

**α 5-containing GABA_A receptor in proximity to
excitatory synapses**

and

**Potential new binding partner of radixin on the
plasma membrane of hippocampal neurons
Mass Spectrometry study**

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Diese Thesis widme ich meiner Familie, deren stetige Liebe und
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This thesis is dedicated to my family, whose constant support and love I
am deeply grateful to experience!

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Abstract

Excitatory and inhibitory synaptic balance plays a crucial role in memory formation within the hippocampus. The latest research shows that these two types of synapses seem to regulate one another to a certain degree. Over the past two decades, increasing evidence of inhibitory synapses forming on spines carrying excitatory synapses has challenged the traditional understanding of synaptic and spine organization. However, the function and development of these spines remain not fully understood.

One GABA_A receptor of particular interest is the $\alpha 5$ -containing GABA_A receptor. Its ability to mediate tonic inhibition at extrasynaptic sites and phasic inhibition at synaptic sites, as well as its activity-dependent diffusion between these regions, suggests it may play a role in the development of dually innervated spines.

In the main project of this thesis, the presence of $\alpha 5$ -containing GABA_ARs on spines in the proximity of excitatory synapses is examined. The data suggest a subpopulation of $\alpha 5$ -containing GABA_ARs near excitatory synaptic protein markers such as PSD95, GluR2, and VGLUT1. Furthermore, using signal particle tracking, the study demonstrates that the diffusion speed of $\alpha 5$ -containing GABA_ARs decreases in PSD95-positive regions. Additionally, a novel phenotype is observed, characterized by an increased density of synaptophysin and VGLUT1 puncta in the absence of $\alpha 5$ -containing GABA_ARs. *Gabra5* knockout-derived neurons also exhibit an increased number of excitatory synapses. A potential role of radixin is indicated as increased colocalization of $\alpha 5$ -containing GABA_ARs and PSD95 cluster is observed in *RDX* knockout-derived dendrites. These findings suggest a new role of $\alpha 5$ -containing GABA_A receptors near excitatory synapses and applying a stronger influence than previously thought.

This thesis's second project involves identifying potential new binding partners of radixin, the scaffold protein of $\alpha 5$ -containing GABA_A receptors, at the plasma membrane of hippocampal neurons. To our knowledge, this study represents the first comparison of radixin immunoprecipitation samples from *RDX* wildtype and knockout hippocampal lysates using mass spectrometry. The analysis identifies 45 significantly more abundant proteins in wildtype samples than in knockout. Promising candidates, including Ap2B1, DLG4, bassoon, and GKAP, are further investigated using co-immunoprecipitation, Western Blot, and immunocytochemistry. The mass spectrometry data reveal several novel interactions related to pre-and postsynaptic organization and structures. These findings could suggest new roles for radixin in excitatory synapses, axonal guidance, and neurodevelopment.

German Abstract

Die Balance zwischen exzitatorischen und inhibitorischen Synapsen spielt eine entscheidende Rolle bei der Gedächtnisbildung im Hippocampus. Die neusten Forschungen zeigen, dass diese beiden Synapsentypen in gewissem Maße gegenseitig regulierend wirken. In den letzten zwei Jahrzehnten haben zunehmende Beweise für inhibitorische Synapsen, die sich an Spines mit exzitatorischen Synapsen bilden, das traditionelle Verständnis von Synapsen- und Spineorganisation infrage gestellt. Die Funktion und Entwicklung dieser Spines ist jedoch nicht vollständig verstanden.

Ein besonderer Fokus liegt auf den $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptor. Seine Fähigkeit, sowohl tonische Inhibition an extrasynaptischen Bereichen als auch phasische Inhibition an synaptischen Stellen zu vermitteln, sowie seine aktivitätsabhängige Diffusion zwischen diesen Bereichen, deutet darauf hin, dass er eine Rolle bei der Entwicklung von dual innervierten Spines spielen könnte.

Im Hauptprojekt dieser Thesis wird das Vorkommen von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren in der Nähe von exzitatorischen Synapsen an Spines untersucht. Die Daten weisen auf eine Subpopulation von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren in der Nähe von exzitatorischen synaptischen Proteinmarkern wie PSD95, GluR2 und VGLUT1 hin. Mithilfe des Single-Particle-Trackings wird zudem gezeigt, dass die Diffusionsgeschwindigkeit von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren in PSD95-positiven Regionen abnimmt. Darüber hinaus wird ein neuartiger Phänotyp beobachtet, der durch eine erhöhte Dichte von Synaptophysin- und VGLUT1-Punkten in Abwesenheit von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren gekennzeichnet ist. Neuronen von *Gabra5*-Knockout-Mäusen zeigen auch eine erhöhte Anzahl an exzitatorischer Synapsen. Eine potenzielle Rolle von Radixin wird durch die erhöhte Kolo-kalisierung von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren und PSD95-Clustern in Dendriten von RDX-Knockout-Mäusen angedeutet. Diese Ergebnisse weisen auf eine neue Rolle von $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren in der Nähe exzitatorischer Synapsen hin, die einen stärkeren Einfluss ausüben könnten als bisher angenommen.

Das zweite Projekt dieser Arbeit befasst sich mit der Identifizierung potenzieller neuer Bindungspartner von Radixin, dem Gerüstprotein der $\alpha 5$ Subtyp enthaltenen GABA_A-Rezeptoren, an der Plasmamembran von Neuronen aus dem Hippocampus. Soweit es uns bekannt ist, stellt diese Studie den ersten Vergleich von Radixin-Immunpräzipitationsproben aus Wildtyp- und Knockout-RDX-Hippocampuslysaten mittels Massenspektrometrie dar. Die Analyse identifiziert 45 signifikant stärker angereicherte Proteine in Wildtyp-Proben im Vergleich zu Knockout-Proben. Vielversprechende Kandidaten, darunter Ap2B1, DLG4, Bassoon und GKAP, werden weiter mittels Ko-Immunpräzipitation, Western Blot und Immunzytochemie untersucht. Die Massenspektrometrie-Daten offenbaren mehrere neuartige Interaktionen im Zusammenhang mit prä- und postsynaptischer Organisation und Strukturen. Diese Ergebnisse könnten auf neue Rollen von Radixin bei exzitatorischen Synapsen, der axonalen Führung und der neuronalen Entwicklung hinweisen.

List of abbreviations

A β ₁₋₄₂	42-amino acid form of amyloid beta
α 1-GABA _A Rs	α 1-containing GABA _A Rs
α 2-GABA _A Rs	α 2-containing GABA _A Rs
α 5-GABA _A Rs	α 5-containing GABA _A Rs
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
AP2b1	β 2-adaptin protein
BSA	Bovine serum albumin
Ca ₂₊	Calcium
CaCl ₂	Calcium chloride
CaMKII	Calmodulin-dependent kinase II
C-ERMAD	C-terminal ERM-association domain
CO ₂	Carbon dioxide
D	Diffusion coefficient
DAB	3,3'-diaminobenzidine
DiS	Dually innervated spines
DLG1	Disc large MAGUK scaffold protein 1
DLG4	Disc large MAGUK scaffold protein 4
DLGAP1	DLG-associated protein 1
DMSO	Dimethyl sulfoxide
DIV	Days in vitro
ddH ₂ O	Double distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF4G1	Eukaryotic translation initiation factor 4 gamma 1
EM	Electron microscopy
EPSPs	Excitatory postsynaptic potentials
ERM-proteins	Ezrin/Radixin/Moesin-proteins
F-actin	Filamentous-actin
GABA	Gamma-aminobutyric acid
GABA _A Rs	γ -aminobutyric acid type A receptor
GFP	Green fluorescent protein
GKAP	Guanylate kinase-associated protein
γ -adaptin	Gamma-adaptin
γ 2-GABAARs	γ 2-containing GABA _A Rs
HBS	HEPES buffered saline
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IPSCs	Inhibitory postsynaptic currents
IPSP	Inhibitory postsynaptic potential
LTD	Long-term depression
LTP	Long-term potentiation
LINGO4	Immunoglobulin-like domain-containing NOGO receptor-interacting

	protein 4
MgCl ₂	Magnesium chloride
MSD	Mean Square Displacement
mRNA	Messenger RNA
Na ⁺	Sodium ion
NMDAR	N-methyl-D-aspartate (NMDA) receptor
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PPI	Prepulse inhibition
PRG-2	Plasticity-related gene 2
PSD	Postsynaptic density
P	Postnatal day
QD	Quantum dot
Rpm	Revolutions per minute
RT	Room temperature
SAP-102	Synapse-associated protein 102 kDA isoform
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SiS	Singly innervated spines
SPT	Single-particle tracking
Taq	Thermus aquaticus
TARP	Transmembrane AMPA receptor regulatory proteins
TBST	Tris-buffered saline with Tween20
TEMED	Tetramethylethylenediamine
TBS	Theta burst stimulation
VGAT	Vesicular GABA transporter
VGLUT1	Vesicular glutamate transporter type 1

1. Introduction

1.1 Hippocampal and neuronal structure

The brain is divided into distinct regions, the cerebellum, brainstem, and cerebrum, each ascribed with different roles. The cerebrum, the brain's largest part, consists of two hemispheres that control the opposite sides of the body and are responsible for more complex functions. It can be further divided into the cerebral cortex, hypothalamus, thalamus, amygdala, and hippocampus (reviewed in (Bear et al., 2016; Squire et al., 2008)). Named after its seahorse-like shape (genus *Hippocampus*), the hippocampus plays a central role in memory navigation learning and spatial perception (reviewed in (W. Jin et al., 2020; Knierim, 2015; Voss et al., 2017)). Memory processing in the hippocampus follows a loop, beginning primarily in the entorhinal cortex and proceeding to the first hippocampal region, the dentate gyrus (DG). From the DG, signals pass to the Cornu Ammonis 3 (CA3) region, followed by the CA2 region, and finally to the CA1 region before returning to the entorhinal cortex (reviewed in (Danieli et al., 2023; Knierim, 2015)).

The primary cell type in the brain next to the glia cells is the neuron, consisting of a cell body (soma) and forming, along with blood vessels, the main component of the grey matter located in the brain's outer areas, like the cortex. In contrast, white matter in the brain's deeper layers contains nerve cell axons that connect neurons for information distribution through neurotransmitters and the generation of electrical currents. Axons are insulated by myelin, produced by oligodendrocytes, a type of glial cell. Other glial cells include astrocytes and microglia, each with distinct roles essential to brain functions (reviewed in (Bear et al., 2016; Squire et al., 2008)).

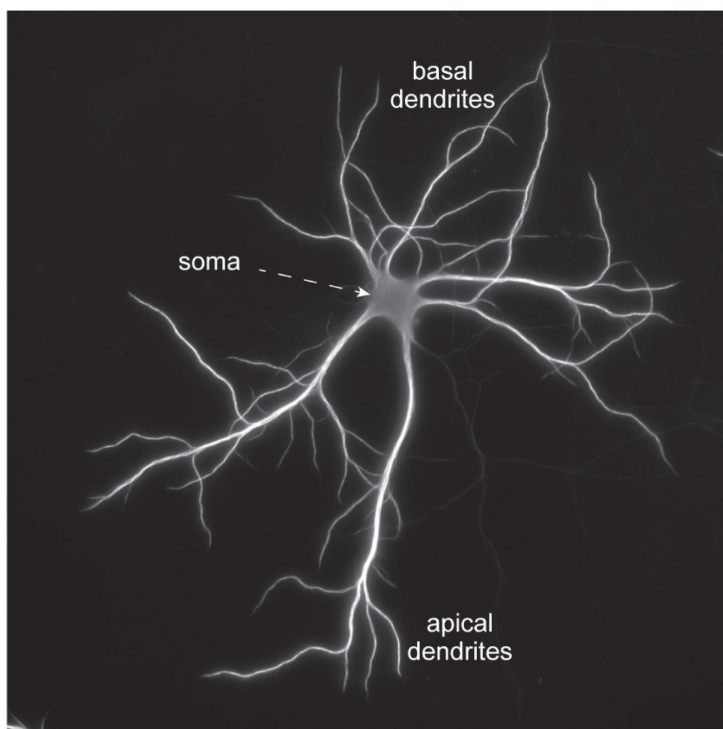


Figure 1: Image of an neuron showing basal and apical dendrites

The nerve cell body, also called soma, consists of the nucleus, cytoplasm, plasma membrane, and different organelles, including mitochondria, the Golgi apparatus, rough and smooth endoplasmic reticulum, and lysosomes (reviewed in (Bear et al., 2016; Squire et al., 2008)). The cell body is covered with branched extensions called dendrites. Dendrites originating from the cell body and spreading horizontally near the soma are known as basal dendrites (Peters & Kaiserman-Abramof, 1970). In contrast, apical dendrites are typically long extensions that reach more peripheral layers of the cortex or hippocampus regions (Peters & Kaiserman-Abramof, 1970). Dendrites exhibit small protrusions called dendritic spines.

1.2 Dendritic spines

Traditionally, dendritic protrusions are divided morphologically into filopodia, stubby, thin, and mushroom type spines. Dendritic filopodia are long, thin protrusions that lack a bulbous head and often do not develop a postsynaptic density (PSD) (reviewed in ((Berry & Nedivi, 2017; Bourne & Harris, 2007; Harris,' et al., 1992; Hering & Sheng, 2001; Peters & Kaiserman-Abramof, 1970; Yoshihara et al., 2009))). The PSD is a region on the postsynaptic side of an excitatory synapse, characterized by a high density of proteins, including scaffold proteins and receptors (reviewed in (Luo et al., 2011)).

Filopodia are highly dynamic, and the appearance of newly formed protrusions as filopodia suggests that they may serve as precursors to more mature spine types (Dailey & Smith, 1996). Over a period of two to four hours, more than 60 % of filopodia were observed to transition into stubby, thin, or mushroom spines, while most thin and mushroom spines retained their morphology (Parnass et al., 2000). In one-month-old mice, approximately 10 % of the spines in cortical regions appear to be filopodia, a proportion that decreases with age to around 2 % to 3 % (Zuo et al., 2005). Stubby spines are short, thick protrusions without bulbous heads and are generally considered immature spines. They are more abundant in developing brains, with only a few present in fully developed brains. Thin spines are long protrusions with a small bulbous head at the tip. They can form synapses but are more likely to disappear or reform in response to synaptic activity. Mushroom spines have long, narrow necks and a large bulbous head, making them easily distinguishable from other spine types. They possess larger PSDs and form stronger synapses compared to thin spines. Additionally, organelles such as smooth endoplasmic reticulum can be observed in mushroom spines (reviewed in ((Berry & Nedivi, 2017; Bourne & Harris, 2007; Harris,' et al., 1992; Hering & Sheng, 2001; Peters & Kaiserman-Abramof, 1970; Yoshihara et al., 2009))). Studies on hippocampal samples from adult rats have shown that approximately 19 % of spines are stubby spines, 9 % to 25% are mushroom spines, and 60 % to 72 % are considered to be thin spines (Harris,' et al., 1992; Peters & Kaiserman-Abramof, 1970).

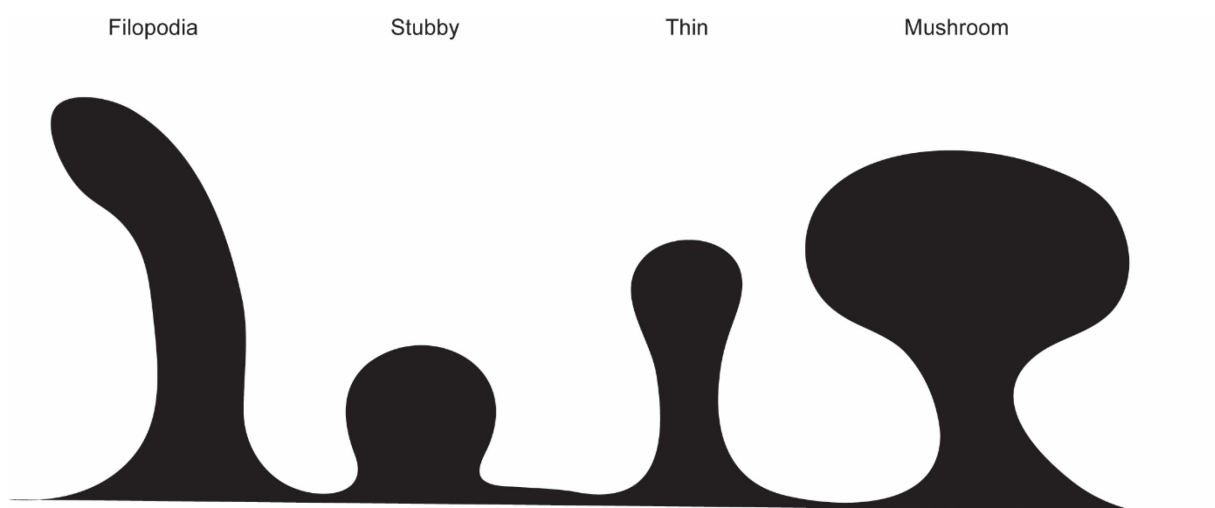


Figure 2: Schematic overview of the different spine types. Filopodia, stubby spine, thin spine, and mushroom spine (from left to right)

Researchers have increasingly defined spines in recent decades based on their dynamic properties. Thus, the different spine subtypes vary in morphology and their distribution and density across the development of nerve cells and different brain regions. Spine stability, often measured by turnover rates, provides a basis for categorizing spines. Early classifications

divided spines by lifespan, describing "transient spines" with lifespans under a day, "semi-stable spines" with a mean lifespan of two to three days, and "stable spines", which persist longer than eight days (Trachtenberg et al., 2002). A population analysis of spines in young adult mouse brains (6-10 weeks) revealed that approximately 17 % were transient, around 23 % were semi-stable, and 60% were considered stable spines (Trachtenberg et al., 2002). Later, spine classification was simplified into two categories: "transient spines", lasting up to four days, and "persistent spines", persisting for more than eight days (Holtmaat et al., 2005). With this redefinition, the survival rate of spines lasting $\leq$ eight days dropped from 60 % (Trachtenberg et al., 2002) to 35 % (Holtmaat et al., 2005). Notable, spine stability appears to correlate with spine size, as larger mushroom spines are highly stable (Holtmaat et al., 2005; Trachtenberg et al., 2002), whereas transient spines are more commonly thin spines (Holtmaat et al., 2005). The size of the spine may also be associated with learning, as it is suggested that thin spines can stabilize during the learning process or disappear more quickly (reviewed by (Hao et al., 2006)). Age-related changes in spine distribution were observed across three age points. Comparing spine density based on their kinetic type, transient spines decreased with age, accounting for approximately 30 % in young adult mice (5-11 weeks), 24 % in three-month-old mice, and 21 % in six-month-old mice. In contrast, the density of persistent spines increased with age, from 53 % in young adult mice to 66 % in three-month-olds and 72 % in six-month-olds (Holtmaat et al., 2005). Despite a high elimination rate, around 60 % of the spines in adult mice were considered stable, with their viability and maintenance potentially projected to be for a lifetime (Zuo et al., 2005). The age-related loss of thin spines can also be observed in samples from monkeys (Dumitriu et al., 2010).

The occurrence of long-term potentiation (LTP), the persistent and prolonged transmission of neurotransmitters that increases synaptic strength, appears to promote further spine maturation. LTP increased filopodia formation in hippocampal slices, which is a sign that synaptic activity stimulates spinogenesis (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999). Additionally, LTP has been shown to increase spine size (Kopec et al., 2006), and larger spine heads were observed after glutamate uncaging (Matsuzaki et al., 2004). Protein distribution has also been studied concerning spine maturation. Dynamic spines were smaller than stable ones, and postsynaptic density proteins 95 (PSD95) recruitment was associated with spine stability. Spines that did not recruit PSD95 or lost PSD95 eventually disappeared. Furthermore, PSD95-positive spines were larger than those lacking PSD95 (Villa et al., 2016). Notable, the recruitment of PSD95 in the synapse resulted in spine stabilization for more than 24 hours and seemed activity-related (De Roo et al., 2008). Interestingly, the recruitment of PSD95 was observed between 12 and 20 hours after the identification of new spines, while the levels for the synapse-associated protein 102 kDa isoform (SAP-102) peaked already at three hours and PSD93 levels around six hours (Lambert et al., 2017).

It was observed that young and thin spines had more N-methyl-D-aspartate (NMDA) receptors (NMDARs) than α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) receptors (Durand et al., 1996). The recruitment of AMPARs is thus considered a marker for spine maturation, which appears to depend on factors such as NMDAR activity (Matsuzaki et al., 2004). SAP102 has been shown to mediate the early enrichment of NMDARs and AMPARs during the early stages of synaptogenesis (Elias et al., 2008). Notable, NMDAR-induced LTP was found to increase spine survivability (Hill & Zito, 2013), whereas long-term depression (LTD), the decrease of synaptic strength, has the opposite effect, reducing spine stability (Zhou et al., 2004). This capacity for activity-dependent spine formation and elimination declines with age (Nägerl et al., 2004).

1.3 Excitatory and inhibitory synaptic system

In the literature, synapses are categorized into excitatory, forwarding signals, and inhibitory synapses, which inhibit signal transmission to other neurons. These synapses differ primarily in their neurotransmitters in the presynaptic terminal and receptors at the postsynaptic terminal. When an action potential (electrical signal) reaches the presynaptic terminal, calcium (Ca^{2+}) influx is triggered, activating various cascades (reviewed in (McCormick, 2014)).

In excitatory synapses (Fig. 3 A), glutamate is the primary neurotransmitter. Through the vesicular glutamate transporter type 1 (VGLUT1), the main glutamate transporter of the three isoforms (VGLUT1-3) (reviewed in (X. Du et al., 2020)), glutamate is loaded into vesicles and then released into the synaptic cleft via fusion of the vesicle with the plasma membrane (exocytosis). After diffusing across the synaptic cleft, glutamate binds to kainate receptors, metabotropic glutamate receptors, NMDARs or AMPARs, which are located in the PSD on the postsynaptic membrane (reviewed in (Egbenya et al., 2021; Traynelis et al., 2010)). Scaffold proteins anchor these receptors on the plasma membrane, with the PSD95 protein being the best-known. PSD95 binds in the PSD directly NMDARs (Kornau et al., 1995), and indirectly, the AMPARs over the transmembrane AMPA receptor regulatory proteins (TARPs), which include proteins such as stargazing, gamma-3, gamma-4, and gamma-8 AMPARs (Tomita et al., 2003). Activation of these receptors results in Ca^{2+} (NMDARs) or sodium ion (Na^+) (APMARs) influx, leading to depolarization of the postsynaptic membrane (reviewed in (McCormick, 2014)). When sufficient excitatory postsynaptic potentials (EPSPs) are generated, their summation triggers a new action potential, which then propagates along the dendrites to the soma and onward through the axon to other connected neurons (reviewed in (Wood, 2017)).

