 2005). Overexpressing BDNF in the cortex *in vivo* accelerates the maturation of GABAergic synapses as well (Huang et al. 1999; Gianfranceschi et al. 2003). Taken

together, all these findings suggest that BDNF is a contributing factor for the normal development of both excitatory and inhibitory synapses. Therefore, changes in BDNF expression levels are likely to lead to an altered excitation/inhibition ratio and diseases resulting from it.

My findings show that BDNF signaling is required for distinct aspects of neuroligin action, namely, that perturbing BDNF signaling reduces the number of synapses formed on the dendrites of NL2-overexpressing neurons and impairs the presynaptic maturation induced by both NL1 and NL2. If neuroligins and BDNF cooperate also *in vivo* to induce presynaptic maturation, and considering that this process can be synapse subtype specific, a pathway in which they act together would be very interesting to explore. Knowing that BDNF application can rescue impaired presynaptic maturation in cultures from NL1 knock-out mice, this might represent the next therapeutic strategy.

It has been proposed that while there is a shift in the excitation/inhibition balance in ASD, there are also homeostatic processes taking place that might reactively alter the balance as a result of primarily increased excitation or inhibition (Nelson and Valakh 2015). Studies in a variety of model systems have identified mechanisms of neuronal and synaptic function that homeostatically regulate network activity (Turrigiano and Nelson 2004; Davis 2006; Pozo and Goda 2010; Turrigiano 2011). These mechanisms are in place in order to attempt to return the impaired system to a predetermined balanced level by changing the intrinsic neuronal excitability or the strength and number of excitatory and inhibitory synapses. These mechanisms can be very potent; therefore, it can be difficult to distinguish primary effects from compensatory changes in network function. Using sparse cell-type or pathway-specific studies is necessary to understand the primary deficits, but they do not represent any behavioral phenotype. However, full knock-out approaches *in vivo* do capture behavioral aspects but lack the neuron subtype specificity and are obscured by network and homeostatic effects (Nelson and Valakh 2015).

Such an imbalance in the excitation/inhibition ratio in the neocortex is thought to be the cause of network hyperexcitability in fragile X syndrome (Gibson et al. 2008; Castrén and Castrén 2014; Morin-Parent et al. 2019). Fragile X syndrome is a monogenetic disease of the autism spectrum, which is caused by mutation in a gene called *Fmr1*, encoding a protein called FMRP (Fragile X Mental Retardation Protein). Mice lacking this gene (Fmr1-knockout mice) are a model for studying fragile X syndrome (Oostra and Hoogeveen 1997). This mouse model for autism could be of particular interest, taking into account my findings that BDNF signaling is required

for NL-induced presynaptic maturation. Spine formation and elimination are increased in the barrel cortex of Fmr1-knockout mice, which is characteristic of immature neurons, and these abnormalities are likely the result of impaired synaptic stabilization (Pan et al. 2010). Intriguingly, in Fmr1-knockout mice, the levels of NL1 are reduced and overexpressing NL1 improves the social behavior in these mice (Dahlhaus und El-Husseini 2010). This reduced NL1 function could result in impaired BDNF expression and, as a consequence, reduced synaptic maturation in these mice. What makes this even more interesting is the finding that BDNF actions are indeed implicated in the abnormal synapse function in fragile X syndrome: BDNF application in acute hippocampal slices from Fmr1-knockuot mice can fully restore defective long-term potentiation in the CA1 region (Lauterborn et al. 2007).

With this study, I have made the first steps to uncovering the cooperation of neuroligins and BDNF in promoting presynaptic maturation. This could be a new converging pathway worth exploring on the quest to treat neurodevelopmental disorders.

Summary

Mutations in genes for postsynaptic cell adhesion molecules called neuroligins – in particular the isoforms neuroligin-1, neuroligin-3, and neuroligin-4 – have been implicated in autism spectrum disorders. Based on these genetic findings, genetically modified mouse models have been generated and their phenotype studied to check for features similar to the manifestation of autism spectrum disorders in humans.

Interestingly, neuroligin isoforms are neuron-subtype specific, meaning that neuroligin-1 is expressed in excitatory neurons, neuroligin-2 and neuroligin-4 in inhibitory ones, and neuroligin-3 has been found in both subtypes. Thus, mutating one of these isoforms, resulting in a loss of its function, could have a dramatic effect on the excitation/inhibition ratio. In cultured neurons, neuroligin-1 mediates structural and functional maturation of presynaptic terminals. What still remains untested, however, are the transsynaptic signals that allow neuroligins, being postsynaptic, to regulate presynaptic maturation. So far, it has been expected that the interaction of neuroligins and their presynaptic partners, the neurexins, is involved. However, the precise mechanism has not been established.

Brain-derived neurotrophic factor is an activity-dependent secreted molecule that has been implicated in many mental disorders, including autism spectrum disorders. It has been shown to support the survival and differentiation of neurons, induce synapse formation and maturation, and modulate synaptic transmission. In addition, brain-derived neurotrophic factor also has distinct properties in regard to excitatory and inhibitory neuronal subtypes.

Here, I introduce brain-derived neurotrophic factor as a potential mediator of neuroligin-induced presynaptic maturation. Using neuronal rat and mouse cultures from either wild-type or genetically modified animals, as well as organotypic hippocampal slice cultures from rats, I explored the link between these proteins. I employed several assays to test the structural and functional maturation state of the presynaptic active zones – depolymerization of F-actin using latrunculin A, temporary protein synthesis inhibition using anisomycin, and synaptotagmin-1 antibody uptake. Taking advantage of these methods, I found that brain-derived neurotrophic factor, neuroligin-1, and neuroligin-2 display similar properties in regard to inducing presynaptic maturation. By perturbing brain-derived neurotrophic factor signaling, I discovered that it is required for neuroligin-induced presynaptic maturation and that specifically postsynaptic brain-derived neurotrophic factor is required for neuroligin-1-induced presynaptic maturation. Remarkably, application of brain-derived

neurotrophic factor was able to restore impaired presynaptic maturation in cultures from neuroligin-1 knock-out mice.

Taken together, my results show that neuroligins and brain-derived neurotrophic factor act in the same pathway to induce presynaptic maturation. The ability of brain-derived neurotrophic factor to rescue defective maturation in neuroligin-1 knock-out animals could provide a potential therapeutic target for autism spectrum disorders.

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