In inhibitory presynaptic terminals (Fig. 3 B), glutamate is converted by the enzyme glutamate decarboxylase 67 kDa isoform into gamma-aminobutyric acid (GABA) (reviewed in (Schousboe & Waagepetersen, 2016)), the primary inhibitory neurotransmitter (Roberts & Frankel, 1950). The vesicular GABA transporter (VGAT) facilitates the GABA transport into vesicles (Chaudhry et al., 1998; McIntire et al., 1997). After release from the presynaptic membrane, GABA diffuses across the synaptic cleft to bind receptors on the postsynaptic side, in the central nervous system primarily targeting the γ -aminobutyric acid type A receptor (GABA_ARs) (reviewed in (Olsen & Tobin, 1990)). These receptors are ligand-gated chloride channels, which lead to chloride ion (Cl^-) influx and membrane hyperpolarization (reviewed in (McCormick, 2014)). The resulting inhibitory postsynaptic potential (IPSP) decreases the likelihood of generating a new action potential, thereby inhibiting further signal transmission (reviewed in (Wood, 2017)). GABA_A receptors are associated with rapid inhibitory responses (reviewed in (Goetz et al., 2007)) and are mainly anchored at the plasma membrane by its scaffold protein gephyrin (Kneussel et al., 1999).

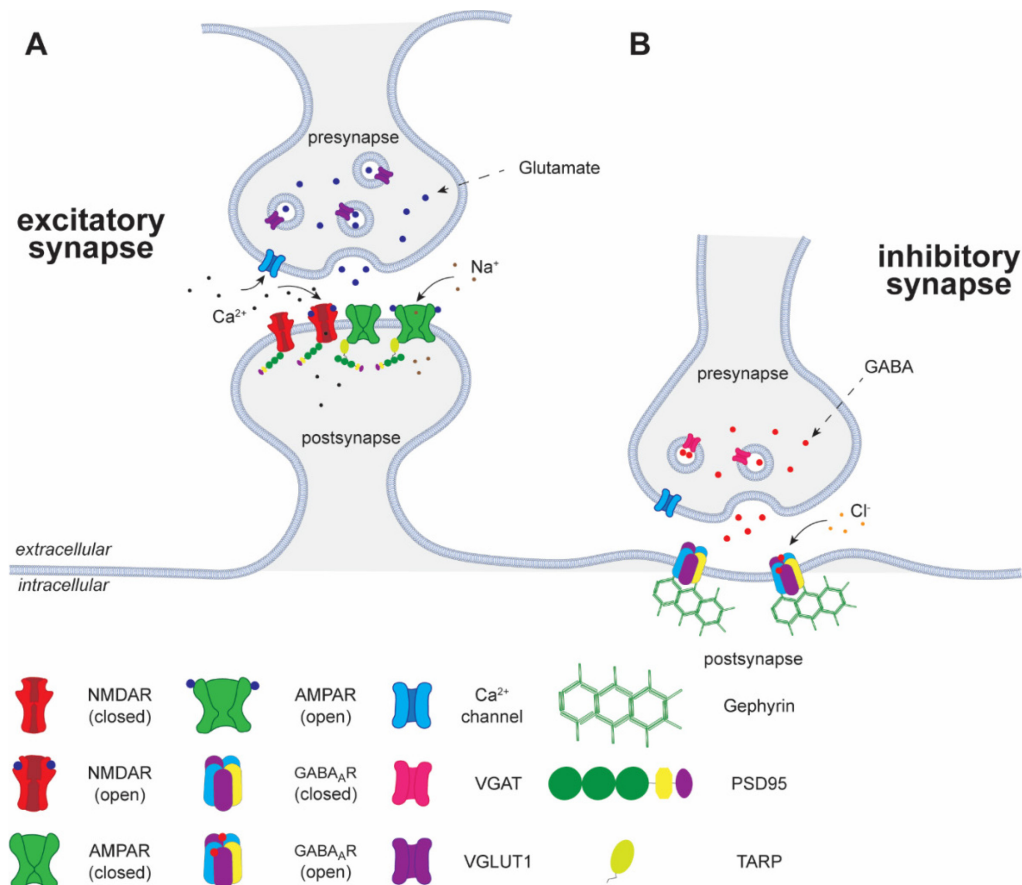


Figure 3: Schematic representation of the two synaptic types. Illustration of an excitatory synapse on a mushroom spine (A) and an inhibitory synapse on a dendritic shaft (B), with associated protein architecture

In subregions of the dendritic spine, the postsynaptic terminal often begins to form and connects with the presynaptic terminal, which originates from an axonal bouton of another nerve cell. The literature suggests that each spine typically contains a type 1 asymmetric synapse, identifiable by its large PSD and an opposing presynaptic terminal filled with round vesicles. Electron microscopy (EM) analysis has shown that presynaptic terminals containing the neurotransmitter glutamate exclusively form type 1 synapses with postsynaptic terminals. In contrast, type 2 synapses lack a PSD, making the PSD a distinct marker for type 1 synapses (reviewed by (Berry & Nedivi, 2017)). Consequently, excitatory synapses are primarily located on spines rather than dendritic shafts (reviewed by (Berry & Nedivi, 2017)). However, not every spine has a synapse, as research shows that approximately 3,6% of spines in the mouse neocortex lack synaptic connections (Arellano et al., 2007). During the first postnatal week in the rat CA1 region, about 21 % of synaptic contacts occur on filopodia, which decreases after 10 days to around 7 %. Approximately 50 % of synaptic contacts were observed on dendritic shafts, which decreased to around 32 % with ongoing development. In contrast, the synapse occurrence on spines increased from around 4 % to approximately 37 % (Fiala et al., 1998).

1.4 Dually innervated spines

In recent decades, new observations have been made about the organization of excitatory and inhibitory synapses. One new observation is the localization of gephyrin clusters on PSD95-positive spines (Fig. 4) (J. L. Chen et al., 2012), which are part of a functional inhibitory synapse (Villa et al., 2016). Another spine classification system was created based on the presence and type of synapses. Spines lacking PSD95 cluster, singly innervated spines (SiS) with an excitatory synapse, and dually innervated spines (DiS) that contain both an excitatory synapse and inhibitory synapses (Villa et al., 2016). These three spine types differ in stability and size, with SiS observed to be smaller than DiS (Villa et al., 2016). Additionally, PSD95 clusters on DiS are more stable than those on SiS (Villa et al., 2016), and the size of the gephyrin cluster appeared to correlate with the stability of the correlate inhibitory synapse (van Versendaal et al., 2012). Increased stability of DiS has been linked to reduced structural plasticity (Kleinjan et al., 2023). Furthermore, the formation of DiSs is associated with the early stages of spine development in neurons, which does not seem to depend on neuronal activity (Kleinjan et al., 2023). Higher dynamics were found for gephyrin clusters on spines compared to those on dendritic shafts, which also seem to correlate with cluster size, as smaller clusters tend to be less stable (van Versendaal et al., 2012). A later study confirmed that approximately 65 % of inhibitory synapses on DiSs exhibit higher dynamics (higher turnover rate) than inhibitory synapses on dendritic shafts (Villa et al., 2016). In contrast, PSD95 clusters on DiSs were highly stable (~98 %) compared to PSD95 clusters on SiSs (~26 %) (Villa et al., 2016).

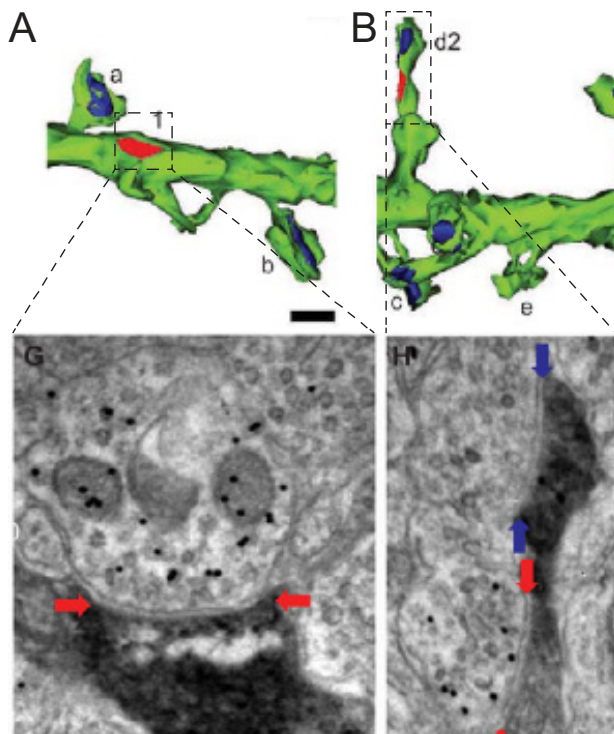


Figure 4: Synaptic localization of the inhibitory synapse. **A:** SSEM reconstruction of an inhibitory shaft synapse (red, GABAergic synapse) (top left panel) and associated EM image (bottom left panel). **B:** SSEM reconstruction of a dually innervated dendritic spine with an excitatory synapse (blue, non-GABAergic synapse), an inhibitory synapse (red, GABAergic synapse) (top right panel), and associated EM image (bottom right panel). Arrows mark the synaptic cleft of the inhibitory synapse (red) and the excitatory synapses (blue). Adapted and modified from (J. L. Chen et al., 2012)

The influence of sensory input on the stability of DiSs and synapses has been tested with varying results. Sensory deprivation in the visual system led to a more significant loss of inhibitory synapses on spines than those on dendritic shafts (van Versendaal et al., 2012). In contrast, later experiments showed that PSD95 clusters and inhibitory synapses on spines were unaffected by altered sensory input, while a higher density of dynamic inhibitory synapses was observed on dendritic shafts (Villa et al., 2016).

In addition to testing the stability of DiSs, further research explored the function of inhibitory synapses on spines. Experiments suggest that GABA uncaging can reduce the calcium influx in apical spines by around 51 % (Chiu et al., 2013). It was also shown that inhibitory postsynaptic currents (IPSCs) co-occurring with excitatory synaptic events could reduce excitatory currents by approximately 50 % (Kubota et al., 2015). Boivin and Nedivi propose that inhibitory synapses on spines with co-existing excitatory synapses do not act as strict on/off switches but instead function as local regulators of excitatory currents (reviewed in (Boivin & Nedivi, 2018)). However, initial data suggest that GABA_B receptors rather than GABA_A receptors may modulate the

reduction of NMDARs-mediated excitatory postsynaptic current while AMPAR currents remain unaffected (Kleinjan et al., 2023).

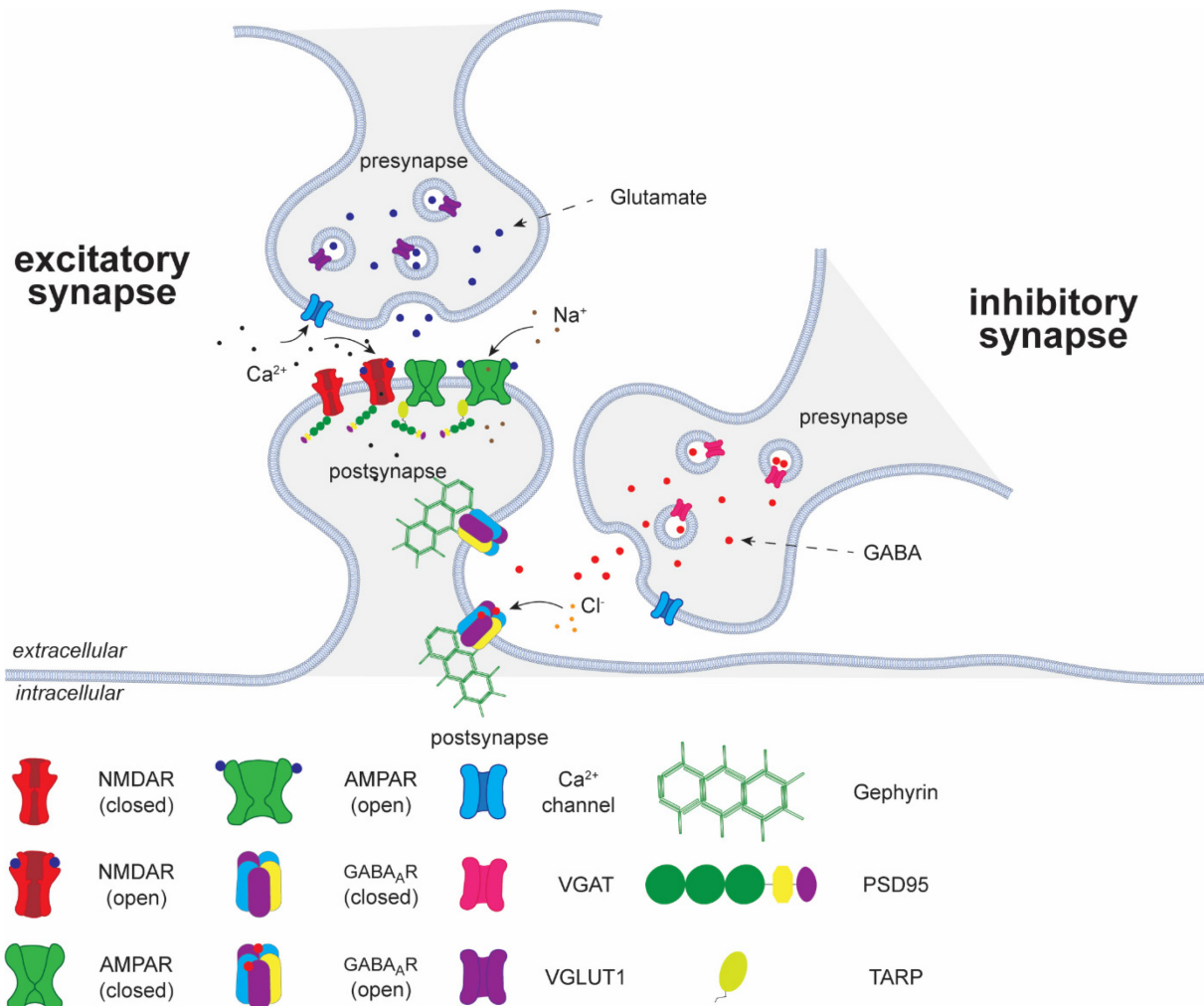


Figure 5: Schematic representation of an dually innervated spine. Illustration of an dually innervated spine opposed by an excitatory and inhibitory presynaptic terminal, with associated protein architecture

1.5 GABA_A receptors

GABA is present in approximately 30 % of synapses (Bloom & Iversen, 1971). GABA_ARs are heteropentameric, meaning their structure consists of various combinations of subunits, including α 1-6, β 1-3, γ 1-3, ρ 1-3, δ , ϵ , π and θ (reviewed in (Olsen & Tobin, 1990)). Due to this diversity in subunit composition, GABA_A receptors can mediate different functions.

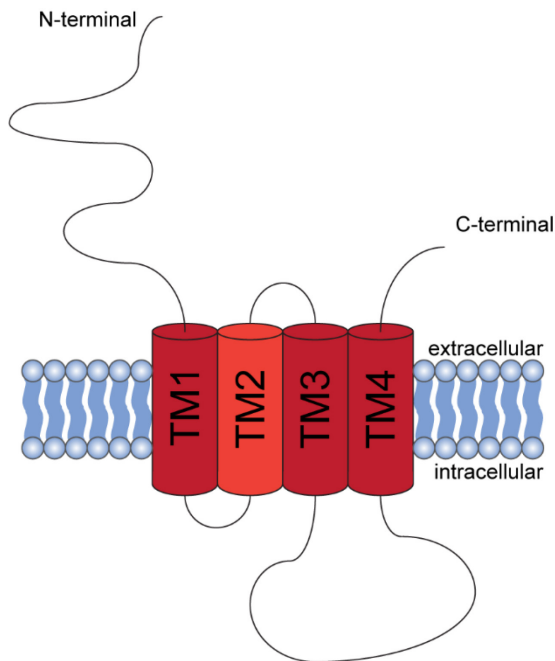


Figure 6: Schematic illustration of a GABA_A receptor subunit. Each subunit contains an extracellular N-terminus (N-tail), four transmembrane domains (TM1-TM4), and an intracellular C-terminus (C-tail) (reviewed in (Olsen & Tobin, 1990)). Between the TM3 and TM4 domains, a cytoplasmic region functions as binding site for various proteins, including the scaffold proteins gephyrin (Kneussel et al., 1999) and radixin (Loebrich et al., 2006).

The genes encoding the 19 subunits of GABA_ARs are located on different chromosomes in humans. On chromosome 4 p12 (Chr4p12), the genes of the γ 1, α 2, α 4 and β 1 subunits are located. The genes for the β 2, α 6, α 1 and γ 2 subunits are located on chromosome 5 q34 (Chr5q34). Genes for the ρ 1, and ρ 2 subunits are clustered on chromosome 6 q15 (Chr6q35.1), while the β 3, α 5, and γ 3 subunits are located on chromosome 15 q13 (Chr15q13). The last chromosome with clustered subunits genes is chromosome X q28 (ChrX q28), where the genes for the ϵ , α 3, and θ subunits are found. The remaining subunit genes are isolated across other chromosomes, with π on Chr3q35.1, ρ 3 at Chr3q12.1, and δ at Chr1p36.3 (Simon et al., 2004). Gene expression of several subunits has been measured across the brain. Eleven genes were shown to contribute to approximately 95 % of the total GABA_AR mRNA. Specifically, 48 % of the mRNA derived from the genes encoding α 1, β 2, and γ 2, while 43 % results from the gene expression associated with α 2, δ , β 3, γ 1, α 5 and β 1. Approximately 4 % of the mRNA originates from the genes encoding the α 3- and α 4-subunits. The hippocampus and amygdala notably show the most heterogeneous gene expression (Sequeira et al., 2019).

The pentameric GABA_A receptors exist in a 2:2:1 pattern formed by three subunit families. Most GABA_A receptors consist of two α subunits, two β subunits, and one additional subunit (Sequeira et al., 2019). Until now, the literature has summarized 27 different subunit combinations of GABA_ARs. Eleven of these combinations are categorized as "identified", six as "existence with high probability", and nine as "tentative". However, the authors point out that these are only the combinations identified so far, and additional combinations may be discovered in the future (Olsen & Sieghart, 2008). The highest positive correlations across the human brain were detected, for example, for the subunit pairs α 1/ β 2, α 2/ β 3, and α 5/ β 3, while the three most negative correlations were observed between α 3 and δ , δ and β 1, and α 1 and β 1 (Sequeira et al., 2019). A significant negative correlation was found for the gene expressions of the α 1- and α 5- subunits. However, a significant positive correlation was found for the α 2- and α 5 - subunits (Sequeira et al., 2019).

The different α -subunits are attributed to distinct roles in neurons. They are distributed differently across the cell membrane. α 1- and α 2-containing GABA_ARs (α 1-GABA_ARs; α 2-GABA_ARs) colocalize with gephyrin at the postsynaptic site of the inhibitory synapses, whereas α 4- and α 6 -containing GABA_ARs are found at extrasynaptic sites. α 3-containing GABA_ARs can be located at both synaptic and extrasynaptic sites. α 5-containing GABA_ARs (α 5-

GABA_ARs) are primarily localized at extrasynaptic sites, although a small population is also found at inhibitory synapses and axon initial segments (Brünig et al., 2002; Christie & De Blas, 2002; Serwanski et al., 2006). This thesis's primary receptor of interest is the $\alpha 5$ -subunit, and therefore the $\alpha 5$ -containing GABA_A receptor.

1.6 $\alpha 5$ -containing GABA_A receptors

12 % of benzodiazepine binding sites in the hippocampus can be attributed to $\alpha 5$ -GABA_ARs. Additionally, 4.2 % were identified in the cortex, followed by 2.7 % in the striatum and 1.8 % in the thalamus (Quirk et al., 1996). High concentrations of the $\alpha 5$ -subunit have been observed in the olfactory bulb, neocortex, hypothalamus, and hippocampus of rat brains (Fritschy & Mohler, 1995; Heldt & Ressler, 2007). It is suggested that approximately 5 % of all GABAergic receptors contain the $\alpha 5$ subunit (reviewed by McKernan & Whiting, 1996; Quirk et al., 1996). However, later studies reported a higher percentage. Specifically, in the hippocampus, $\alpha 5$ -GABA_ARs contribute 19-26 % of the benzodiazepine binding site population in the CA1 to CA3 regions. Around 10 % of GABAergic receptors in the DG are associated with $\alpha 5$ -GABA_ARs (Sur et al., 1999). However, slight variations in $\alpha 5$ -subunit expression are observed between different organisms. In rat hippocampal immunostainings, high expression levels were detected in the dendritic regions of CA1 and CA3, whereas lower concentrations were measured in the molecular layer of the dentate gyrus (Pirker et al., 2000; Sperk et al., 1997; Wisden et al., 1992). In humans, dendritic expression of the $\alpha 5$ -subunit was the highest in the granule cell layer of the DG but was also present in CA2 and CA3, with the weakest expression observed in CA1. Persistent somatic expression of the $\alpha 5$ -subunit was detected in the granule cell layer of the dentate gyrus, CA2 to CA4, and the entorhinal cortex, with the strongest expression found in CA2 (Stefanits et al., 2018). Interestingly, no age-related changes in the density of $\alpha 5$ -GABA_ARs were observed in the mouse hippocampal regions CA1, CA2/3, and the DG region (Palpagama et al., 2019).

In the whole mouse brain, a peak of mRNA levels for $\alpha 5$ -GABA_ARs was observed around P3 (Liu et al., 1998). $\alpha 5$ -GABA_AR mRNA levels in the hippocampus of rat brains peak at postnatal day 5 (P5) and begin to decline by postnatal day 10 (P10), reaching levels similar to those in adult rats. Interestingly, between P5 and P10, $\alpha 5$ -GABA_AR levels are concentrated in the soma of pyramidal cells. This distribution shifts around postnatal day 15, with $\alpha 5$ -GABA_ARs becoming more abundant at dendritic sites (Ramos et al., 2004). A study on inhibitory synapse formation between hippocampal interneurons and pyramidal neurons showed that dendrite-preferring interneurons exhibit higher $\alpha 5$ -GABA_ARs activity compared to inhibition from interneurons targeting areas closer to the soma (Ali & Thomson, 2008).

The functional roles of $\alpha 5$ -GABA_ARs depend on their location and the type of neuron involved. Most $\alpha 5$ -GABA_ARs are found at extrasynaptic sites (Brünig et al., 2002; Christie & De Blas, 2002; Serwanski et al., 2006), where they contribute to tonic inhibition (Manzo et al., 2021; Pangratz-Fuehrer et al., 2016). In contrast, synaptic $\alpha 5$ -GABA_ARs are associated with GABA_A,^{slow} inhibitory postsynaptic currents (X. Chen et al., 2017; Pangratz-Fuehrer et al., 2016; Zarnowska et al., 2009). Notably, different isoforms of $\alpha 5$ -GABA_ARs are associated with distinct locations. The isoform $\alpha 5\beta 1\gamma 2$ is primarily found synaptically, while $\alpha 5\beta 2\gamma 2$ - and $\alpha 5\beta 3\gamma 2$ isoforms are more likely to localize perisynaptically (X. Chen et al., 2017). Furthermore, the $\alpha 5\beta 3\gamma 2$ isoform and $\alpha 5$ -GABA_ARs containing the δ -subunit are linked to tonic current generation (X. Chen et al., 2017; Glykys et al., 2008). The significance of tonic currents is highlighted by findings that only 25 % of total time-averaged inhibitory conductance arises from synaptic (phasic) inhibition, whereas 75 % originates from tonic inhibition by extrasynaptic GABA_A pools (Mody & Pearce, 2004). However, the distribution of $\alpha 5$ -GABA_ARs between synaptic and extrasynaptic sites can vary through a cascade of anchoring mechanisms involving the scaffold proteins radixin and gephyrin (Hausrat et al., 2015; Loebrich et al., 2006).

Also, NMDAR-mediated LTP and the associated increase in cytoplasmic Ca^{2+} are necessary to increase the $\alpha 5$ -GABA_ARs concentration at the postsynaptic terminal (Davenport et al., 2021). Additionally, dopamine modulation in the hippocampus has been shown to depend on the extrasynaptic, rather than synaptic, localization of $\alpha 5$ -GABA_ARs (McCoy et al., 2024).

A critical function of $\alpha 5$ -GABA_ARs is directly responding to increased neuronal activity and helping regulate it. The blockade of synaptic $\alpha 5$ -GABA_ARs led to an increase in NMDAR-mediated currents. Conversely, increased $\alpha 5$ -GABA_ARs activity decreased NMDAR-mediated dendritic depolarization (Schulz et al., 2018). Following theta burst stimulation (TBS), relocation of $\alpha 5$ -GABA_ARs towards somatostatin interneuron synapses was observed. The induction and subsequent increase in $\alpha 5$ -GABA_AR activity caused stronger hyperpolarization, which, in turn, suppressed the formation of new excitatory LTP (Davenport et al., 2021). When $\alpha 5$ -GABA_ARs were blocked, this saturation effect did not occur. Similar observations were made in experiments with *Gabra5* knockout mice (Davenport et al., 2021).

Another function of $\alpha 5$ -GABA_ARs is their involvement in neuronal development, as tonic GABA_AR-mediated currents have been shown to influence early neuronal growth (Marchionni et al., 2007). During the development of granule cells of the DG, the contribution of synaptic $\alpha 5$ -GABA_ARs was analyzed. In young cells, $\alpha 5$ -GABA_ARs showed higher involvement in synaptic areas, which declined as the cell matured and integrated into the hippocampal network, and $\alpha 5$ -GABA_ARs increasingly contribute to tonic inhibition. Interestingly, in adult granule cells, LTP appeared to increase synaptic $\alpha 5$ -GABA_ARs contributions while reducing tonic inhibition (Davenport et al., 2021). Additionally, the localization of $\alpha 5$ -GABA_ARs plays a critical role in dendritic development. Synaptic localization of $\alpha 5$ -GABA_ARs has been shown to reduce dendritic outgrowth during development, whereas extrasynaptic localization promotes dendritic outgrowth (Brady & Jacob, 2015). Altered $\alpha 5$ -GABA_AR activity has also been linked to reduced dendritic length (Giusi et al., 2009) and delayed spine maturation (Nuwer et al., 2021).

One of the primary functions associated with $\alpha 5$ -GABA_ARs is learning and memory (Collinson et al., 2002; Crestani et al., 2002; Davenport et al., 2021). Reducing $\alpha 5$ -GABA_AR activity has improved mice's performance in cognitive tasks (Atack et al., 2006). Blocking of $\alpha 5$ -GABA_ARs also led to spontaneous gamma oscillation linked to learning and memory (Glykys et al., 2008). Interestingly, dichotomous effects on memory patterns have been observed. Treatment with a low concentration of a positive allosteric modulator of $\alpha 5$ -GABA_ARs over 24 hours improved spatial memory. However, high concentrations worsened short-term spatial memory and long-term memory. Mild facilitation of the receptor activity reduced short-term object memory, while strong facilitation improved it (Santrač et al., 2022). Despite these variations, increasing $\alpha 5$ -GABA_ARs activity remains a potential therapeutic approach for treating memory impairments (Koh et al., 2013). Differences in $\alpha 5$ -GABA_ARs-associated learning mechanisms have also been observed between female and male rats (Le et al., 2022). In addition, the scaffold protein radixin is also associated with $\alpha 5$ -GABA_ARs and their role in learning mechanisms (Hausrat et al., 2015; Loebrich et al., 2006).

1.7 Radixin

Radixin and the proteins ezrin and moesin belong to the Ezrin/Radixin/Moesin-proteins (ERM-proteins) (Sato et al., 1992). The FERM domain of ERM proteins shows approximately 24-32 % homology to the erythroid protein 4.1, classifying ERM-proteins as part of the protein 4.1 superfamily (Chishti et al., 1998; Funayama et al., 1991; Gould et al., 1989). Among the ERM-proteins, there is a significant sequence homology, as ezrin and radixin share around 75 % (Funayama et al., 1991), moesin and ezrin approximately 72 % (Lankes & Furthmayr, 1991; Sato et al., 1992) and moesin and radixin around 80 % (Sato et al., 1992) amino acid sequence identity. ERM proteins consist of three key domains. At the N-terminus is the FERM-domain, which can be further divided into three subdomains (F1, F2, F3) (reviewed in (Fehon et al., 2010)). This domain is highly conserved among the ERM proteins, with approximately 85 % sequence identity (reviewed in (Tsukita & Yonemura, 1999)). An α -helical domain follows the FERM-main and terminates at the C-terminal ERM-association domain (C-ERMAD), also known as the actin-binding domain (Fig 7) (reviewed in (Fehon et al., 2010)).

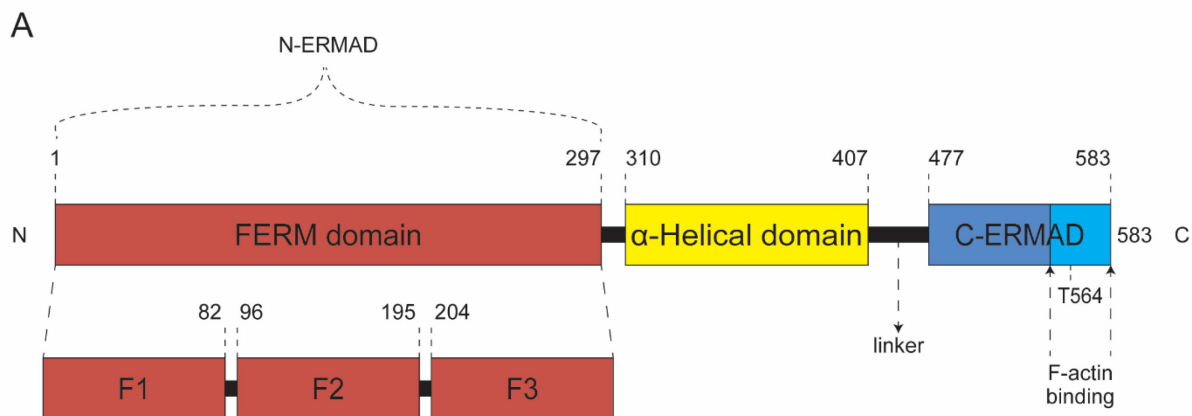


Figure 7: Overview of the different domains of the ERM-protein radixin. Illustration of the FERM domain (red) at the N-terminal with its three subdomains F1, F2, F3, the α -helical domain (yellow), and the C-terminal ERM- association domain (blue). Adapted and modified based on (Hamada et al., 2000)

The different domains of ERM proteins, next to their binding function, play a role in their conformational state. In the closed or inactive conformation, parts of the FERM domain are masked by regions of the α -helical domain or the C-ERMAD. In contrast, the open and active conformation enables ERM proteins to bind directly to the plasma membrane and associated proteins, including CD43, CD44, NHERF1, and ICAM2. Indirect binding mechanisms were observed with the ERM-binding phosphoprotein 50 (EBP50), also known as the Na⁺/H⁺ exchanger regulatory factor 1 (reviewed by (Chong & Souayah, 2024; Fehon et al., 2010; Ponuwei, 2016))

As mentioned above, it has been shown that radixin anchors $\alpha 5$ -GABA_AR at extrasynaptic sites of the neurons and stabilizes it at the plasma membrane by linking it to the actin-cytoskeleton (Hausrat et al., 2015; Loebrich et al., 2006). Figure 8 illustrates the two-step process that leads to the open conformation of radixin and the subsequent anchoring of $\alpha 5$ -GABA_AR, whereas Figure 9 shows the diffusion of $\alpha 5$ -GABA_ARs into the inhibitory synapses resulting from radixin's closed conformation.

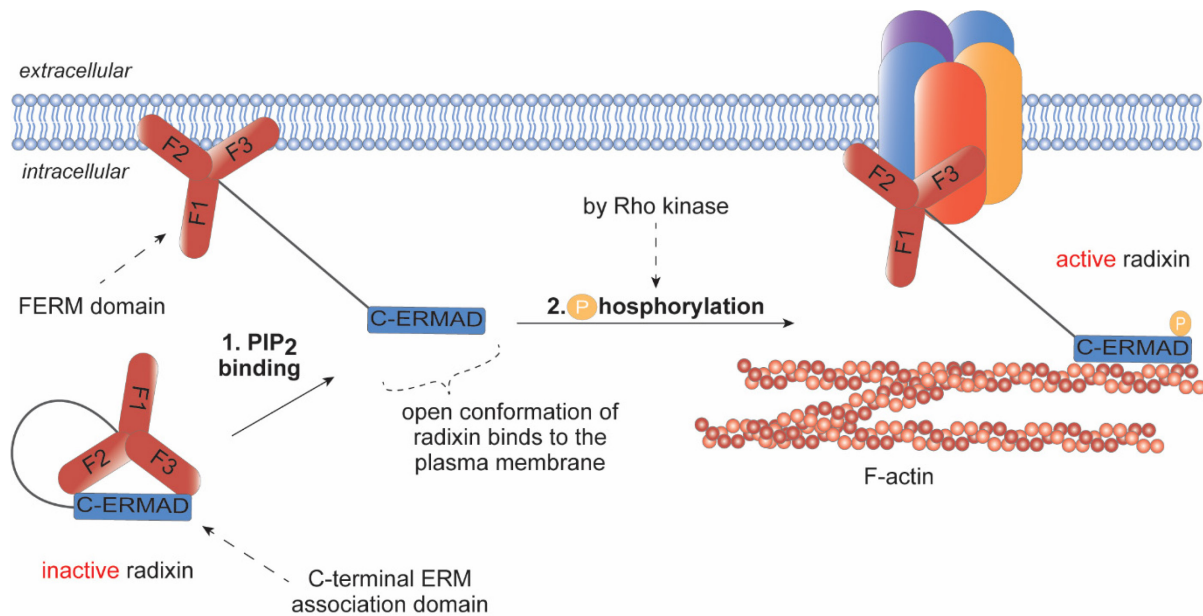


Figure 8: Illustration of the two-step activation process of radixin and the subsequent anchoring of $\alpha 5$ -GABA_A R. The anchoring of the $\alpha 5$ -GABA_A R on the plasma membrane and their linkage to the actin cytoskeleton is conducted in two steps. First, the FERM domain of the inactive radixin undergoes unmasking. The free FERM domain binds to phosphatidylinositol 4,5-bisphosphate (PIP₂) at the plasma membrane, leading to radixin's open conformation. In the second step, the phosphorylation of the threonine residue (Thr576 in ezrin, Thr564 in radixin, and Thr 558 in moesin) by kinases such as Rho kinase occurs at the C-ERMAD, facilitating binding to the actin cytoskeleton. It should be noted that several kinases can be associated with the phosphorylation of the ERM proteins (Hausrat et al., 2015; Loebrich et al., 2006).

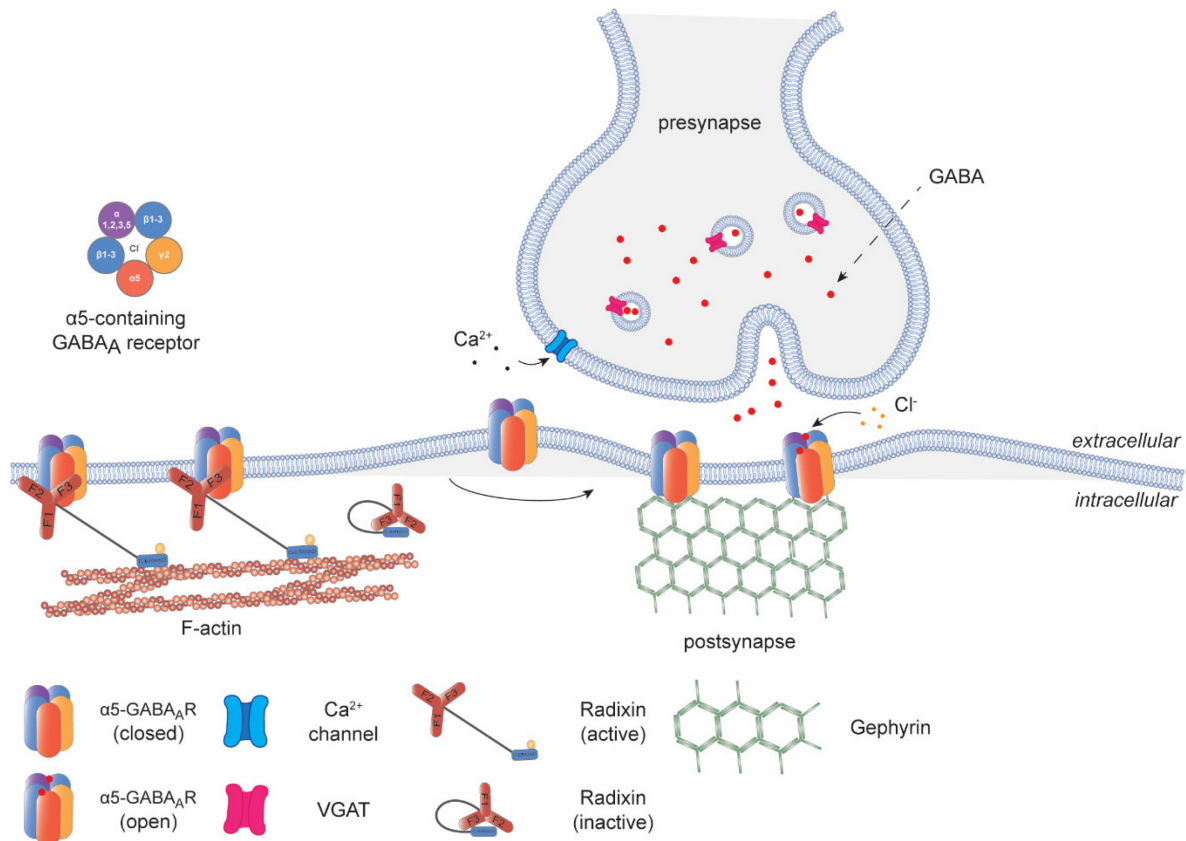


Figure 9: Schematic representation of the diffusion of $\alpha 5$ -GABA_ARs from the extrasynaptic site toward the inhibitory synapse. An increase in excitatory activity results in $\alpha 5$ -GABA_ARs redistribution mediated by a rise of radixin dephosphorylation. The detachment of $\alpha 5$ -GABA_ARs from the scaffold protein enables a higher diffusion into the synapse, where they are anchored again by the scaffold protein gephyrin.

1.8 Diseases and disorders associated with $\alpha 5$ -containing GABA_A receptors and radixin

Since $\alpha 5$ -GABA_ARs and radixin are linked to various functions such as learning and memory (Collinson et al., 2002; Davenport et al., 2021; Hausrat et al., 2015; Zurek et al., 2014), synaptic and extrasynaptic architecture (Christie & De Blas, 2002; Davenport et al., 2021; Hausrat et al., 2015; Serwanski et al., 2006), as well as axonal (Castelo & Jay, 1999; Marsick et al., 2012), dendritic (Brady & Jacob, 2015), and spinal outgrowth (Brady & Jacob, 2015; Nuwer et al., 2021), their association with various diseases and disorders have been observed.

Autism spectrum disorder (ASD) is among the disorders linked to $\alpha 5$ -GABA_ARs and radixin. Interestingly, both reduced and elevated levels of $\alpha 5$ -GABA_ARs have been associated with ASD development. The reduced number of benzodiazepine binding sites in post-mortem hippocampal samples of ASD subjects has led to the hypothesis that $\alpha 5$ -GABA_ARs may play a role in ASD (Blatt et al., 2001). In fact, lower expression levels of $\alpha 5$ -GABA_AR have been observed in ASD patients (Mendez et al., 2013; Zurek et al., 2016). Furthermore, a 50 % reduction in the frontal cortex (Brodmann area 9) and a 51 % reduction in the parietal cortex (Brodmann area 40) was observed in subjects with ASD. Additionally, a significant decrease of $\alpha 5$ -GABA_ARs mRNA was measured in Brodmann area 9 region (Fatemi et al., 2010). Deletion of the *Gabra5* gene led to autism-like behavior in mice (Mesbah-Oskui et al., 2017; Zurek et al., 2016), and reduced $\alpha 5$ -GABA_ARs activity was associated with altered EEG-activity and sleep-wake behavior linked to ASD (Mesbah-Oskui et al., 2017). Reduced prepulse inhibition (PPI), which is also observed in ASD patients (C. H. Cheng et al., 2018), was shown to correlate with decreased $\alpha 5$ -GABA_ARs levels (Hauser et al., 2004), and might be connected to radixin (Hausrat et al., 2022).

Conversely, duplication of the human chromosome 15q11-13, frequently observed in ASD, has been recreated in the patDp/+ mouse model, where upregulation of genes, including *Gabra5*, was noted (Nakatani et al., 2009). The patDp/+ mouse model is also one of the three ASD models exhibiting non-syndromic ASD traits, used by Isshiki et al. to analyze spine architecture. No change in spine density was observed in pyramidal neurons of the rodent frontal cortex, which is considered one of the initial areas showing dysfunction in some ASD patients. However, in all the ASD mouse models, PSD95-positive spines exhibited an increased rate of addition and subtraction (Isshiki et al., 2014). Mutations in *Gabra5* and RDX have also been linked to ASD (Zurek et al., 2016), although the impacts of these mutations are not fully understood. Nonetheless, radixin's known interaction and regulatory role forward $\alpha 5$ -GABA_ARs (Davenport et al., 2021; Hausrat et al., 2015) suggests that it may play a role in ASD development.

Mutations of the $\alpha 5$ -GABA_AR have been identified in patients with early-onset epileptic encephalopathies. These mutations can lead to increased sensitivity of Zn²⁺, reduced amplitude of $\alpha 5$ -GABA_AR-mediated mIPSCs due to decreased receptor activity and synaptic location, and altered distribution of $\alpha 5$ -GABA_ARs both intracellular and on the cell surface (Hernandez et al., 2019). Another study investigated a variant of mutant $\alpha 5$ -GABA_AR with increased GABA sensitivity attributed to enhanced receptor desensitization and found elevated intracellular and cell surface levels (K. M. Butler et al., 2018). A shift from GABA_ARs containing the $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -subunits toward an increased proportion of $\alpha 5$ -GABA_ARs has also been observed in association with epilepsy (McGinnity et al., 2021).

Outside the hippocampus, the nucleus accumbens is another area with high *Gabra5* gene expression, a region linked to depression, addiction, and the reward system (reviewed by (L.

Xu et al., 2020)). Reduced $\alpha 5$ -GABA_AR levels have been observed in several regions, including the nucleus accumbens, the right side of the hippocampus, and the amygdala, in patients with alcohol-related issues. However, it remains unclear whether this reduction arises from coping mechanisms or originates from neurotoxicity (Lingford-Hughes et al., 2010). Increased mRNA levels of $\alpha 5$ -GABA_ARs have been found in the dentate gyrus of human alcoholics (Z. Jin et al., 2012). Later, a study reported elevated $\alpha 5$ -GABA_AR levels in the prefrontal and motor cortices of cirrhotic alcoholics compared to controls and alcoholics without complications. Furthermore, sex-related differences in $\alpha 5$ -GABA_AR levels associated with alcohol addiction have also been suggested (Janeczek et al., 2020).

A potential role of extrasynaptic $\alpha 5$ -GABA_AR in the antinociceptive effect observed in neuropathic female rats was indicated. Interestingly, the author further suggests that estrogen-associated changes in extrasynaptic $\alpha 5$ -GABA_ARs following nerve injury may shift receptor function from hyperpolarizing to depolarizing, leading to tonic excitation and tactile allodynia in female rats (Franco-Enzástiga et al., 2021). Another study demonstrated that the absence of $\alpha 5$ -GABA_ARs in the spinal cord dorsal horn results in hyperalgesia, suggesting that tonic inhibition by extrasynaptic $\alpha 5$ -GABA_ARs may reduce pain sensitivity (Perez-Sanchez et al., 2017). Formalin injection, which stimulates pain response, increased $\alpha 5$ -GABA_ARs mRNA in the spinal cord and dorsal root ganglia (Bravo-Hernández et al., 2016). Several findings show a positive correlation between $\alpha 5$ -GABA_ARs and pain. Cognitive impairment linked to chronic neuropathic pain appears to correlate with increased $\alpha 5$ -GABA_ARs expression (Cai et al., 2022). $\alpha 5$ -GABA_ARs have been implicated in increased pain sensitivity in diabetic rats (Hernández-Reyes et al., 2019). Inhibition of $\alpha 5$ -GABA_AR activity improved problem-solving and memory in mice with traumatic brain injury (Khodaei et al., 2020). Regarding radixin, increased levels of phosphorylated ERM proteins were observed following nerve injury (Kashimoto et al., 2013). Additionally, ERM-proteins are present in myelinating Schwann cells (Melendez-Vasquez et al., 2001; Scherer et al., 2001), which are associated with neuropathic pain (reviewed in (Z. Wei et al., 2019)).

$\alpha 5$ -GABA_ARs are also linked to memory impairments after anesthesia (Amiri et al., 2020). Increased cell-surface expression of $\alpha 5$ -GABA_ARs and elevated tonic inhibition were observed after anesthesia (Zurek et al., 2014). Known anesthetics can activate GABA_ARs, particularly $\alpha 1$ - and $\alpha 4$ -subunit-containing receptors, in astrocytes, resulting in crosstalk with neurons that increases tonic inhibition (D. S. Wang et al., 2024). This rise in neuronal tonic inhibition appears to be associated with a cascade leading to increased expression of $\alpha 5$ -GABA_ARs (D. S. Wang et al., 2012). A recent study found that the anesthesia sevoflurane leads to increased radixin dephosphorylation, resulting in an increased diffusion rate of $\alpha 5$ -GABA_ARs and long-term impairments in learning and memory (S. Wang et al., 2024). Furthermore, post-anesthesia implications may also differ between male and female patients (reviewed in (Cabrera et al., 2020)).

Increased neuronal activity, which can alter dopamine levels, appears to be linked to schizophrenia-like symptoms (Aguilar et al., 2015; Lodge & Grace, 2007). Lower levels of $\alpha 5$ -GABA_ARs in the hippocampus were observed in antipsychotic-free schizophrenia patients (Marques et al., 2020). Additionally, $\alpha 5$ -GABA_ARs are associated with the development of latent inhibition (Gerdjikov et al., 2008), which is a potential biomarker for schizophrenia (reviewed in (Granger et al., 2020)). Research suggests that $\alpha 5$ -GABA_ARs may serve as therapeutic targets for schizophrenic conditions, as modulation of their activity has been shown to influence dopamine activity and attenuate schizophrenia-like symptoms (Donegan et al.,

2019; Perez et al., 2023). The decrease in PPI in mouse models with reduced $\alpha 5$ -GABA_AR expression also suggests a potential role in schizophrenia research (Hauser et al., 2004).

Alzheimer's disease (AD) accounts for approximately 70 % of dementia cases (reviewed in (Barker et al., 2002; Lane et al., 2018)). However, the onset of the disease is estimated to begin approximately 15 years before the first onset of its hallmark symptom, memory impairment (reviewed in (Ju et al., 2014)). Alongside memory loss, AD progression is marked by changes in neuronal activity, such as hyper-excitability and epileptic seizures (Martinsson et al., 2022; Scala et al., 2015; Vossel et al., 2013; W. Xu et al., 2015; Zott et al., 2019), and altered sleep patterns (reviewed in (C. Wang & Holtzman, 2019; F. Zhang et al., 2019)). Synapses are among the first areas affected by AD pathology (reviewed in (Blennow & Zetterberg, 2018; Takahashi et al., 2002)).

In hippocampal mouse cultures treated with the 42-amino acid form of amyloid beta ($A\beta_{1-42}$), increased mRNA expression of the $\alpha 2$ -, $\alpha 5$ -, $\beta 2$ -, and $\beta 3$ -subunits was observed. Subsequent treatment with an inverse agonist of $\alpha 5$ -GABA_ARs reversed this phenotype, reducing $A\beta_{1-42}$ -mediated cell loss by approximately 13 % after short-term treatment and around 17 % after 5 days (Vinnakota et al., 2020). The highest $\alpha 5$ -GABA_AR levels were found in the CA1 region and the dentate gyrus in human patients. In cases of severe pathology, $\alpha 5$ -GABA_AR levels were reduced by about 13 % in the CA1/CA2 and CA3 regions compared to mild pathology cases (Rissman et al., 2003). In another study, increased $\alpha 5$ -GABA_AR levels were observed in the CA1 region and decreased levels in the superior temporal gyrus in human AD brain samples (Kwakowsky et al., 2018). The viability of $\alpha 5$ -GABA_ARs as a therapeutic target for AD remains debated (Petrache et al., 2020), though the observed memory improvement following increased $\alpha 5$ -GABA_AR activity in aged rats with cognitive impairment highlights their potential importance in dementia research (M. T. Koh et al., 2013). While radixin has not yet been directly linked to AD, studies suggest a relationship between AD and moesin, the homolog of radixin (Y. Du et al., 2023; Rayaprolu et al., 2020).

Radixin is also associated with the auditory system and deafness (Hausrat et al., 2022; Kitajiri et al., 2004; Prasad et al., 2020). Beyond neurodevelopmental diseases, ERM proteins, including radixin, have been linked to cancer (Tokunou et al., 2000). Associations between radixin and tumor growth have been observed (S. D. Chen et al., 2012; Kobori et al., 2021; Qin et al., 2014; Tang et al., 2023), with indirect association also reported in studies examining ERM-binding proteins such as EBP50/NHERF1 (Bartholow et al., 2011; Pan et al., 2006) and ICAM2 (Tang et al., 2023). Additionally, ERM proteins have been linked to diabetes mellitus (Bach et al., 2005; Lopez et al., 2010; McRobert et al., 2003).

1.9 Aims of the thesis

The thesis focuses on two proteins: the $\alpha 5$ -containing GABA_A receptor and its scaffold protein, radixin. However, the two projects were conducted independently, and each project's results and discussion chapters are presented consecutively.

Project 1: $\alpha 5$ -containing GABA_A receptor in proximity to excitatory synapses

$\alpha 5$ -containing GABA_A receptors, primarily located at extrasynaptic sites, have also been identified in postsynaptic regions of inhibitory synapses. Associated with inducing tonic inhibition when located extrasynaptic and phasic inhibition at synaptic areas of neurons, their diffusion appears to be influenced by NMDA-mediated long-term potentiation. In the past decade, several studies have shown that excitatory and inhibitory synapses often appear in mutual proximity, sometimes within the same spine. In such cases, the interaction between these two types of synapses seems to influence their activity, dendritic growth, and spines' formation or elimination.

Dually innervated spines, which contain an additional inhibitory synapse alongside the excitatory synapse, offer new insights into known synaptic dynamics. The discovery of DiSS raises questions about whether $\alpha 5$ -GABA_ARs may play a role in their formation, given their unique diffusion pattern between extrasynaptic and synaptic regions. Considering the potential role of $\alpha 5$ -GABA_ARs in various diseases, it is crucial to explore how likely GABA_A receptors containing the $\alpha 5$ -subunit are located near excitatory synapses, such as on DiS. This thesis will examine the potential localization of $\alpha 5$ -GABA_ARs in proximity to excitatory synaptic proteins. Additionally, it will focus on the organization of potentially related post- and presynaptic protein densities.

Project 2: Mass Spectrometry study: Potential new binding partner of radixin on the plasma membrane of hippocampal neurons

Radixin is known to bind to various plasma membrane- and plasma membrane-associated proteins and links the plasma membrane to the cortical F- actin cytoskeleton via its N-terminal interaction with PIP₂ and its C-terminal interaction with F-actin. Given its essential role in the anchoring, pooling, and distribution of $\alpha 5$ -GABA_ARs and other membrane and membrane-associated proteins, the identification of new potential binding partners of radixin at the plasma membrane could advance the knowledge of the plasma membrane's organization and the arrangement of proteins in extrasynaptic regions. Immunoprecipitation of radixin from *RDX* wildtype- and knockout-derived hippocampal lysates was followed by mass spectrometry analysis to screen for potential RDX-interacting proteins. Novel potential binding partners could introduce new hypotheses regarding radixin and its role in the organization of the plasma membrane.

2. Material and methods

2.1 Animals

The euthanasia of the mice at the Center of Molecular Neurobiology of Hamburg (ZMNH), Hamburg, Germany, in the course of the experiments mentioned in this thesis was compiled in accordance with the European Communities Council Directive (10110/63/EU), the approval of the ethic committees of the city-state of Hamburg, Germany (Behörde für Justiz und Verbraucherschutz, Fachbereich Lebensmittelsicherheit und Veterinärwesen), and with the animal care committee of the University Medical Center Hamburg-Eppendorf, Germany. The maintenance and care of the mice were conducted in the ZMNH animal facility.

The *RDX* mouse line has already been implemented at the animal facility and the Institute for Molecular Neurogenetics of the ZMNH. The generation of the *RDX* knockout mice has been previously described. Briefly, the *RDX* knockout mice were created by deleting a 1 kb fragment (KpnI and SacI cut sites) containing exon 3 of the *RDX* gene. Male chimeras were then bred with C57BL/6 wildtype mice to produce heterozygous offspring. These heterozygous mice were interbred to generate homozygous knockout mice (Kikuchi et al., 2002). In this thesis, control wildtype cultures were conducted from *RDX* wildtype littermates (*RDX* +/+).

For the $\alpha 5$ -GABA_ARs related part of this thesis, a new mouse line was introduced at the animal facility and the Institute for Molecular Neurogenetics of the ZMNH. Four heterozygous embryos were obtained from Taconic Biosciences (Taconic Biosciences, Inc., Germantown, NY 12526, USA). After undergoing the rederivation/quarantine breeding processes in the UKE's mouse quarantine, the new *GABRA5* lines were established at the ZMNH mouse facility. The generation of the *Gabra5* knockout mice was previously described (Collinson et al., 2002). Briefly, the targeting vector was engineered to partially delete exons 2 and 4. This vector was then introduced into embryonic stem cells, and chimeric mice were created by injecting these cells into blastocysts derived from C57/Bl6 mice. Breeding between the chimeras and wildtype mice resulted in the production of heterozygous and wildtype offspring. Mating of heterozygous animals was used to generate wildtype, heterozygous and homozygous knockout mice.

2.2 Genotyping

To determine the genotype of the mice, tail biopsies were taken by an animal caretaker of the ZMNH animal facility and stored in 1.5 ml reaction tubes at -20 °C until further use.

Quick Extract was diluted 1:1 with ddH₂O, and 25 µl of this solution was added to each tail sample. The tubes were then placed on a heat block and incubated for 10 minutes at 65 °C, followed by an additional 3 minutes at 95 °C. PCR reagents and DNA were mixed for *RDX* genotyping, as outlined in Table 1 A, and for *Gabra5* genotyping, as described in Table 2 A. The primers used for *RDX* are listed in Table 1 B, and those for *Gabra5* in Table 2 B. PCRs were carried out using a thermocycler, following the *RDX*-PCR thermal protocol as shown in Table 1 C and the *Gabra5* protocol in Table 2 C.

The *RDX* samples and 10 µl FastGene1 kb ladder were loaded into the wells of a 2 % agarose gel containing 0.00004 % (v/v) ethidium bromide and run for 30-40 minutes at 120 V. The *Gabra5* samples and 10 µl FastGene1 kb ladder were loaded into a 3% agarose gel with the same ethidium bromide concentration and run for approximately 25 minutes at 120 V. The gels were subsequently analyzed using an ultraviolet transillumination/digital camera system (Intas Science Imaging Instruments GmbH, 37079 Göttingen, DE).

DNA fragments of 800 base pairs (bp) identified *RDX* wildtype samples, while fragments of 210 bp indicated knockout samples. Heterozygous *RDX* samples were identified by the presence of 800 bp and 210 bp fragments. For *Gabra5*, wildtype samples were identified by a 499 bp DNA fragment, heterozygous samples by two fragments of 449 bp and 375 bp, and knockout samples by a 375 bp fragment.

Table 1: Genotyping protocol for *RDX*. A: PCR reagent mix protocol. **B:** Genotyping PCR primer list **C:** PCR thermal protocol

A	Stock solution	Volume (μl)
	H ₂ O	13.6
	10x Buffer	2.50
	2 mM dNTPs	1.00
	K10 (10 μM)	2.00
	K18 (10 μM)	1.50
	K17 (10 μM)	0.20
	Taq-Polymerase	0.20
	DNA	4.00

B	Primer name	Oligonucleotide sequence	
	K10	CAATTTAAGCCATGTAGAAATATCC	WT allele
	K18	GGAATTTTGGCAGTACATATTCAG	
C	K17	GGTTCCTCTTCCCATGAATTC	KO allele
	Program step	Temperature	Time (min)
	1	95°C	10:00
	2	94°C	00:30
	3	66°C	01:00
	4	72°C	00:30
	5	72°C	10:00
	6	4°C	infinite

Table 2: Genotyping protocol for *Gabra5*. A: PCR reagent mix protocol. **B:** Genotyping PCR primer list. **C:** PCR thermal protocol

A	Stock solution	Volume (μl)
	H ₂ O	6.75
	10x Buffer	2.50
	2 mM dNTPs	0.50
	Primer GABRA-WF (10 μM)	2.50
	Primer GABRA-R (10 μM)	2.50
	Primer N752S (10 μM)	2.50
	Primer N1131AS (10 μM)	2.50
	Taq-Polymerase	0.25
	DNA	5.00

B	Primer name	Oligonucleotide sequence	
	GABRA-WF	GTGTGCACCAAAGCCCTT	WT allele
	GABRA-R	CTGGTAACATAGATGTCTGTTTCGC	WT allele
	N752S	GATGGATTGCACGCAGGTTCT	KO allele
	N1131AS	AGGTAGCCGGATCAAGCGTAT	KO allele

C	Program step	Temperature	Time (min)	
	1	95°C	15:00	35 cycle
	2	94°C	00:45	
	3	60°C	01:00	
	4	72°C	01:00	
	5	72°C	05:00	
	6	4°C	infinite	

2.3 Hippocampal/cortical primary neuronal culture

Embryos were removed at embryonic stage 16 (E16). The mother mouse was placed in a chamber and anesthetized using O₂/CO₂. Afterward, cervical dislocation was performed to euthanize the animal, and the embryos were taken out, placed in an ice-cold glucose solution, and decapitated. The hippocampi were isolated and washed once with the cold glucose solution. After aspiration of the solution, 500 μl of ice-cold Hanks' balanced salt solution (HBSS) was added to the reaction tubes. 500 μl pre-warmed 0.05 % trypsin/EDTA was added to the hippocampi, which were then incubated at 37 °C for 5 minutes to dissociate the cells. The reaction was stopped by adding 500 μl pre-warmed PBS/glucose/FBS solution (10 mM glucose, 10 % FBS in PBS). After aspirating the solution, the hippocampi were washed with 500 μl pre-warmed HBSS. Following the washing step, HBSS was aspirated, and a fresh volume of HBSS was added. The hippocampi were dissociated using fire-polished glass Pasteur pipettes of three different diameters. Subsequently, cell density was calculated using a Neubauer counting chamber (Marienfeld, Lauda-Königshofen), and 60,000 cells were plated on 12 mm glass coverslips. These coverslips had been coated overnight with poly-L-lysine (50 μg/ml) at 37 °C. The next day, after two washes with ddH₂O, the wells were filled with approximately 1.5 ml of primary neuron growth medium. During neuronal growth, the 24-well plates were stored at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂ in a cell incubator (HERAccl 150i CO₂ Incubator, Thermo Fisher Scientific, Waltham, MA 02451, USA).

For hippocampal primary neuronal cultures used to compare the different genotypes of *RDX* or *Gabra5*, tail biopsies were taken from the embryos, and the hippocampi were dissected and

stored in 1.5 ml reaction tubes containing 500 µl of ice-cold Hibernate solution until the genotyping was completed. After determining the desired genotype, the procedure continued without performing a cell-counting step and using fire-polished glass Pasteur pipettes. After washing one time with HBSS following the step pre-warmed PBS/glucose/FBS, HBSS was removed, and 120 µl of HBSS was added to the tube. The hippocampi were dissociated by 8 strokes using a 100 µl pipette. For a cell density of approximately 53,000 cells, 15 µl of the cell suspension was added to 1.5 ml cell medium in a 24-well plate.

For cortical primary neuronal cultures later used to create S1 lysates expressing the radixin TD-mutant, 400,000 cells per well were plated in a 6-well plate coated overnight with poly-L-lysine (50 µg/ml), following the same procedure as described above.

2.4 Hippocampal neuronal lysates

The plasmamembrane-enriched P2 lysate was obtained using differential centrifugation. Hippocampi from male *RDX* wildtype (+/+; N4, age 31-39 weeks) and knockout (-/-; N4, age 31-40 weeks) mice were placed into a small chamber and anesthetized using O₂/CO₂. Following the anesthesia step, the mice underwent cervical dislocation and decapitation. The hippocampi were dissected and placed in 500 µl of Sucrose buffer 1 (320 mM sucrose, 1 mM MgCl₂, 1 mM NaHCO₃, 500 µM CaCl₂, 100 mM PMSF, protease inhibitor (1x), phosphatase inhibitor (1x) in H₂O) in a 2 ml Potter-Elvehjem Tissue Grinder (Wheaton®-357969, DWK Life Sciences Inc., Wertheim, Germany). Throughout the procedure, the samples were kept on ice. Subsequently, the hippocampi were homogenized with 12 strokes at 900 rpm. The lysates were transferred to 1.5 ml Eppendorf tubes using a single-use transfer pipette. After this initial homogenization step, the samples were centrifuged at 1,480 rpm for 10 minutes at 4 °C. The supernatant was collected, and the pellet was resuspended again in 500 µl of Sucrose buffer 1, followed by another homogenization step with 8 strokes at 900 rpm. The samples were centrifuged at 780 rpm for 10 minutes at 4 °C. The second supernatant was collected and pooled with the first (S1 lysate), and the pellet was discarded.

The S1 lysate was further centrifuged at 13,800 rpm for 10 minutes at 4 °C. The resulting supernatant (S2) was transferred to a fresh 1.5 ml reaction tube. The P2 pellet was resuspended with the same volume of Sucrose buffer 2 (320 mM sucrose, 6 mM NaHCO₃ in H₂O) to create the P2 lysate. Finally, the S1, S2, and P2 lysates were diluted 1:1 in Triton X-100 buffer and incubated on a rotation wheel for 15 minutes at 4 °C. For long-term storage, the samples were kept at -80 °C.

A bicinchoninic acid assay (BCA) was performed using a Pierce BCA Protein Assay Kit to measure the total protein concentration of the different samples.

2.5 SDS-PAGE and Western Blot

One day before the SDS-PAGE, 8% Tris-glycine SDS-polyacrylamide gels were cast, consisting of an 8 % resolving gel (Table 3 A) and a 5 % stacking gel (Table 3 B). A comb, 1.5 mm wide with ten teeth, was placed into the stacking gel. After 45 minutes, the stacking gel was polymerized, wrapped in H₂O-moistened paper and transparent film, and stored at 4 °C in the fridge overnight until further use.

Table 3: Protocol for the resolving (A) and the stacking gel (B). The specified volumes are sufficient to produce four gels

A	4 x 8% resolving gel (1.5 mm)	Volume (ml)
	40% Polyacrylamide	8.00
	1.5 M Tris (pH 8.8)	10.00
	10% SDS	0.40
	10% APS	0.40
	TEMED	0.024
	ddH ₂ O	21.20
B	4 x stacking gel (1.5 mm)	Volume (ml)
	40% Polyacrylamide	2.50
	1 M Tris (pH 6.8)	2.50
	10% SDS	0.20
	10% APS	0.20
	TEMED	0.02
	ddH ₂ O with Orange G	14.60

Before the samples were subjected to the SDS-PAGE, hippocampal lysates (see section 2.4) were mixed in a 5:1 ratio with 6x protein loading buffer (30 % glycerol, stacking buffer, 6 mM EDTA, 10 % SDS, 60 mM dithiothreitol (DTT), Bromphenol Blue in H₂O). To denature the proteases in the lysate, the samples were heated at 95 °C for 5 minutes before being shortly centrifuged using the bench centrifuge at room temperature (RT). The gel was then placed into the electrophoresis chamber containing an SDS-running buffer. Samples and 9 µl BlueStar Plus Prestained Protein Marker (MWP 04, Nippon Genetics Co., Ltd., Tokyo, 112-0004, JP) were loaded. First, the gel was run at 90 V until the proteins migrated into the stacking gel. After 15 minutes, the voltage was increased to 120 V for an additional 70 minutes, allowing the sample to run through the resolving gel.

A Western Blot was conducted to transfer the proteins from the gel to an Immobilon-P PVDF membrane (0.45 µm pore size, IPVH00010, Merck Millipore, Burlington, MA 1803, USA). Before starting the Western Blot, the membrane needed to be activated. It was placed in 99.99 % methanol (MeOH) for 20 seconds, washed in ddH₂O for 2 minutes, and then stored in transfer buffer (48 mM Tris, 39 mM glycine, 0.037 % SDS, 20 % MeOH in ddH₂O) for a short term (e.g., up to 15 minutes). The gel and the membrane were placed between Whatman 3 mm CHP chromatography paper (#3030-704) in the wet transfer chamber containing ice-cold transfer buffer and cooling pack. The transfer was conducted at 100 V for 70 minutes. After the transfer, the membrane was washed once in Tris-buffered saline with Tween20 (TBST)

For the immune analysis, the membrane was blocked with a 25 ml 5 % skim-milk (w/v; Roth, Karlsruhe, DE) in TBST at RT for 60 minutes on a shaker. For incubation with the primary antibody, the membrane was placed in a 50 ml Falcon tube containing the primary antibodies diluted in 5 ml 5 % skim-milk (w/v) in TBST and incubated on a rotating shaker at 4 °C overnight. The next day, the membrane was washed 3 times in TBST on a shaker at RT for 5 minutes each. The membrane was then incubated with 1 µl of fluorophore-coupled secondary antibody diluted (1:15,000) in 15 ml of 5 % skim-milk (w/v) in TBST on a shaker at RT for 45 minutes. Subsequently, the membrane was washed 3 times in TBST on a shaker at RT for 5 minutes each. Afterward, the membrane was placed in a Odyssey CLx Imaging System (LI-COR Biosciences, Lincon, NE 68504-1357, USA) for analysis. Two proteins with different host

origins could be images simultaneously. Image Studio Lite Version 5.2.5 (LI-COR Biosciences, Lincoln, NE 68504-1357, USA) was used for the analysis.

2.6 Immunocytochemistry (ICC)

For the analysis of the $\alpha 5$ -subunit-containing GABA_A receptor, a live surface immunostaining protocol was performed. Primary hippocampal neuronal cells were cultured on coverslips for 10 to 20 days (DIV 10-20). Before application of the antibody, 1 μ l anti- $\alpha 5$ -containing GABA_A receptor antibody (224503, Synaptic Systems GmbH, 37079 Goettingen, DE), 1 μ l 10 % casein solution, and 8 μ l of 1 % BSA in PBS were mixed using a vortex mixer for 15 minutes. For the live surface immunostaining, the premixed antibody was diluted in the conditions culture medium of the hippocampal neurons and the medium from the coverslips at a ratio of 1:100 to achieve a final concentration of 1:1,000. Subsequently, 100 μ l of this antibody solution was applied to the coverslips in a wet chamber and incubated at 37 °C for 10 minutes. Next, the coverslips were dipped once in three wells filled with warm HEPES buffer, followed by a fixation step in 4 % PFA, 4 % sucrose in PBS for 12 minutes at RT. After fixation, the coverslips were washed three times in 1,000 μ l of PBS for 5 minutes each. After washing, the cells were blocked with 100 μ l of 1 % BSA in PBS in a wet chamber at RT for 60 minutes. This was followed by an incubation step with 50 μ l of 1 % BSA in PBS containing the secondary antibody for 45 minutes. Afterward, the coverslips were washed four times with 100 μ l of PBS for 5 minutes each. The cells were then permeabilized with 0.25 % Triton-X-100 in PBS for 4 minutes, followed by two short wash steps with PBS. Subsequently, the cells were blocked again with 100 μ l of blocking buffer in a wet chamber for 60 minutes. 50 μ l of 1 % BSA in PBS containing primary antibodies was added to the coverslips, and the wet chamber was placed at 4 °C overnight. The next day, the coverslips were washed four times with 100 μ l of PBS for 5 minutes each. Secondary antibodies were diluted in 1 % BSA in PBS, and 50 μ l were added to the coverslips in the wet chamber at RT for 45 minutes. After the incubation with the secondary antibody, the coverslips were washed twice with 100 μ l PBS, once with 100 μ l of PBS containing diamide-2 phenylindole (DAPI, 1:1,000), and finally with 100 μ l of PBS for 5 minutes each. The coverslips were dipped once in ddH₂O for the last step before being mounted in Aqua-Poly Mount on glass microscope slides and dried overnight in the dark at RT. Finally, the coverslips were stored at 4 °C until further analysis.

To analyze the $\alpha 2$ -containing GABA_A receptor, the antibody was used in a surface immunostaining protocol as follows. The cells were fixed and washed according to the live immunostaining protocol. 100 μ l of 1 % BSA in PBS was added to the coverslips in the wet chamber at RT for 60 minutes. The anti- $\alpha 2$ -containing GABA_A receptor antibody was diluted in 1 % BSA in PBS (1:250), and 50 μ l of the primary antibody solution was added to the cells and incubated in the wet chamber at 4 °C for 120 minutes. Afterward, the coverslips were washed four times with 100 μ l of PBS for 5 minutes each. The protocol proceeded as described in the live immunostaining protocol.

Localizations and signal intensities of the immune-stained proteins were acquired using a Carl Zeiss LSM 900 laser scanning confocal microscope (Carl Zeiss AG, 73447 Oberkochen, DE) equipped with a 63x objective. For STED microscopy, the Abberior STED infinity line microscope (Abberior Instruments GmbH, 37077 Göttingen, DE) was used at the UKE Microscopy Imaging Facility led by Dr. Antonio Virgilio Faila, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

An ICC protocol was used to analyze various proteins identified in the mass spectrometry analysis. Therefore, the nerve cells were fixed in 4 % PFA, 4 % sucrose in PBS for 8 minutes at RT. After the washing step, they were blocked for 30-60 minutes with a blocking buffer. 50 µl of 1 % BSA in PBS containing primary antibodies were added to the coverslips, and the wet chamber was placed at 4 °C for 1,5 to 2 hours or overnight. The protocol continues from this point, as described in the live surface immunostaining protocol. Images were acquired using an Olympus FluoView FV100 confocal laser scanning microscope (Olympus, 20097 Hamburg, DE) equipped with a 60x objective and the Olympus Fluoview Software Version 2.1b (Olympus, 20097 Hamburg, DE).

2.7 Immunohistochemistry (IHC)

Brains from *Gabra5* wildtype (+/+; N1, age >12 weeks) and *Gabra5* knockout (-/-; N1, age >12 weeks) mice were placed into a chamber and anesthetized using O₂/CO₂. Following this step, cervical dislocation and decapitation were performed to euthanize the animal. The brains were post-fixed in 4 % PFA in PBS for 2 hours, then placed in 30 % sucrose in PBS (w/v) for dehydration overnight at 4 °C. The next day, once the brains sank to the bottom of the tube, they were embedded in Tissue-Tek (Cat. 4583, Sakura Fitnetek, 2408 AV Alpheen aan den Rijn, The Netherlands) and stored at -80 °C. Using a cryostat (CryoStarT NX70 Cryostat, Thermo Fisher Scientific, Waltham, MA 02451, USA), sagittal sections of the hippocampus from the left hemisphere of each brain were obtained. The sections were then placed in ice-cold PBS, mounted on SuperFrost glass slides (Epredia SuperFrost Plus Adhesion slides, Thermo Fisher Scientific, Waltham, MA 02451, USA), and allowed to dry for several hours before being stored at -20 °C.

Before the immunostaining steps, the hippocampal sections were outlined with a Hyrdophobic Pap Pen (Agilent Dako, Santa Clara, CA 95051, USA). For permeabilization, 400 µl of 0.1 % Tween in TBS was added to the sections and incubated for 10 minutes at room temperature. Blocking was performed by adding 400 µl of 10 % normal goat serum in TBS to the tissue sections and incubating for 1 hour in a wet chamber at RT. Subsequently, 400 µl of primary antibody solution containing the anti-α5-containing GABA_A receptor antibody (224503, Synaptic Systems GmbH, 37079 Goettingen, DE) diluted in 10 % normal goat serum in PBS was added to each tissue slide, and the wet chamber was placed at 4 °C overnight.

The following day, the glass slides were placed in a staining trough containing TBST and washed 3 times for 5 minutes each. Then, 400 µl of secondary antibody solution, containing the secondary antibody diluted in 10 % normal goat serum in TBST, was added to the sections and incubated in a wet chamber for 1 hour at RT. Another washing step in TBST followed 3 times, 5 minutes each. The slides were then placed in the wet chamber, and 400 µl DAPI in TBST (1:1,000) was added to the sections for 5 minutes, followed by another washing step in TBST, 3 times for 5 minutes each. After this final washing step, the slides were dipped in ddH₂O and mounted using Aqua Poly Mount.

Localizations and signal intensities of the immune-stained proteins were acquired using the Olympus FluoView FV100 confocal laser scanning microscope (Olympus, 20097 Hamburg, DE) equipped with 20x and 60x objectives and the Olympus Fluoview Software Version 2.1b (Olympus, 20097 Hamburg, DE).

2.8 DAB staining

Preparation of Aclar foil was required to use neuronal cultures for the DAB staining. Therefore, pre-punched 12 mm Aclar coverslips were placed in acetone on a shaker for 20 minutes. Afterward, the coverslips were washed three times with ddH₂O and incubated in 70 % ethanol on a shaker für 3 times, 5 minutes each. The coverslips were placed in glass cuvettes and then in an oven at 50- 60 °C for 3 hours to dry. Once dried, the Aclar coverslips were transferred to 24-well plates, where they were treated with 100 % ethanol for 15 minutes, followed by three washing steps with ddH₂O. The coverslips were then coated with approximately 0.5 ml of PLL/laminin in borate buffer per well and left overnight at 4 °C. The coverslips were washed three times with ddH₂O the next day, and the primary neuronal growth medium was added. As previously described, the well plates were then used to cultivate primary hippocampal neurons.

At DIV 14, the coverslips were fixed with 4% PFA and 0.1% glutaraldehyde (GA) in 0.1 M phosphate buffer for 30 minutes on a shaker. Following fixation, the coverslips were washed with 500 µl of PBS per well 3 times, 5 minutes each. Permeabilization was carried out with an ethyl alcohol (EtOH) series (500 µl/well) on a shaker, with each step lasting 10 minutes in the following order: 10 % EtOH → 20 % EtOH → 40 % EtOH → 20 % EtOH → 10 % EtOH. After this, the coverslips were washed 3 times more, 5 minutes each, with 500 µl of PBS per well. Next, 500 µl of 0.5 % sodium borohydride in PBS was added to each well for 10 minutes, followed by another wash step of 3 times, 5 minutes each with PBS. The coverslips were then treated with 0.5 % H₂O₂ in PBS (500 µl/well) for 30 minutes on a shaker, after which they were washed again 3 times for 5 minutes each with PBS. 500 µl of 0.3 % BSA and 10 % horse serum in PBS were added to every well and incubated for 45 minutes on a shaker at RT to block the neurons. anti-α5-containing GABA_A receptor antibody (224503, Synaptic Systems GmbH, 37079 Goettingen, DE) were diluted in 0.2 % BSA in 1 % horse serum in PBS and was then added to the coverslips (500 µl/well) and incubated overnight at 4 °C. The next day, the coverslips were washed 3 times for 5 minutes each with 500 µl of PBS per well. The secondary antibody was diluted in 0.2 % BSA, and 1 % horse serum in PBS was added to the wells (450 µl/well) and incubated for 45 minutes at RT. This was followed by three more washing steps with PBS (500 µl/well), 5 minutes each. Subsequently, the neurons were incubated with 500 µl/well of ABC reagent (1:1,000) for 60 minutes at RT. After this incubation, the coverslips were washed again, 3 times for 5 minutes each with PBS, followed by another 3 washing steps with 500 µl/well of 50 mM Tris (pH 7.9). For the DAB staining, 500 µl of DAB solution per well was added to the cells for 10 minutes, followed by three more washing steps with PBS (500 µl/well) for 5 minutes each. The coverslips were dried overnight and finally embedded in Eukitt. Images were acquired using a JEM-2100Plus Transmission Electron Microscope (JEOL Ltd., Tokyo 196-8558, JP). Dr. Michaela Schweizer from the MNH Core Facility Morphology, University Medical Center Hamburg-Eppendorf performed the EM analysis.

2.9 Quantum Dot (QD)-based single particle tracking (SPT)

The quantum dot (QD)-based single particle tracking (SPT) experiments were conducted at the Institute for Developmental Biology and Neurobiology, in the research group Functional Neurobiology of Prof. Dr. Martin Heine, at the Johannes Gutenberg University Mainz, Germany. Pregnant C57/6J mice were ordered from JAVIER LABS to prepare primary hippocampal neuron cultures. Embryos were collected at embryonic stage 16, and the cultures were prepared by Dr. Anita Heine at Johannes Gutenberg University Mainz, Institute for

Developmental Biology and Neurobiology, Mainz, Germany. The tracking experiments were conducted by Oliver Stange.

At DIV 11, the cultures were transfected with Xph15-GCaMP6f expressing plasmids and incubated for several days before the coverslips were prepared for SPT at DIV 18-21. pCMV_Xph15-GCaMP6f was a gift from Matthieu Sainlos (Addgene plasmid # 135538; <http://n2t.net/addgene:135538>; RRID:Addgene_135538, Watertown, MA 02472, USA). The CMV promoter in the pCMV-Xph15-GCaMP6f construct with the hSyn promoter, generating the hSyn_Xph15-GCaMP6f construct. A pre-binding step was performed before adding the anti- $\alpha 5$ -GABAA receptor antibody to the neuron culture. In a 1.5 ml Eppendorf tube, 1 μ l quantum dot labeled secondary antibody (sc-32233, Thermo Fisher Scientific, Waltham, MA 02451, USA), 8 μ l 1 % BSA in PBS, 1 % Casein and 1 μ l anti-rabbit $\alpha 5$ -GABAA_R (224503, Synaptic Systems GmbH, 37079 Goettingen, DE) antibody were mixed and vortexed for 15 minutes. Subsequently, the conditioned growth medium of the wells was transferred into a new tube and mixed with the antibody solution to achieve a 1:1000 concentration. The coverslips were transferred from the 24-well plate into a wet chamber, and 100 μ l of the primary antibody solution was applied to each coverslip, incubating at 37°C for 10 minutes. Afterward, the coverslips were dipped 3 times in warm Hibernate solution and placed into a Ludin Chamber (Life Imaging Services GmbH, 4057 Basel, CH), where they were covered with Ringer's solution containing 2 mM CaCl₂ and 2 mM MgCl₂.

The Ludin Chamber was then placed on a Nikon Eclipse-Ti2-E inverted microscope equipped with a 60 x oil-immersion TIRF objective at a temperature of 37°C. Fluorophores were excited at 488 nm (for PSD95-GFP) with an exposure time of 50 ms for 200 frames (200 x 50 ms = 10 s) and at 561 nm (with QD-labeled $\alpha 5$ -GABAA_Rs) with an exposure time of 20 ms for 1000 frames (1,000 x 20 ms = 20 s). To ensure that the recorded signal represents only the PSD95-GFP, a GFP filter was used during the PSD95 analysis to detect the fluorescence emitted from the GFP-tagged PSD95 proteins selectively. For the $\alpha 5$ -GABAA_R receptor analysis, the filter was removed.

For the chemical induction of LTP, the coverslips were transferred to a new 24-well plate containing 1000 μ l ringer solution with 50 μ M forskolin and 0.1 μ M rolipram (Otmakhov et al., 2004). The coverslips were incubated at 37 °C for 10 minutes. Afterward, they were transferred back to 500 μ l of conditioned growth medium and placed at 37 °C for an additional 20 minutes. After this recovery period, the protocol was followed as described above.

The images were acquired using TIRF illumination mode and the Orca-Fusion BT sCMOS camera (Orca-Flash 4.0 Hamamatsu Photonics), equipped with the NIS-Elements advanced research acquisition software (Nikon). Five recordings of different regions on the coverslips were performed in a maximum time of 15 minutes. The trajectories were analyzed using the PALMTracer (C. Butler et al., 2022) software package for Metamorph (BioVisions Technologies, version 7.10.3.297).

2.10 Immunoprecipitation for Mass Spectrometry analysis

The whole procedure was conducted with increased contamination safety conditions to ensure a keratin-free environment during the experiment.

30 μ l magnetic dynabeads protein A were added to low-binding 1.5 ml Eppendorf tubes and washed 3 times with 800 μ l/tube ice-cold 1 % Tritonx-100 in PBS. After the final wash, the washing buffer was removed, and 800 μ l of ice-cold antibody buffer was added. Next, 1.105 μ g radixin antibody (Boster Bio, M01926, Pleasanton, CA 94566, US) or rabbit IgG isotype control (02-6102, Thermo Fisher Scientific, Waltham, MA 02451, USA) was added to the

antibody buffer and incubated on a rotation wheel at 4 °C for 2 hours. Following the incubation, the antibody solution was aspirated, and the beads were washed twice with 1 % Triton-X-100 in IMAC buffer on the rotation wheel at 4 °C for 5 minutes each. The washing buffer was then removed, and ice-cold lysate buffer and 60 µg of P2 hippocampal lysate was added to a total volume of 800 µl/tube. The lysate was incubated on the rotation wheel at 4°C for 2 hours. After the incubation, the beads were washed 5 times with 1 % Triton-X-100 in ice-cold IMAC buffer and placed on the rotation wheel at 4 °C for 10 minutes each. Finally, the washing buffer was aspirated, and 50 µl of IMAC buffer, along with 10 µl of 6x protein loading buffer, were added to the beads. The sample was heated at 95 °C for 5 minutes before being loaded onto the gel or stored at -80 °C.

The protein ladder, 30 µl (~30 µg) of the IP sample and the IgG control sample were loaded into the wells of an 8% nUView Tris-Glycine precast gel (NG10-008, NuSep, Germantown, MD 20879, USA) in an electrophoresis chamber containing SDS-running buffer. The gel was run for approximately 20-25 minutes at 90 V. Afterward, the gel was cut with a scalpel above and below the focused samples (fine line), and the gel samples were placed into 2 ml Eppendorf tubes filled with 500-1,000 µl 20 % ethanol.

The IP and IgG samples were analyzed by mass spectrometry by Dr. Hannah Voss and Antonia Gocke at the University Medical Center Hamburg-Eppendorf (UKE), Institute of Clinical Chemistry and Laboratory Medicine, in the group of Mass Spectrometric Proteomics led by Prof. Hartmut Schlüter.

2.11 Transduction of GFP-tagged radixin TD mutant

The generation of the RdxT564D construct was previously described (Hausrat et al., 2015). The adeno-associated virus (AAV) serotype 9 Rdx-TD-GFP and the AAV9CMV-GFP control were generated by Dr. Ingke Braren at the Institute for Experimental Pharmacology and Toxicology, Vector Facility, University Medical Center Hamburg-Eppendorf Hamburg, Germany.

Cortical neuron cultures were prepared as described earlier, and AAV transduction was conducted at DIV 12. The transduced genes were then expressed for two to seven days (DIV 14 to DIV 19).

For the three experiments, the following concentrations were used to transduce the neurons:

- **Experiment 1:** 7.5 µl/ well AVV-GFP (3.7×10^{14} virus/ µl) and 15 µl/well AAV-RDX-TD-GFP (5.1×10^{13} virus/ µl).
- **Experiment 2:** 4.1 µl/ well AVV-GFP (3.7×10^{14} virus/ µl) and 8.2 µl/well AAV-RDX-TD-GFP (5.1×10^{13} virus/ µl)
- **Experiment 3:** 5.5 µl/ well AVV-GFP (4.6×10^{13} virus/ µl) and 5 µl/well AAV-RDX-TD-GFP (5.1×10^{13} virus/ µl)

To produce the lysates, the nerve cells were washed once with PBS, scraped, and collected in 500 µl 1 % Triton-X-100 in IMAC or 1 % Triton-X-100 in PBS with protease inhibitor (1x). The solutions were incubated on ice for 30 minutes with occasional mixing. After incubation, the Eppendorf tubes were centrifuged at 1,000x g for 10 minutes, and the supernatant was transferred to new tubes. The protein concentration was then measured using a Dot Blot assay.

2.12 Immunoprecipitation for protein validation

Before performing the immunoprecipitation, the input for SDS-PAGE was prepared by collecting 100 µl of each sample and transferring it into separate Eppendorf tubes with 20 µl of 6x loading buffer. The remaining sample volume was divided into 200 µl aliquots, each containing approximately 100 µg of protein. Next, 120 µl of protein A Dynabeads were washed twice with 1 ml of PBS and resuspended with 400 µl 1 % Triton-X-100 in IMAC buffer. To pre-clean the samples, 200 µl of 1 % Triton-X-100 in IMAC buffer and 100 µl of washed beads were added to the 200 µl sample aliquots. The remaining 200 µl of bead solution was diluted with 300 µl of 1 % Triton-X-100 in IMAC buffer. Then, 2 µl of rabbit anti-GFP antibody (5 µg/µl) was added to the diluted bead solution. The Eppendorf tubes containing the sample/bead solution and the anti-GFP antibody/bead solution were incubated on a rotation wheel at 4 °C for 1 hour. After incubation, the antibody-coupled beads were washed twice with 1 ml of PBS and resuspended in 200 µl of 1 % Triton-X-100 in IMAC buffer. The pre-cleaned samples were transferred into new tubes, and 100 µl of antibody-coupled beads were added. The solution was incubated overnight on a rotation wheel at 4 °C.

The following day, the IP samples were washed twice with 1 ml of 1 % Triton-X-100 in IMAC buffer. The samples were then eluted in 90 µl of 1x sample buffer and boiled along with the input samples for 5 minutes at 95 °C. For SDS-PAGE, 15.4 µl (15 %) of the input and 30 µl of the IP samples were loaded into the wells of an 8 % SDS-gel. The gel was run at 140 V for approximately 45 minutes.

The gel was placed in a blotting chamber for the Western Blot, and the protein transfer was performed at 70 V for 2.5 hours. For immune analysis, the membrane was blocked in 5 % skim-milk (w/v; Roth, Karlsruhe, DE) in TBST. Afterward, the membrane was incubated overnight at 4 °C with primary antibodies in 5 % milk in TBST. The next day, the protocol was conducted using the standard Western Blot protocol described above.

2.13 Analysis with ComDet

Detection, colocalization, and quantification of ICC images obtained through confocal microscopy were performed using the ComDet v.0.5.5 plugin for ImageJ (<https://github.com/UU-cellbiology/ComDet>). The spot colocalization method (ComDet) was developed by Eugene Katrukha at Utrecht University, The Netherlands (<http://katpyxa.info>, Katrukha E. 2020, ComDet plugin for ImageJ, v0.5.3, Zenodo, doi:10.5281/zenodo.4281064). After identifying the different puncta parameters, the resulting Excel tables were manually screened to eliminate false positive detections of colocalizations between channels and then processed for further statistical analysis. A colocalization was confirmed valid when the centers of both puncta were within three pixels or fewer of each other.

2.14 Statistics

Statistical analysis and graph generation were conducted with GraphPad Prism software (GraphPad, version 10.2.3). Outliers were detected with the ROUT method (1%) and were removed from the dataset. Normality and Lognormality tests were conducted using the Shapiro-Wilk test for data size numbers under 50. Kolmogorov-Smirnov tests were conducted for sample sizes over 50. Normal distributed datasets were tested for differences with the two-tailed unpaired t-test. An ordinary one-way ANOVA was used to compare three different sets of data. Nonparametric data sets were tested using the Mann-Whitney test. A significant difference was achieved with $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****).

2.15 List of Antibodies

Table 4: List of primary antibodies

Antibody	Catalog number	Company	Dilution
Mouse anti-human/mouse/rat actin	A5441	Sigma-Aldrich, St. Louis, MO 63103-2530, USA	WB: 1:3000
Mouse anti-human/mouse/rat γ -adaptin	610386	BD Bioscience, Franklin Lakes, New Jersey, 07417, USA	WB: 1:2000
Rabbit anti- human/mouse/rat AP2B1	15690-1-AP	Proteintech Group, Inc., Rosemont, IL 60018, USA	ICC: 1:200
Mouse anti-human/mouse/rat bassoon	ADI-VAM-PS003-F	Enzo Biochem, Inc., Farmingdale, NY 11735, USA	ICC: 1:400
Mouse anti-human/mouse/rat NCadherin	610921	BD Bioscience, Franklin Lakes, New Jersey, 07417, USA	ICC: 1:500
Rabbit anti-human/mouse/rat α 2-containing GABA _A receptor	224103	Synaptic Systems GmbH; 37079 Goettingen, Germany	ICC: 1:250
Rabbit anti-human/mouse/rat α 5-containing GABA _A receptor	224503	Synaptic Systems GmbH; 37079 Goettingen, Germany	ICC: 1:1000 IHC: 1:500 WB: 1:500
Rabbit anti-human/mouse/rat GAPDH	sc-32233	Abcam plc, Cambridge, CB20AX, UK	WB: 1:2000
Chicken anti-Gephyrin	ab13970	Abcam plc, Cambridge, CB20AX, UK	WB: 1:2500
Mouse anti-human/mouse/rat GKAP/SAPAP	832601	BioLegend, San Diego, CA 92121, USA	WB: 1:1000
Mouse anti-mouse/rat GluR2 (2mikrog/ml)	MAB397	MilliporeSigma, Burlington, MA 1803, USA	ICC: 1:250
Rabbit anti-human/mouse/rat Myo 18a	14611-1-AP	Proteintech Group, Inc., Rosemont, IL 60018, USA	WB: 1:2000
Guinea pig anti-mouse PSD95	124014	Synaptic Systems GmbH; 37079 Goettingen, Germany	WB: 1:1000
Mouse anti-human/mouse/rat PSD95	MA1-046	Thermo Fisher Scientific, Waltham, MA 02451, USA	ICC: 1:100
Rabbit anti-human radixin (predicted also for mouse and rat)	ab52495	Abcam plc, Cambridge, CB20AX, UK	ICC: 1:200 WB: 1:1000
Rabbit anti-human/mouse/rat radixin	M01926	Boster Biological Technology, CA, 94566, USA	IP: 5 μ l (1.105 μ g) WB: 1:1000
Rat anti-mouse radixin (R21)	not commercial	Sachiko Tsukita, Kyoto University, Japan (*)	ICC: 1:25
Rabbit anti-human/mouse/rat Synapsin 1/2	106002	Synaptic Systems GmbH; 37079 Goettingen, Germany	WB: 1:1000
Guinea pig anti-human/mouse/rat Synaptophysin 1	101004/ 101308	Synaptic Systems GmbH; 37079 Goettingen, Germany	ICC: 1:1000
Guinea pig anti-mouse/rat VGAT	131011	Synaptic Systems GmbH; 37079 Goettingen, Germany	ICC: 1:500
Guinea pig anti-rat VGLUT1	AB5905	MilliporeSigma, Burlington, MA 1803, USA	ICC: 1:1000
* Kikuchi, S., Hata, M., Fukumoto, K., Yamane, Y., Matsui, T., Tamura, A., Yonemura, S., Yamagishi, H., Keppler, D., Tsukita, S., & Tsukita, S. (2002). Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. <i>Nature Genetics</i> 2002 31:3, 31(3), 320–325.			

Table 5: List of secondary antibodies

Antibody	Catalog number	Company	Dilution
Donkey Alexa 488 anti-mouse IgG	715-546-150	Jackson ImmunoResearch, Cambridgeshire, CB7 4EX, UK	ICC: 1:500
Donkey Cy3 anti-rabbit IgG	711-166-152	Jackson ImmunoResearch, Cambridgeshire, CB7 4EX, UK	ICC: 1:500
Donkey Cy5 anti-guinea pig IgG	706-175-148	Jackson ImmunoResearch, Cambridgeshire, CB7 4EX, UK	ICC: 1:500
Donkey Cy5 anti-rat IgG	712-175-153	Jackson ImmunoResearch, Cambridgeshire, CB7 4EX, UK	ICC: 1:500
Donkey Cy3 anti-rat IgG	712-165-150	Jackson ImmunoResearch, Cambridgeshire, CB7 4EX, UK	ICC: 1:500
Phalloidin-Atto 488	49409	Sigma-Aldrich, St. Louis, MO 63103, USA	ICC: 1:1000
DAPI	D1306	Thermo Fisher Scientific, Waltham, MA 02451, USA	ICC: 1:1000
Goat F(ab') ₂ - anti rabbit IgG (H+L) secondary antibody, Qdot TM 655	sc-32233	Thermo Fisher Scientific, Waltham, MA 02451, USA	pre-coupling: 1:10 SPT: 1:1000
IRDye 680RD Goat anti-mouse IgG (H+L)	926-68070	LI-COR Biosciences; Lincoln, NE 68504-1357, USA	WB: 1:15000
IRDye R 800CW Goat anti-rabbit IgG (H + L)	926-32211	LI-COR Biosciences; Lincoln, NE 68504-1357, USA	WB: 1:15000
IRDye 680RD Donkey anti-chicken IgG (H+L)	926-68075	LI-COR Biosciences; Lincoln, NE 68504-1357, USA	WB: 1:15000
IRDye 680RD Goat anti-rabbit IgG (H+L)	926-68071	LI-COR Biosciences; Lincoln, NE 68504-1357, USA	WB: 1:15000
IRDye 680RD Donkey anti-guinea pig IgG (H+L)	926-68077	LI-COR Biosciences; Lincoln, NE 68504-1357, USA	WB: 1:15000
Alexa Fluor 594 Goat anti-mouse IgG (minimal x-reactivity)	405326	BioLegend, Inc San Diego, CA 92121, USA	ICC: 1:1250
Alexa Fluor 594 Donkey anti-rabbit IgG (minimal x-reactivity)	406418	BioLegend, Inc San Diego, CA 92121, USA	ICC: 1:1250
abberior STAR RED, goat anti-mouse IgG	STRED-1001-500UG	abberior GmbH Göttingen, 37077, DE	ICC: 1:1250
abberior STAR RED, goat anti-guinea pig IgG	STRED-1006-500UG	abberior GmbH Göttingen, 37077, DE	ICC: 1:1250

2.16 List of solutions

Table 6: List of solutions of the different experiments

Solutions	
<i>Genotyping</i>	
50x Stock TAE	1 liter = 242g Tris-Base, 57.1 ml glacial Ac. Acid, 0.5 M EDTA pH = 8
<i>Primary neuronal cultures</i>	
PBS	1:10 = 10x Stock (1.37 M NaCl, 27 mM KCl, 100 mM Na ₂ HPO ₄ , 18 mM KH ₂ PO ₄) diluted in ddH ₂ O
Glucose solution	10 mM glucose in PBS
PBS/glucose/FBS	10 mM glucose, 10% FBS in PBS
<i>Hippocampal lysates</i>	
Sucrose Buffer 1 (SP1)	320 mM sucrose, 1 mM MgCl ₂ , 1 mM NaHCO ₃ , 500 µM CaCl ₂ , 100 mM PMSF, protease inhibitor (1x), phosphatase inhibitor (1x), ddH ₂ O
Sucrose Buffer 2 (SP2)	320mM sucrose, 1 mM NaHCO ₃ , ddH ₂ O
Triton-X-100 Buffer	1% Triton-X, 6 mM Tris/HCL, ddH ₂ O
<i>SDS-PAGE and Western Blot</i>	
6x Protein Loading Buffer	30% glycerol, stacking buffer, 6 mM EDTA, 10% SDS, 60 mM dithiothreitol (DTT), Bromphenol Blue in H ₂ O
SDS-running buffer	1:10 = 10x Stock (250mM Tris, 2.5M glycine, 1% SDS in ddH ₂ O, pH 8.3) diluted in ddH ₂ O
Transfer buffer	1:1:8 = 10xTransfer buffer (480 mM Tris, 390 mM glycine, 0.37% SDS), with 10% MeOH in ddH ₂ O
TBST	1:10 = 10xTBST (200mM Tris, 1.5 M NaCl, 20% Tween 20, in ddH ₂ O, pH 7.5) diluted in ddH ₂ O
Blocking Buffer	5% (w/v) milk in TBST
Antibody solution	5% (w/v) milk in TBST
<i>Immunocytochemistry and STP</i>	
HEPES buffer	10 mM HEPES, 135 mM NaCl, 5 mM KCl, 2 mM CaCl ₂ , 2 mM MgCl ₂ , 15 mM glucose, pH 7.4
Fixation solution	4% Paraformaldehyde (PFA), 4% sucrose, in PBS
Antibody solution	1% BSA in PBS
Permeabilisation buffer	0.25% Triton-X-100 in PBS
Blocking Buffer	1% BSA in PBS
Ringer solution	145 mM NaCl, 2.5 mM KCL, 10 mM HEPES, 10 mM Glucose, pH 7.4, osmolarity 300-315
<i>Immunohistochemistry</i>	
Permeabilisation buffer	1 x TBST in ddH ₂ O
Blocking Buffer	10% normal goat serum in TBS (10 x Stock: 200 mM Tris, 1.5 M NaCl in ddH ₂ O)
Antibody solution	10% normal goat serum in TBST

Solutions	
<i>Immunoprecipitation for Radixin</i>	
IMAC buffer	20 mM Hepes, 100 mM CH ₃ CO ₂ K, 40 mM KCl, 5 mM EGTA, 5 mM MgCl ₂
Washing buffer	1% Triton X-100 in PBS
Antibody buffer	0.1% Triton X-100, 50 µl 1 M DTT, 100 µl 1 M PMSF, protease inhibitor complete (1x) in IMAC
Lysate buffer	1% Triton X-100, 50 µl 1 M DTT, 100 µl 1 M PMSF, protease inhibitor complete (1x) in IMAC
<i>DAB staining</i>	
Blocking buffer	0.3% BSA, 10% horse serum in PBS
Antibody buffer	0.2% BSA, 1% horse serum in PBS

2.17 List of Reagents

Table 7: List of chemicals and products

Reagent	Catalog number	Company
VECTASTAIN ELite ABC-HRP Kit, Peroxidase (Standard)	PK-6100	BIOZOL Diagnostica Vertrieb GmbH, 85386 Eching, DE
Molecular Biology Grade Agarose	EP-0010-05	Kaneka Eurogentec S.A., 4102 Seraing, BE
Ammonium persulfate (APS)	9592.1	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
Acrylamide/Bisacrylamide ROTIPHORESE® 40 %	T802.1	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
Auq-polymount	18606-20	Polysciences, Inc., Warrington, PA, 18976, USA
Pierce™ BCA Protein Assay Kit	23227	Thermo Fisher Scientific, Waltham, MA 02451, USA
Boric acid	11607	Honeywell Research Chemicals, Morris Plains, NJ 07950, USA
Bovine Serum Albumin (BSA)	A7303-100G	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
3,3'-Diaminobenzidine (DAB)	D5637	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
Diamidino-2-phenylindole (DAPI)	D9542	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
FastGene 1kb DNA Ladder plus	B64	Thermo Fisher Scientific, Waltham, MA 02451, USA
1,4-Dithiothreitol (DTT)	6908.2	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
Magnetic Dynabeads Protein A	10002D	Thermo Fisher Scientific, Waltham, MA 02451, USA
3,3'-Diaminobenzidine (DAB)	D-4293	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
PNGM™ Primary Neuron Growth Medium BulletKit	CC-4461	Lonza Group AG, 4052 Basel, CHE
Ethanol (ROTIPURAN≥99.8%, p. a.)	9065.4	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
38-DIAMINO-5-ETHYL-6-PHENYLPHENANTHRIDIN-5-IUM BROMIDE	2218.3	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
Goat serum	ENG9010-10	BIOZOL Diagnostica Vertrieb GmbH, 85386 Eching, DE
Hanks' balanced salt solution (HBSS)	14170-088	Thermo Fisher Scientific, Waltham, MA 02451, USA
Hibernate-E Medium	A1247601	Thermo Fisher Scientific, Waltham, MA 02451, USA
Horse Serum	16050130	Thermo Fisher Scientific, Waltham, MA 02451, USA
Hydrogen peroxide 30% SDS	107209	Merck Millipore, Burlington, MA 1803 USA
Isopropanol	6752.2	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
Methanol	P717.1	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
dNTPs	U1240	Promega Corporation, Madison, WI 53711, USA

Reagent	Catalog number	Company
Paraformaldehyde (PFA)	335.3	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
PNGM™ Primary Neuron Growth Medium BulletKit	CC-4461	Lonza Group AG. 4052 Basel, CHE
Poly-D-Lysine	P2636	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
Phalloidin-Atto 488	49409	Sigma-Aldrich, St. Louis, MO 63103, USA
Phenylmethylsulfonyl fluoride (PMSF)	6367.1	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
phosstop Easypack	REF 04906837001	F. Hoffmann-La Roche Ltd.S, 4070 Basel, CHE
nUView Tri-Glycine SDS_PAGE Precast Gel 8% 10well	NN10-008	NuSep, Germantown, MD 20876, USA
protease inhibitor complete tablets, EDTA-free, Easy-pack	REF 04693132001	F. Hoffmann-La Roche Ltd.S, 4070 Basel, CHE
BlueStar Plus Prestained Protein Marker	MWP04	Nippon Genetics Co., Ltd., Tokyo, 112-0004, JP
Quick extract	101098	Lucigen LGC Biosearch Technolo- gies, Middleton, WI 53562 USA
Sodium dodecyl sulfate (SDS)	L4509	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
Sodium borhydride	S-9125	Thermo Fisher Scientific, Waltham, MA 02451, USA
NNN'N'-Tetramethylethylenediamin (TEMED)	T9821	Sigma-Aldrich, St. Louis, MO 63103-2530, USA
Triton-X-100	3051.2	Carl Roth GmbH and Co. KG., Karlsruhe, 76189, DE
0.05% trypsin / EDTA	25300-054	Thermo Fisher Scientific, Waltham, MA 02451, USA

3. Project 1: Results

3.1 Validation of the $\alpha 5$ -containing GABA_A receptor antibody in *GABRA5* knockout-derived cell cultures

This study was conducted to determine if $\alpha 5$ -GABA_ARs can be found on spine necks or even in subregions of the head, or even near excitatory synapses. To explore this hypothesis, validating the anti- $\alpha 5$ -GABA_AR antibody (Fig. 10 A) for various methods is crucial, as the research's integrity and accuracy depend on this.

The antibody was previously validated using immunohistochemistry (Perez-Sanchez et al., 2017), necessitating a more detailed validation for Western Blot and ICC.

Figure 10 A illustrates the scheme of a GABA_A receptor with its extracellular-, transmembrane- and intracellular domains (reviewed in (Olsen & Tobin, 1990)). Furthermore, the extracellular binding site of the antibody and the intracellular binding domain of gephyrin and radixin, two known GABA_AR scaffold proteins, are implied (reviewed in (Loebrich et al., 2006; Tretter et al., 2012)). This scheme shows the theoretical usability of live-immunostaining with this antibody for the project.

Western Blot was performed as a first step of validation. Therefore, the antibody was tested in S1 lysates of wildtype and *Gabra5* knockout-derived hippocampi.

A band at around 53kDa was detected in lysates derived from wildtype samples, while no signal was observed in the *Gabra5* knockout sample. Equal protein volume across samples was confirmed by gamma-adaptin (γ -adaptin) (Fig. 10 B).

An additional antibody validation using IHC was also performed (Fig. 10 C). A strong signal was observed in wildtype-derived hippocampal CA2 sections (upper image), compared to an unresolved signal in the *Gabra5* knockout condition (lower image). The IHC immunostaining demonstrates that based on the antibody, a clear identification of $\alpha 5$ -GABA_ARs cluster is possible, consistent with previously published data (Perez-Sanchez et al., 2017).

This study's elemental method is immunocytochemistry, making a thorough analysis of the antibody in primary neuronal cultures essential. Defined clusters of $\alpha 5$ -GABA_ARs were detected in wildtype-derived hippocampal dendritic regions (Fig. 10 D), whereas no signal was detected in the *Gabra5* knockout condition (Fig. 10 D). Synaptophysin is commonly used in immunostainings to highlight presynaptic terminals, offering additional details of the dendritic arrangement. The explicit depiction of the puncta providing a clear reference for identifying and tracking dendrite locations within the samples strengthens the antibody's positive validation in ICC.

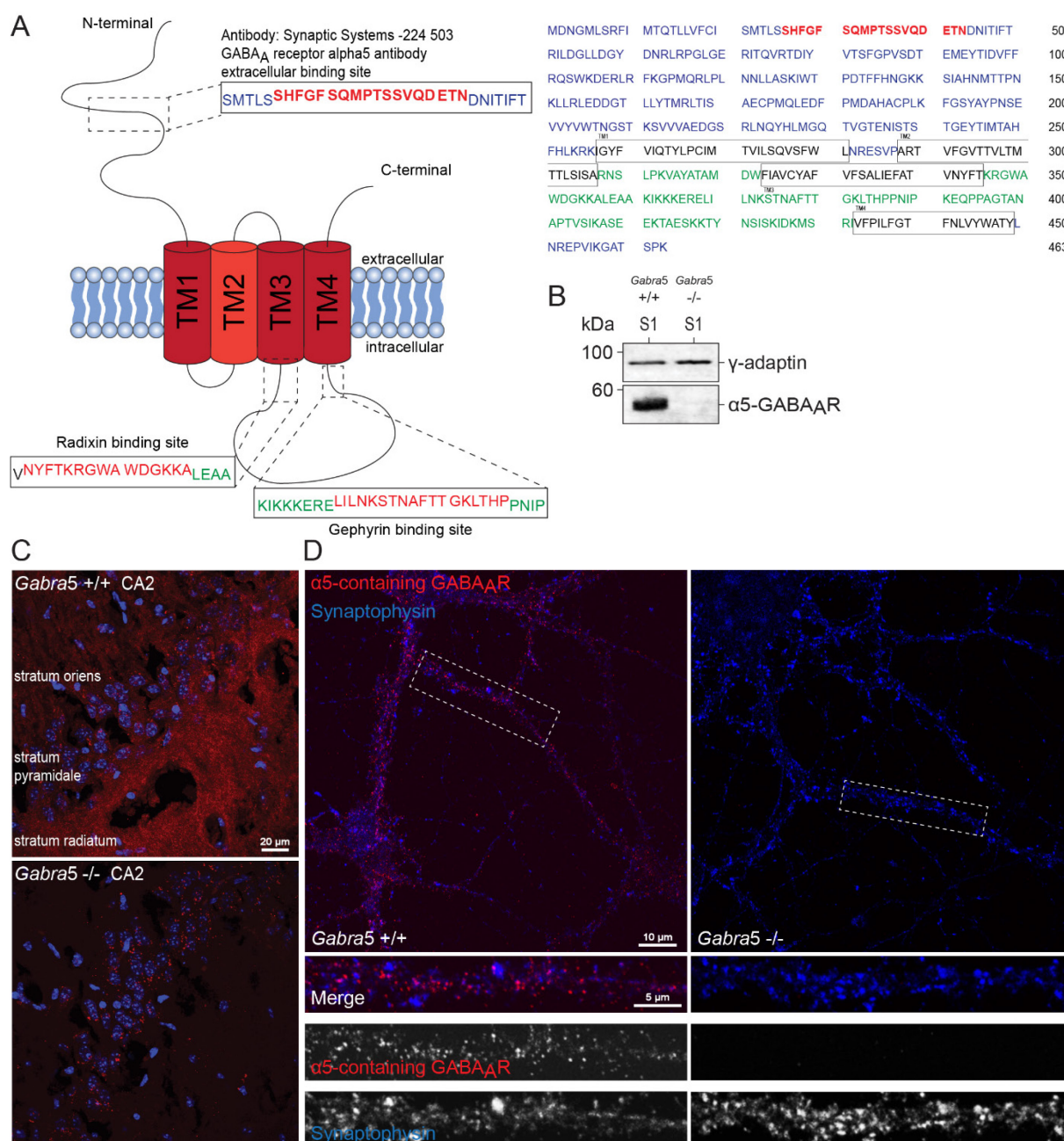


Figure 10: Validation of the anti- $\alpha 5$ -GABA_A receptor antibody (SynapticSystems, 224503). **A:** Scheme (left) and amino acid sequence (right) of an $\alpha 5$ -GABA_AR. Extracellular- (blue), transmembrane- (boxed), and intracellular (green) domains are shown in colored letters in the amino acid sequence (right). Antibody extracellular binding domain, as well as radixin and gephyrin binding sites, are marked. **B:** Analysis of protein abundance for $\alpha 5$ -GABA_AR and γ - adaptin in S1 *Gabra5* wildtype (+/+) and knockout-derived (-/-) hippocampal lysates. **C:** Immunohistochemistry (IHC) staining of $\alpha 5$ -GABA_AR (red) and DAPI (blue) in *Gabra5* wildtype (+/+) and knockout-derived (-/-) hippocampal sections (CA2). **D:** Live-immunostainings of $\alpha 5$ -GABA_AR (red) were conducted in secondary dendrites of wildtype (+/+) and *Gabra5* knockout-derived (-/-) primary hippocampal neurons (DIV18). In addition, synaptophysin (blue) was probed after fixation. Enlargements of a 40 μ m-long dendrite section, highlighted by gray boxes, are displayed in all two channels.

3.2 Triple-colocalization of $\alpha 5$ -containing GABA_A receptors, PSD95, and synaptophysin

Prior validation of the anti- $\alpha 5$ -GABA_ARs antibody (SynapticSystems 224 503) in IHC, Western Blot, and ICC confirmed the specificity of the antibody. Therefore, the first question of the hypothesis can be addressed. Do $\alpha 5$ -GABA_ARs colocalize with an excitatory postsynaptic marker in the presence of a presynaptic marker, i.e., at or in the proximity of excitatory synapses?

ICC analysis in cultured primary hippocampal neurons was conducted to determine if $\alpha 5$ -GABA_ARs are present at postsynaptic terminals close to excitatory marker proteins. Therefore, the colocalization of $\alpha 5$ -GABA_ARs, PSD95 as an excitatory postsynaptic marker (Tomita et al., 2003), and synaptophysin as a highly abundant presynaptic protein (Jahn et al., 1985; Navone et al., 1986; Takamori et al., 2006) were analyzed.

PSD95 was proven suitable for mapping excitatory synapses (Isshiki et al., 2014; Villa et al., 2016). In addition, PSD95 serves as a marker for the degree of spine maturation, as only stable and mature spines exhibit PSD95 puncta (De Roo et al., 2008; Prange & Murphy, 2001; C. E. Taft & Turrigiano, 2014). Therefore, potential colocalizations of the $\alpha 5$ -GABA_ARs and PSD95 puncta could indicate their presence on stable, mature spines.

Figure 11 exemplifies the triple-immunostaining of dendrites derived from wildtype (Fig. 11 A-C), *Gabra5* heterozygous (Fig. 11 D-E), and *Gabra5* knockout-derived neuronal cultures (Fig. 11 F-H). Furthermore, an enlarged section of a dendritic region is displayed for every genotype (boxes with dotted lines). Line-scan analysis of an example triple-colocalization in wildtype neurons suggests that all three channels' peak intensities are approximately 0.2 μm apart (Fig. 11 C). In *Gabra5* heterozygous-derived neurons, the intensity peaks from the different channels were also detected within a maximum distance of approximately 0.2 μm from each other (Fig. 11 F). The final analysis performed in *Gabra5* knockout-derived dendrites revealed no signal for $\alpha 5$ -GABA_ARs, while intensity peaks for PSD95 and synaptophysin were observed (Fig. 11 I).

To further quantify these potential triple-colocalizations, the overlap of the proteins was detected and counted using the ComDet plugin of the ImageJ software. The ComDet plugin identifies colocalizations by detecting the puncta's intensity and measuring the distance between cluster centers. The plugin recommends setting the pixel parameter between 3 and 20 pixels. For this analysis, the parameter was adjusted so that colocalization is considered valid if the puncta centers are within 3 pixels or less of each other (lowest pixel setting). With a scale of 10.0974 pixel/micron, this 3-pixel threshold corresponds to a distance of approximately 297 nm, which remains within the diffraction limit for resolving fine details of the confocal microscopy (reviewed in (Elliott, 2020; MacDonald et al., 2015)).

The triple-colocalization cluster density (clusters/ μm^2) in the *Gabra5* knockout-derived dendrites is significantly decreased compared to *Gabra5* wildtype and heterozygous conditions (Fig. 11 J). No significant difference in cluster density was detected in dendrites of *Gabra5* wildtype and heterozygous-derived neurons.

Quantification of the total intensity (Fig. 11 K) and total area (Fig. 11 L) of $\alpha 5$ -GABA_ARs puncta found in triple-colocalizations with PSD95 and synaptophysin shows a significant decrease for both parameters in *Gabra5* heterozygous conditions compared to wildtype conditions.

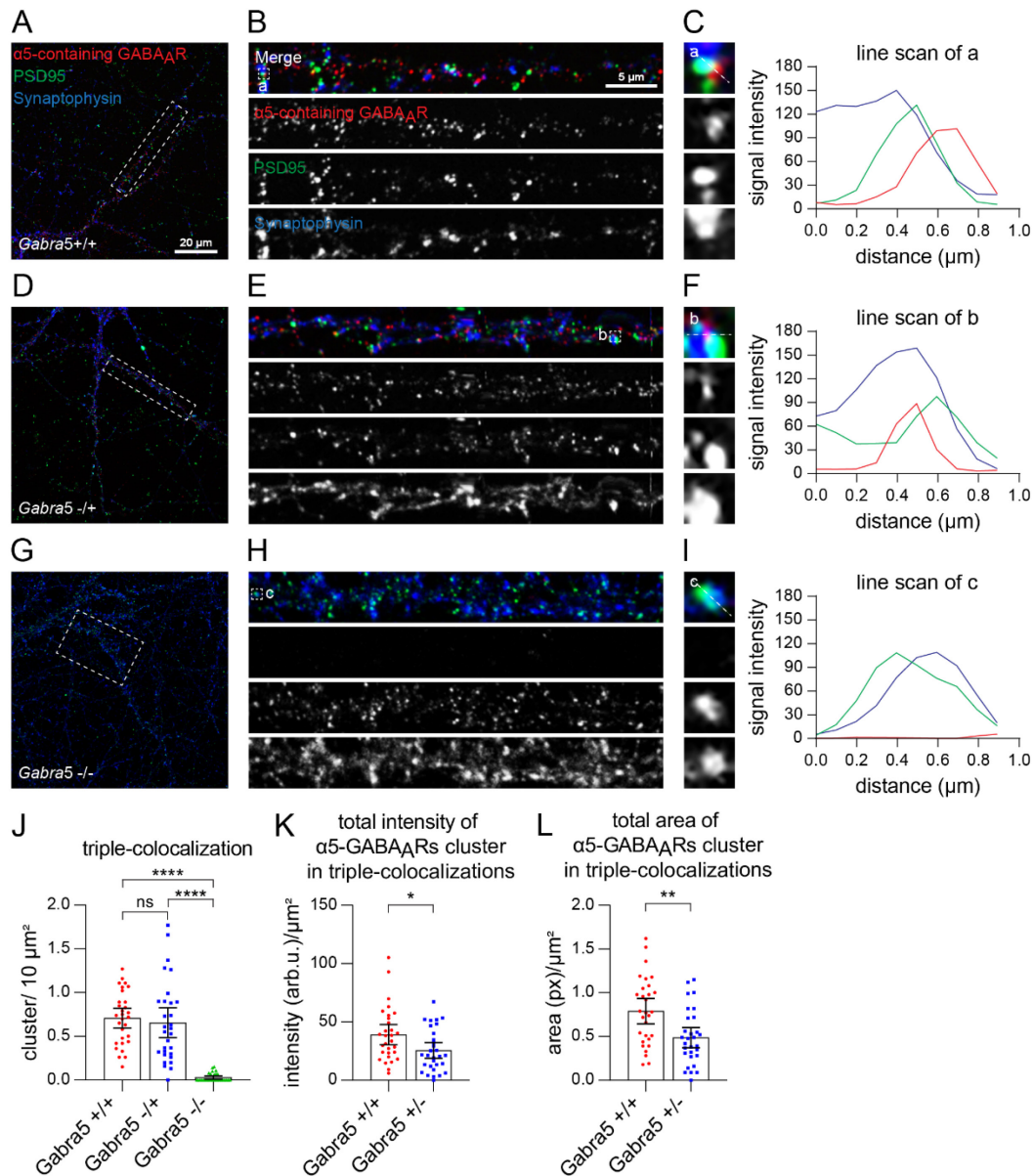
Calculating the percentage of $\alpha 5$ -GABA_AR puncta in triple-colocalizations, relative to the total number of $\alpha 5$ -GABA_AR clusters detected in a 40 μm dendrite section, indicates that in dendrites of both wildtype and *Gabra5* heterozygous-derived neurons, approximately 8 to 9 % of $\alpha 5$ -GABA_ARs colocalize with synaptophysin and PSD95 (WT vs. HET = mean $\pm$ SEM =

8.550% $\pm$ 0.5137% (n = 30), vs. 8.618% $\pm$ 0.8674% (n = 30); t-test p = 0.9465) (Supplementary data Table 1).

Since the total intensity of $\alpha 5$ -GABA_AR puncta in triple-colocalization seems to differ between the two genotypes, a more detailed analysis was performed. Calculating the proportion of the total intensity of $\alpha 5$ -GABA_ARs puncta in triple-colocalizations relative to the total intensity $\alpha 5$ -GABA_ARs cluster per dendrite revealed no significant differences (WT vs. HET = mean $\pm$ SEM = 10.99% $\pm$ 0.8755% (n = 30), vs. 9.535% $\pm$ 1.158% (n = 30); t-test p = 0.3210) (Supplementary data Table 1). However, the total area of $\alpha 5$ -GABA_ARs appears to be proportionally decreased in triple-colocalizations in heterozygous conditions (WT vs. HET = mean $\pm$ SEM = 9.557% $\pm$ 0.6343% (n = 30) vs. 7.294% $\pm$ 0.8192% (n = 30); t-test p = 0.0330) (Supplementary data Table 1).

In addition, further calculations revealed that there is approximately 22% to 27% of the total number of excitatory synapses in colocalization with $\alpha 5$ -GABA_ARs and this number does not significantly differ between wildtype and *Gabra5* heterozygous conditions (WT vs. HET = mean $\pm$ SEM = 27.20% $\pm$ 1.846% (n = 30) vs. 22.39% $\pm$ 2.003% (n = 30); Mann-Whitney-test p = 0.1033) (Supplementary data Table 1).

These results suggest potential triple-colocalizations of $\alpha 5$ -GABA_ARs, PSD95, and synaptophysin. Additionally, they indicate that the size of the $\alpha 5$ -GABA_AR fraction in colocalization with PSD95 and synaptophysin is relatively small.



3.3 $\alpha 5$ -containing GABA_A receptors and PSD95 puncta on the same dendritic spines

The data obtained by confocal microscopy alone is insufficient to fully confirm the exact localization of $\alpha 5$ -GABA_ARs on spines. The ZEISS LSM 900 confocal microscope used in this study has a diffraction limit for resolving fine details of synaptic structures. Confocal microscopes do have a maximum feature size of approximately 200nm in the XY plane and approximately 600 nm in the Z dimension (reviewed in (Elliott, 2020; MacDonald et al., 2015)). In comparison, the STED microscope can achieve structural resolutions down to approximately 50 nm (XY direction) (reviewed in (MacDonald et al., 2015)).

Therefore, $\alpha 5$ -GABA_ARs and PSD95 were labeled in wildtype-derived hippocampal primary neurons with STED-compatible secondary antibodies to validate further the confocal data showing close localization within a maximal distance of 200 nm. In addition, the cells were stained for F-actin filaments using phalloidin to visualize dendritic spines and to highlight the morphology using classical confocal microscopy.

Figure 12 A-I displays three examples of neuronal dendrites with high magnification of their dendritic spines. The images reveal that $\alpha 5$ -GABA_ARs puncta are in nanoscale proximity to PSD95 puncta on the same dendritic spine. The locations of the $\alpha 5$ -GABA_AR clusters seem to vary, with some situated directly on the spine head and others closer to the neck.

Additionally, to further substantiate the previous data, initial DAB-based-immunostaining images of wildtype-derived cell cultures were acquired using JEM-2100Plus Transmission Electron Microscope (EM) (JEOL Ltd., Tokyo 196-8558, JP), which has a guaranteed resolution of approximately 0.14 nm (*JEM-2100Plus: A New 200kV Transmission Electron Microscope* | JEOL, n.d.). The images obtained from EM also indicate the presence of $\alpha 5$ -GABA_ARs on spines (Fig. 13)

These results further suggest the presence of $\alpha 5$ -GABA_ARs on dendritic spines and provide new insight into $\alpha 5$ -GABA_ARs localization in potential subregions within or adjacent to excitatory synapses.

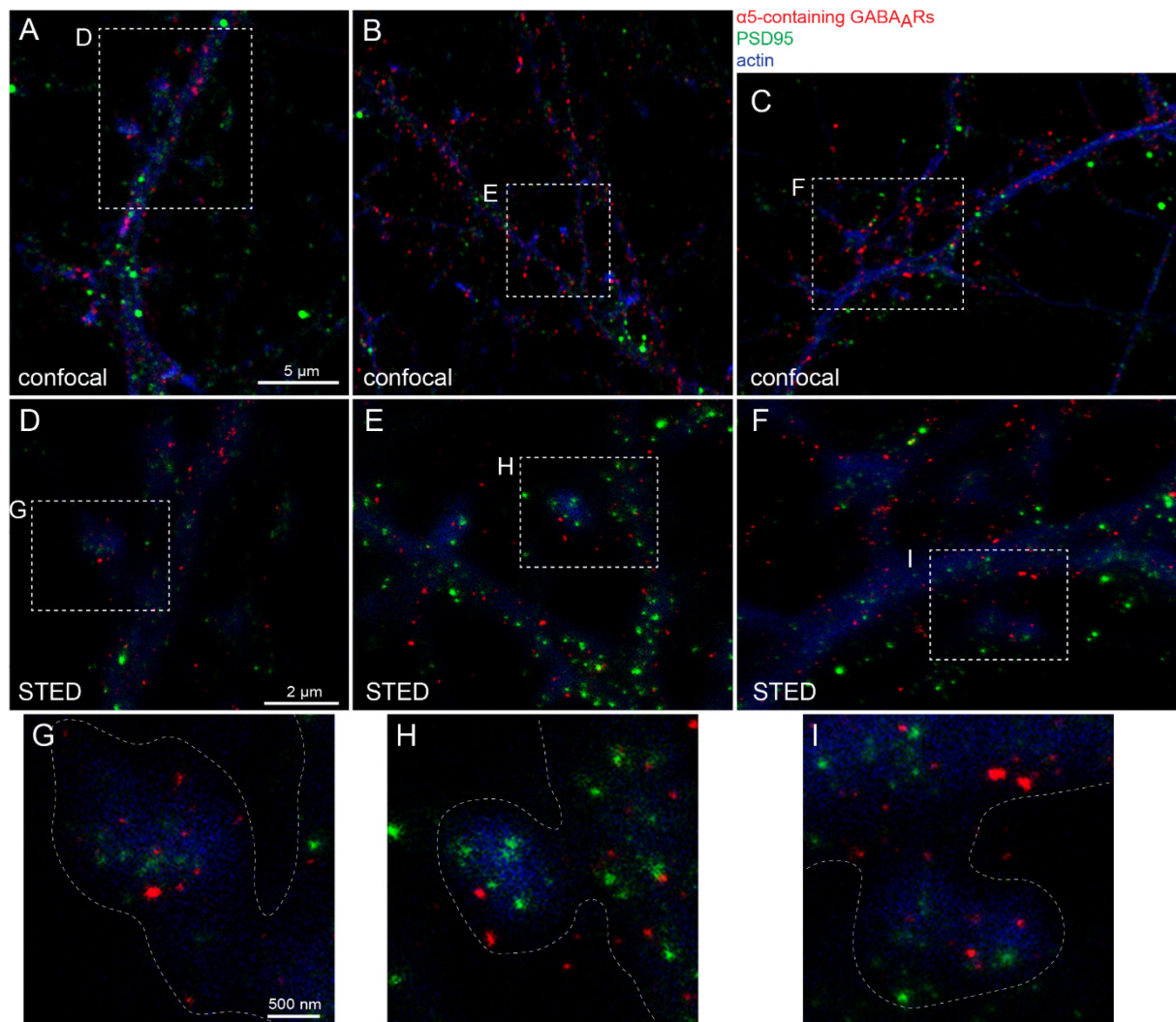


Figure 12: Representative Stimulated Emission Depletion (STED) image of immunofluorescence staining of $\alpha 5$ -GABA_ARs and PSD95 in combination with phalloidin (F-actin) staining. **A, B, C:** Confocal images of live $\alpha 5$ -GABA_ARs immunostaining (red) in wildtype (+/+) hippocampal primary neurons (DIV18). After fixation, PSD95 (green) and phalloidin (F-actin, blue) were stained. **D, E, F:** Images of dendritic spine regions with higher magnification highlighted with a white box in A, B, and C. $\alpha 5$ -GABA_ARs and PSD95 were imaged using STED microscopy and merged with images of F-actin filaments imaged using confocal microscopy. **G, H, I:** Enlargements of dendritic spines display spine shaft, neck, and head, highlighted by boxed regions in D, E, and F.

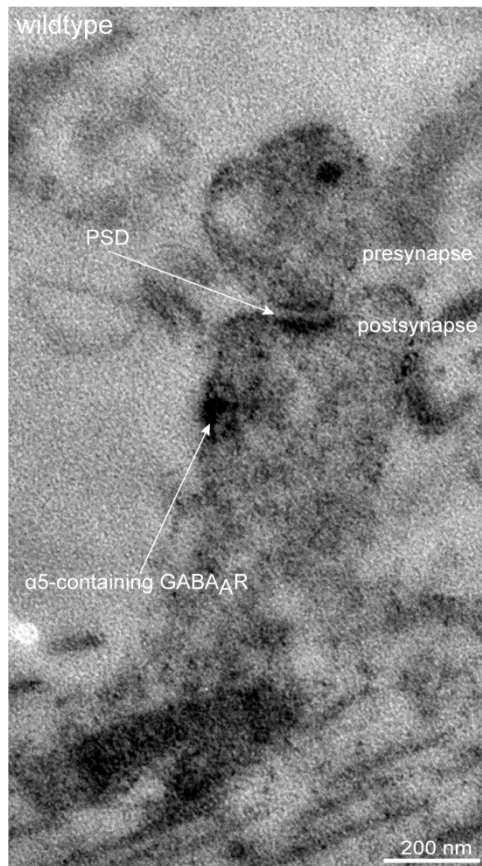


Figure 13: Representative electron microscopy (EM) image of DAB based-immunostaining of a dendritic spine containing $\alpha 5$ - GABA_AR cluster. EM image of $\alpha 5$ -GABA_ARs DAB-based immunostaining in wildtype (+/+) hippocampal primary neurons (DIV18). Dr. Michaela Schweizer from the ZMNH Core Facility Morphology, University Medical Center Hamburg-Eppendorf performed the EM analysis.

3.4 $\alpha 5$ -containing GABA_ARs and PSD95 puncta in nanometer-scale proximity in the presence of synaptophysin

PSD95 plays a crucial role in spine maturation. Overexpression of PSD95 has been shown to promote spine maturation, increase spine density, and enlarge spine size (De Roo et al., 2008; El-Husseini et al., 2000; Meyer et al., 2014; C. E. Taft & Turrigiano, 2014). In addition, enhanced postsynaptic clustering of PSD95 positively affects the maturation of presynaptic terminals (El-Husseini et al., 2000). The former results indicate that $\alpha 5$ -GABA_AR clusters are located near

PSD95 clusters in the same spine. However, this observation does not clarify whether these spines are aligned with presynaptic terminals and form active synapses. Synaptophysin, known for its significant roles in the exocytosis of synaptic vesicles, serves as a presynaptic marker and can be used to clarify further the potential functionality status of the presynaptic terminals (Kokotos et al., 2019; Kwon & Chapman, 2011). Therefore, additional immunostainings were performed to determine whether spines with $\alpha 5$ -GABA_AR puncta in nanometer-scale proximity to PSD95 puncta are in contact with a presynaptic terminal.

$\alpha 5$ -GABA_ARs and PSD95 were labeled in wildtype-derived hippocampal primary neurons with STED-compatible secondary antibodies. Additionally, synaptophysin was labeled with a secondary antibody for confocal microscopy.

The images analyzed using STED microscopy indicate $\alpha 5$ -GABA_AR puncta in nanoscale proximity to PSD95 puncta near synaptophysin clusters. Furthermore, performing two different line scans of potential triple-colocalizations for the various proteins suggests that the intensities of all three proteins reach their peak within the same area, approximately 100 nm in size (Fig. 14 A-E).

This distribution analysis strengthens the previous results obtained by confocal microscopy and shows that $\alpha 5$ -GABA_ARs can be found near PSD95 when a potential functional presynaptic terminal is present.

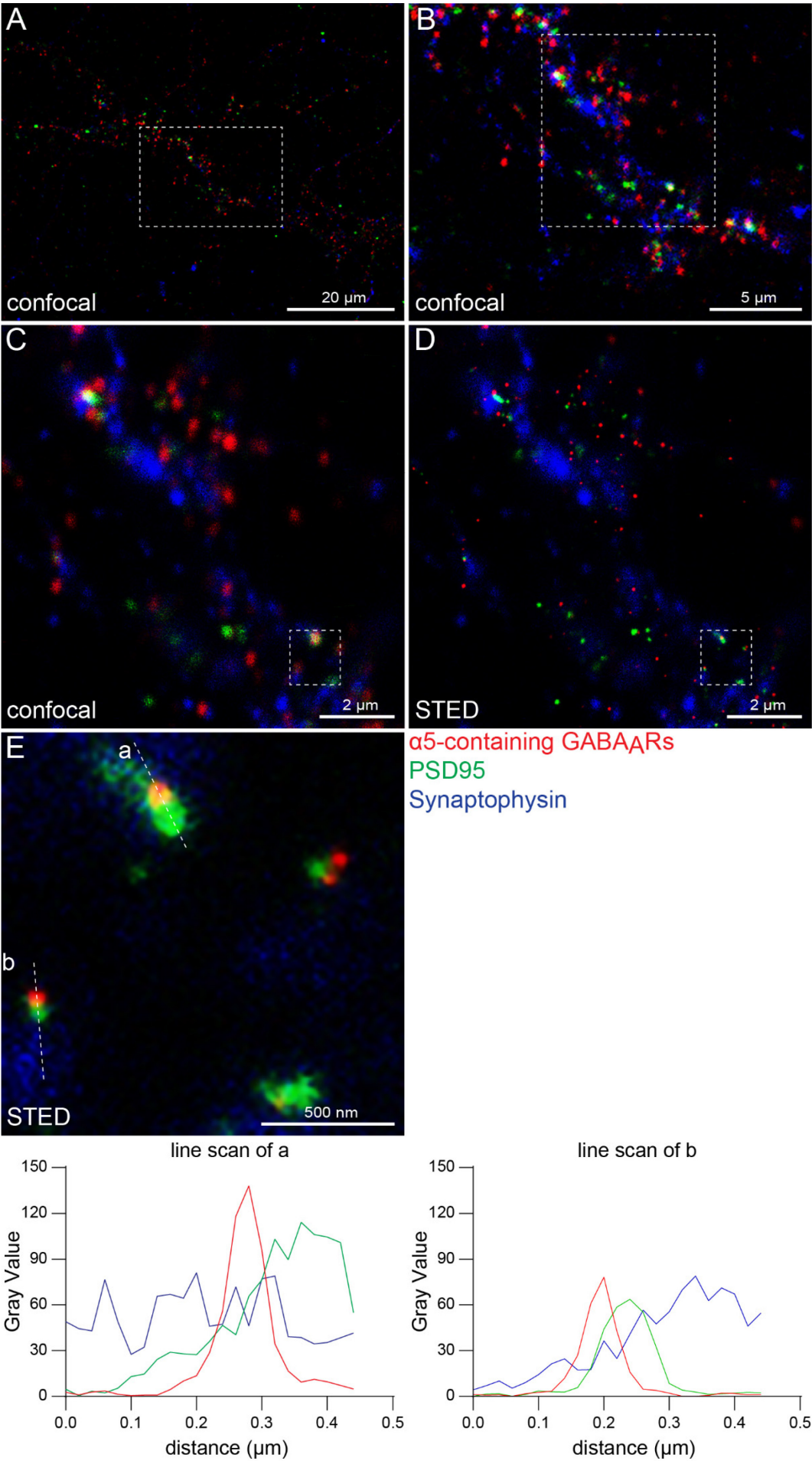


Figure 14: Representative Stimulated Emission Depletion (STED) image of immunofluorescence staining of $\alpha 5$ -GABA_A receptors, PSD95, and synaptophysin. **A-C:** Confocal image of live surface $\alpha 5$ -GABA_ARs immunostaining (red) in wildtype (+/+) hippocampal primary neurons (DIV18). PSD95 (green) and synaptophysin (blue) were stained after fixation. **D-E:** The merged image of live $\alpha 5$ -GABA_ARs immunostaining and PSD95, analyzed with using STED microscopy and synaptophysin, recorded with confocal microscopy. **B:** Enlargement of a dendrite section, highlighted by the white box in A. **C-D:** Enlargement of a dendrite section, highlighted by the white box in B. **E:** Enlargement of synaptic terminals, highlighted by the gray box in D. Signal intensities of individual channels were plotted along the lines (a, b). 2 independent experiments.

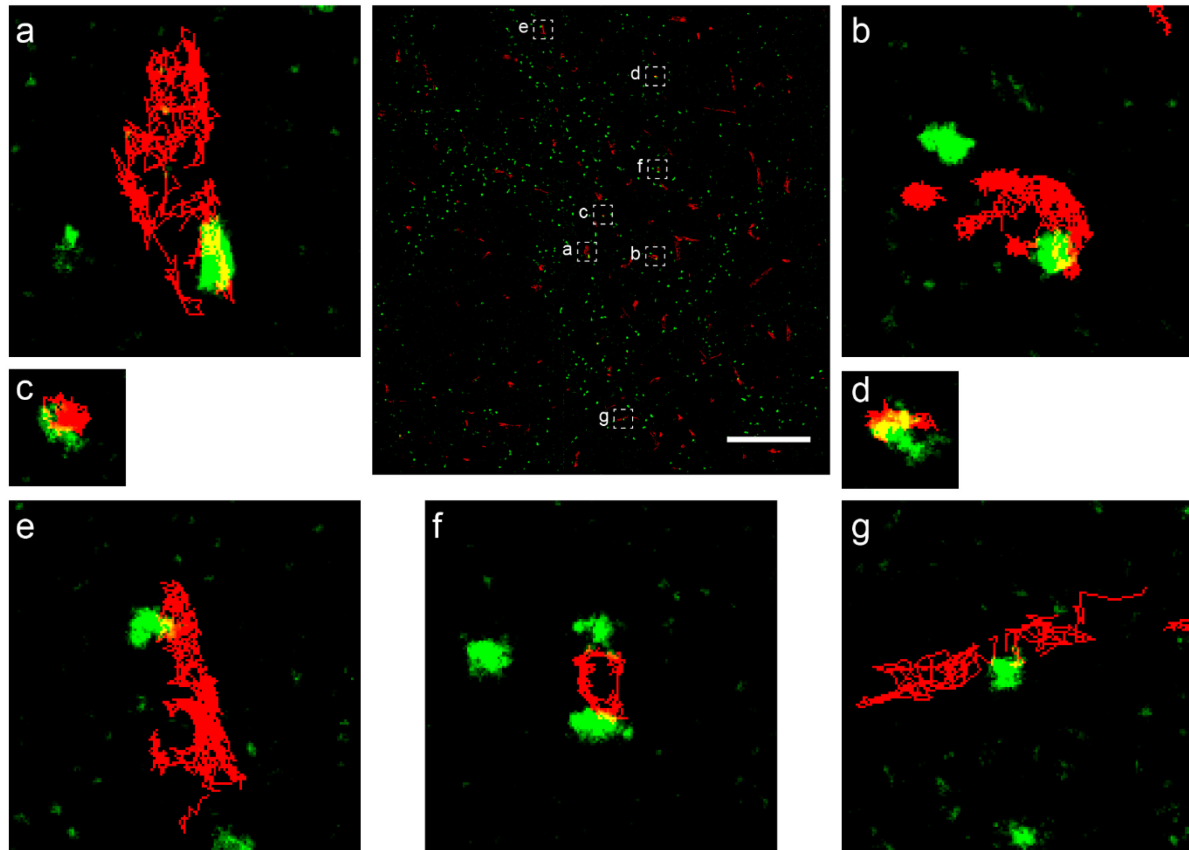
3.5 $\alpha 5$ -containing GABA_AR diffusivity decrease in PSD95-positive regions

The previous data were obtained under fixed conditions in hippocampal neuronal cultures. Further questions arose about whether the potential colocalizations between $\alpha 5$ -GABA_ARs and PSD95 puncta result from the random diffusion of $\alpha 5$ -GABA_ARs passing near spines. For further verification of the colocalization results, $\alpha 5$ -GABA_ARs diffusion was traced inside and outside PSD95-positive regions (green) (Fig. 15 A). This additional validation was achieved by coupling the anti- $\alpha 5$ -GABA_ARs antibody with quantum dots and monitoring the mean square displacement (MSD), diffusion coefficient (D), and confinement radius using STORM microscopy.

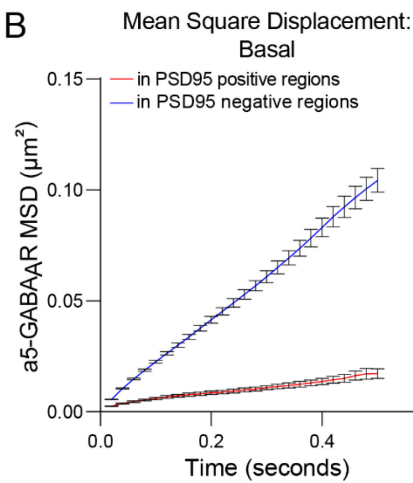
The MSD data indicate that $\alpha 5$ -GABA_ARs in PSD95-positive regions diffuse less from the initial reference point of measurement over a given time than those outside these regions (Fig. 15 B). In addition, the measurements show that $\alpha 5$ -GABA_ARs in PSD95-negative regions have higher diffusion speeds (Fig. 15 C) and travel greater distances (Fig. 15 D) than those in PSD95-positive regions.

The reduced mobility of $\alpha 5$ -GABA_ARs in PSD95-positive regions suggests a potential clustering of $\alpha 5$ -GABA_ARs in PSD95-associated regions.

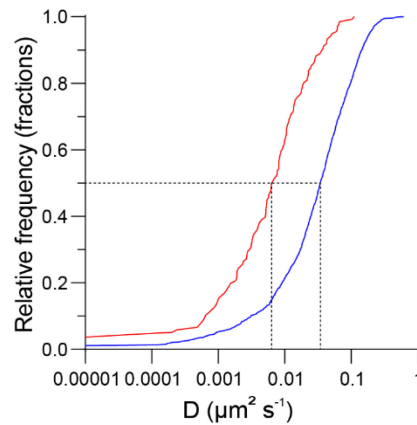
A



B



C



D

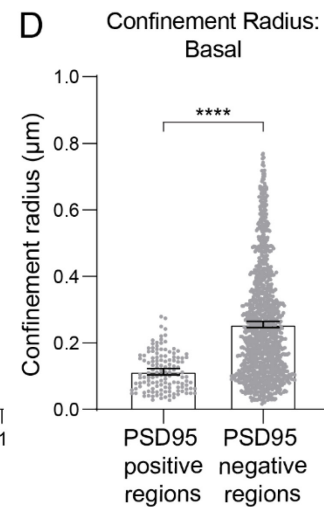


Figure 15: Diffusion properties of $\alpha 5$ -GABA_ARs inside and outside PSD95-positive regions.

A: Example of different trajectories of $\alpha 5$ -GABA_ARs (red) inside and outside PSD95-positive regions (green). **B-D:** $\alpha 5$ -GABA_ARs quantum dot (QD) trajectories inside PSD95-positive regions (red) and PSD95-negative regions (blue) under basal conditions in 0.5s measurement time. **B:** Mean Square Displacement (MSD) versus time plot for $\alpha 5$ -GABA_ARs quantum dot trajectories. **C:** Cumulative probability plot for $\alpha 5$ -GABA_ARs quantum dot diffusion coefficients. **D:** Confinement radius for $\alpha 5$ -GABA_ARs quantum dots (PSD95-pos. vs. PSD95-neg. = 0.1135 ± 0.004951 ($n = 133$), vs. 0.2554 ± 0.004702 ($n = 1247$)). Error bars represent the mean $\pm$ standard error of the mean (SEM). t test: B. **** $p \leq 0.0001$, Man-Whitney test: C, D. **** $p \leq 0.0001$. 3 independent experiments.

3.6 Increased cluster density of PSD95 and synaptophysin on dendrites of *Gabra5* knockout-derived neuronal cells

The activity of $\alpha 5$ -GABA_ARs can influence the dendritic outgrowth and the maturation of spines (Brady & Jacob, 2015). A significant decrease was observed in both the total intensity and total area of $\alpha 5$ -GABA_AR puncta in triple-colocalizations with PSD95 and synaptophysin in *Gabra5* heterozygous-derived neurons compared to wildtype conditions. The subsequent experiment aimed for a more detailed analysis of the proteins involved. Thus, data from the experiment shown in Fig. 11 were further analyzed by comparing the cluster density, total intensity, and total area of $\alpha 5$ -GABA_ARs, PSD95, and Synaptophysin clusters across the different genotypes.

No $\alpha 5$ -GABA_ARs were detected on dendrites of *Gabra5* knockout-derived nerve cells, whereas clear puncta were observed on dendrites derived from *Gabra5* wildtype and heterozygous cell cultures (Fig. 16 A1). The same pattern was also observed for the total intensity (Fig. 16 B1) and total area (Fig. 16 C1), confirming the validity of the $\alpha 5$ -GABA_ARs antibody as presented in the dendrite images of Figures 10 and 11.

Analysis of PSD95 cluster counts reveals a significant increase of cluster density in *Gabra5* knockout-derived neuronal dendrites compared to wildtype and *Gabra5* heterozygous conditions. No notable differences in PSD95 cluster density in *Gabra5* wildtype- and heterozygous-derived dendrites were observed (Fig. 16 A2). The analysis of the total intensity of PSD95 puncta indicates no significant differences among the three genotypes (Fig 16 B2). Furthermore, an increased total area of the PSD95 cluster in *Gabra5* knockout-derived neuronal cultures was observed compared to wildtype and *Gabra5* heterozygous cell cultures. However, no significant differences in the dendrites of *Gabra5* wildtype- and heterozygous-derived neuronal cell cultures regarding the total area of PSD95 were observed (Fig. 16 C2).

The only change in synaptophysin cluster density is a significant increase in dendrites from *Gabra5* knockout-derived neurons compared to wildtype cultures (Fig. 16 A3). No significant differences were seen for the total intensity between all three genotypes (Fig. 16 B3). Analyzing the total area of the synaptophysin cluster reveals the same pattern as seen in Fig. 16 A3. Here, a significant increase can only be seen in dendrites from *Gabra5* knockout-derived neurons compared to wildtype cultures (Fig. 16 C3).

The observed changes in PSD95 and synaptophysin cluster density and total area in the absence of $\alpha 5$ -GABA_ARs could suggest an influence of $\alpha 5$ -GABA_ARs on excitatory synapse formation.

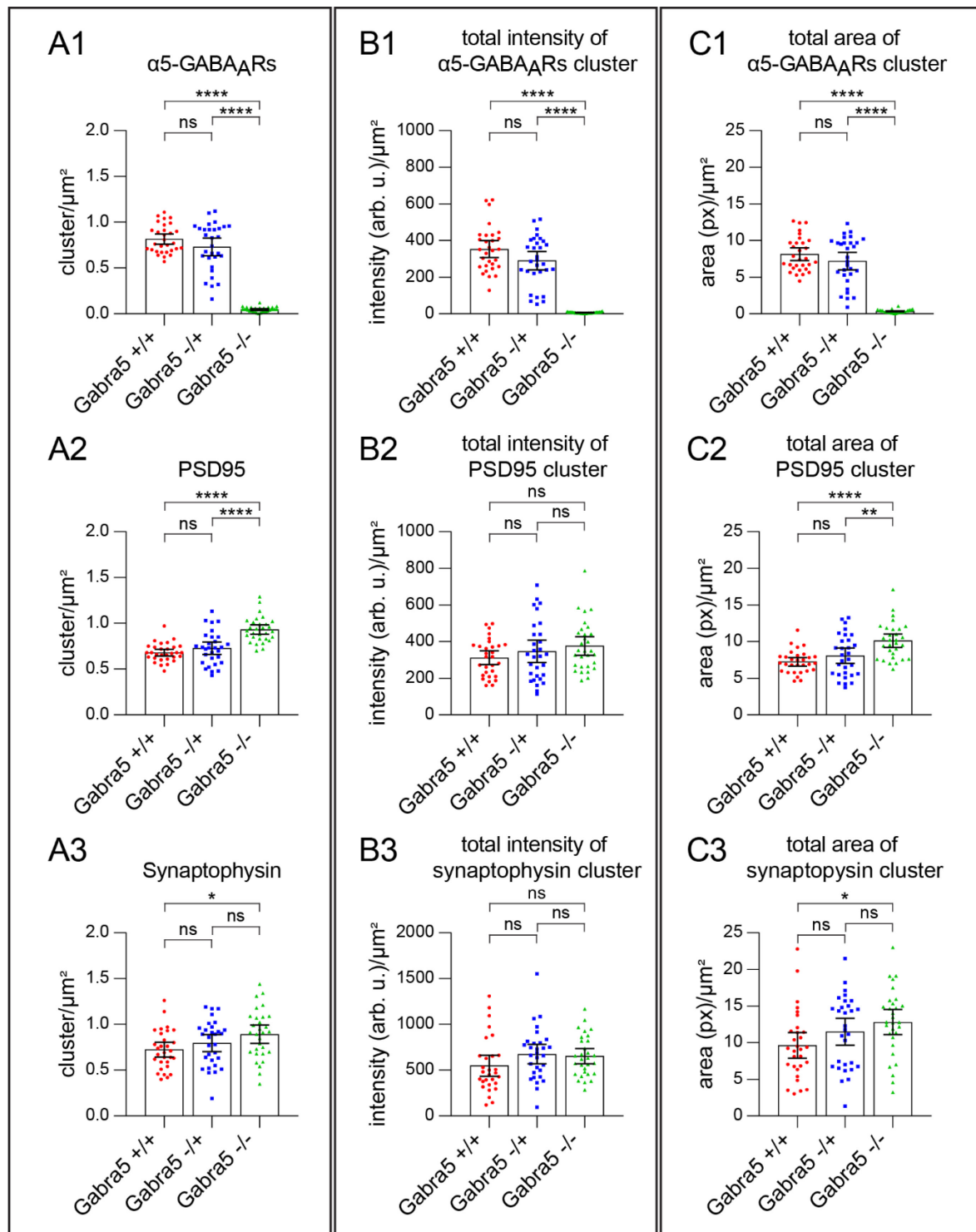


Figure 16: Quantitative analysis of cluster density, total intensity, and total area of $\alpha 5$ -containing GABA_A receptors, PSD95, and synaptophysin in secondary dendrites derived from *Gabra5* wildtype (+/+), heterozygous (-/+) and knockout (-/-) hippocampal primary neuronal cultures. A1-A3: Analysis and comparison of normalized cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs (A1), PSD95 (A2), and synaptophysin (A3) at secondary dendrites derived from wildtype (+/+) (n = 30), *Gabra5* heterozygous (-/+) (n = 30) and *Gabra5* knockout (-/-) (n = 30) hippocampal primary neuronal cultures. **B1-B3:** Analysis and comparison of normalized total intensity (arb. u./ μm^2) of $\alpha 5$ -GABA_ARs (B1), PSD95 (B2), and synaptophysin (B3) at secondary dendrites derived from *Gabra5* wildtype (+/+) (n = 29-30), heterozygous (-/+) (n = 30) and knockout (-/-) (n = 30) hippocampal primary neuronal cultures. **C1-C3:** Analysis and comparison of normalized total area (px/ μm^2) of $\alpha 5$ -GABA_ARs (C1), PSD95 (C2), and synaptophysin (C3) at secondary dendrites derived from *Gabra5* wildtype (+/+) (n = 30), heterozygous (-/+) (n = 30) and knockout (-/-) (n = 30) hippocampal primary neuronal cultures. A1-C3: Error bars

represent mean $\pm$ standard error of the mean (SEM). 3 independent experiments. Ordinary one-way ANOVA: A1-A3, B1-B2, C1-C3; Kruskal-Wallis test: B3. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. Exact p-values and mean $\pm$ SEM are listed in the supplementary data table 1

3.7 Increased excitatory synapse number in *Gabra5* knockout-derived dendrites

The observed phenotype, characterized by an increase in the number and size of PSD95 and synaptophysin clusters in the absence of $\alpha 5$ -GABA_ARs, raises the question of whether this increase leads to a higher number of excitatory synapses.

First, the colocalization of PSD95 and synaptophysin cluster was analyzed. Compared to the colocalization counted on dendrites of wildtype-derived neurons, a significant increase can be detected in *Gabra5* knockout conditions (Fig. 17 A). No significant differences were observed in the number of colocalizations by comparing wildtype- with *Gabra5* heterozygous- and *Gabra5* heterozygous- with knockout-derived dendrites (Fig. 17 A). As before, the percentage of PSD95 and synaptophysin puncta in colocalizations was calculated relative to the total number of PSD95 and synaptophysin clusters detected in a 40 μ m dendrite section. A significantly higher percentage of synaptophysin puncta colocalize with PSD95 in *Gabra5* knockout-derived nerve cells compared to wildtype samples (WT vs. KO = mean $\pm$ SEM = 35.45% $\pm$ 0.9438% (n= 28), vs. 40.47% $\pm$ 1.799% (n= 30); t-test $p = 0.0189$) (Supplementary data Table 1). The proportion of PSD95 puncta in these colocalizations did not differ between the two conditions (WT vs. KO = mean $\pm$ SEM = 38.23% $\pm$ 1.467% (n= 30) vs. 37.66% $\pm$ 1.819% (n= 30); t-test $p = 0.8076$) (Supplementary data Table 1).

Next, the total intensity and total area of the two proteins in the colocalization were analyzed. No significant differences were observed regarding the total intensity (Fig 17 B-C). However, in *Gabra5* knockout-derived dendrites compared to their wildtype counterpart, the total area of PSD95 and synaptophysin puncta was significantly increased (Fig. 17 D-E). Moreover, although the total area of the two proteins increases in colocalizations, the proportion of the total area of PSD95 (WT vs. KO = mean $\pm$ SEM = 44.13% $\pm$ 1.535% (n= 30), vs. 42.71% $\pm$ 1.867% (n= 30); t-test $p = 0.5587$) and synaptophysin puncta (WT vs. KO = mean $\pm$ SEM = 40.22% $\pm$ 1.266% (n= 29), vs. 44.18% $\pm$ 1.893% (n= 30); t-test $p = 0.0897$) relative to the total area of all puncta in the sample remains unchanged across different genotypes (Supplementary data Table 1).

These results reveal more excitatory synapses on the dendrites of *Gabra5* knockout-derived neuronal cells. Furthermore, the altered proportion of synaptophysin puncta found in colocalization with PSD95 compared to the total number of synaptophysin suggests that the increased formation of excitatory synapses may originate from increased synaptophysin cluster density and that the absence of $\alpha 5$ -GABA_ARs may influence presynaptic terminals. Additionally, the data suggest that the increase in the total area of PSD95 and synaptophysin puncta within these colocalizations is directly proportional to the overall increase in area.

Since the synaptophysin can serve as a marker for both inhibitory and excitatory presynapses, further immunostaining with more specific markers could help to clarify whether the potential increase in number and size of the presynaptic side during to the absence of $\alpha 5$ -GABA_ARs is related to excitatory synapses.

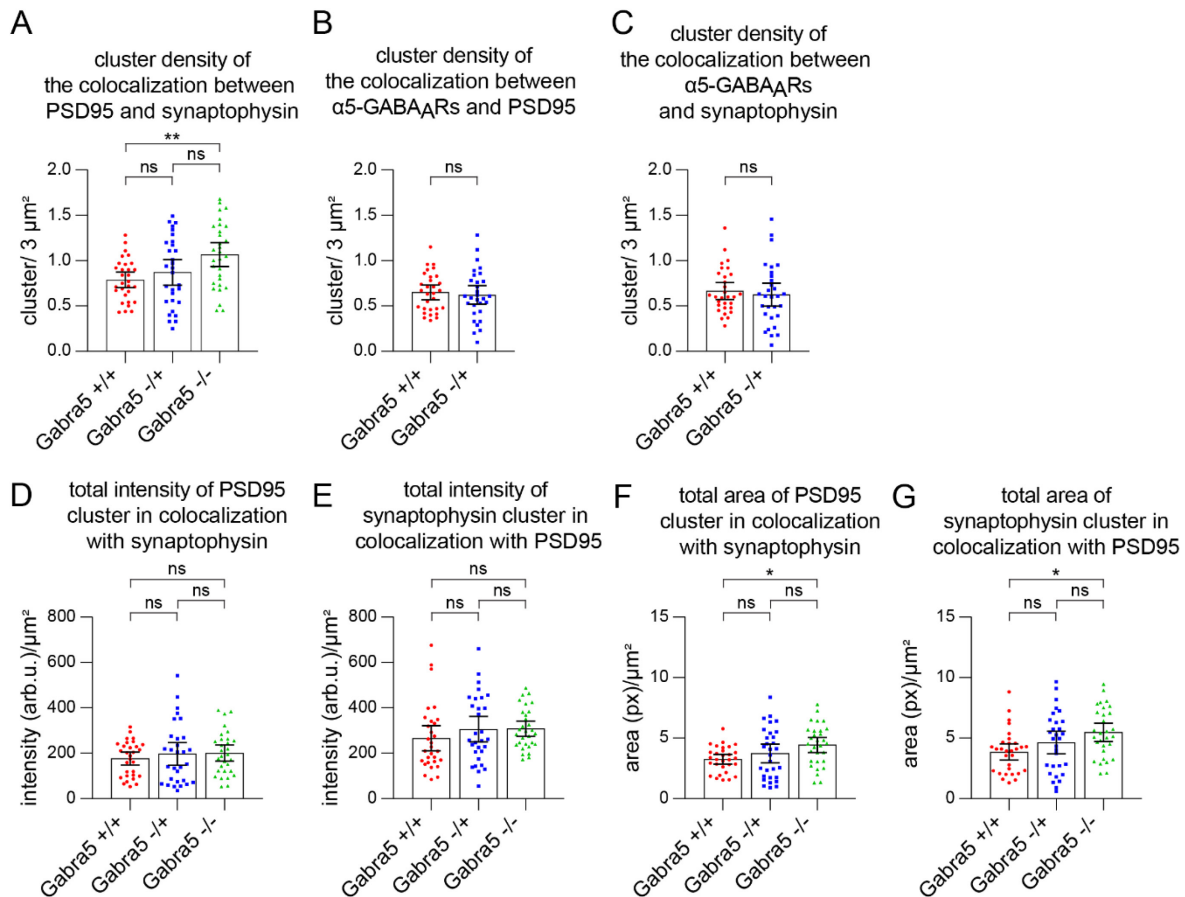


Figure 17: Quantitative analysis of cluster density, total intensity, and total area of the colocalization between PSD95 and Synaptophysin. **A-E:** Analysis and comparisons were performed between secondary dendrites derived from wildtype (+/+) (n = 30), *Gabra5* heterozygous (-/+) (n = 30), and *Gabra5* knockout (-/-) (n = 30) hippocampal primary neuronal cultures (DIV18). **A:** Normalized cluster density (cluster/ μm^2) of colocalization between PSD95 and Synaptophysin. **B:** Analysis of normalized total intensity (arb. u./ μm^2) of PSD95 cluster in the colocalization with synaptophysin. **C:** Normalized total intensity (arb. u./ μm^2) of synaptophysin cluster in the colocalization with PSD95. **D:** Analysis of normalized total area (px/ μm^2) of PSD95 cluster in the colocalization with synaptophysin. **E:** Normalized total area (px/ μm^2) of synaptophysin cluster in the colocalization with PSD95. Error bars represent the mean $\pm$ standard error of the mean (SEM). 3 independent experiments. Ordinary one-way ANOVA: A, D, E. * $p \leq 0.05$. Unpaired t-test: B, C. ns $p > 0.05$. Exact p-values and mean $\pm$ SEM are listed in supplementary data table 1

3.8 Triple-colocalization of $\alpha 5$ -containing GABA_A receptors, GluR2, and VGLUT1

PSD95 and synaptophysin are considered reliable markers used in synapse research (Liebau et al., 2007; Villa et al., 2016). However, synaptophysin as a presynaptic marker does not differentiate between inhibitory or excitatory presynaptic terminals. Therefore, additional immunostainings were required for a more comprehensive analysis. Using antibodies that specifically probe an excitatory receptor and excitatory presynaptic terminal would further strengthen the previous results. Accordingly, the AMPA-receptor GluR2 was used as an excitatory postsynaptic marker, and VGLUT1 as an excitatory presynaptic marker. These proteins were analyzed in the same way as in 3.2.

The following figure exemplifies the triple-immunostaining images of *Gabra5* wildtype- (Fig. 18 A1-A3) and *Gabra5* knockout-derived (Fig. 18 B1-B3) dendrites using confocal microscopy. Furthermore, an enlarged section of a dendrite is shown for every genotype with all three

channels is displayed (Fig. 18 A2, B2). Similar to previous triple-colocalization analyses, a line scan was performed using an example of superimposed protein clusters from a wildtype-derived dendrite. The intensities of all three proteins seem to peak within an area of around 0.2 μm (Fig. 18 A3). As a control, an additional line scan analysis was performed on the *Gabra5* knockout samples. No signal was detected for $\alpha 5$ -GABA_ARs, whereas for GluR2 and VGLUT1, a peak in intensity was detected within an area of 0.2 μm (Fig. 18 B3).

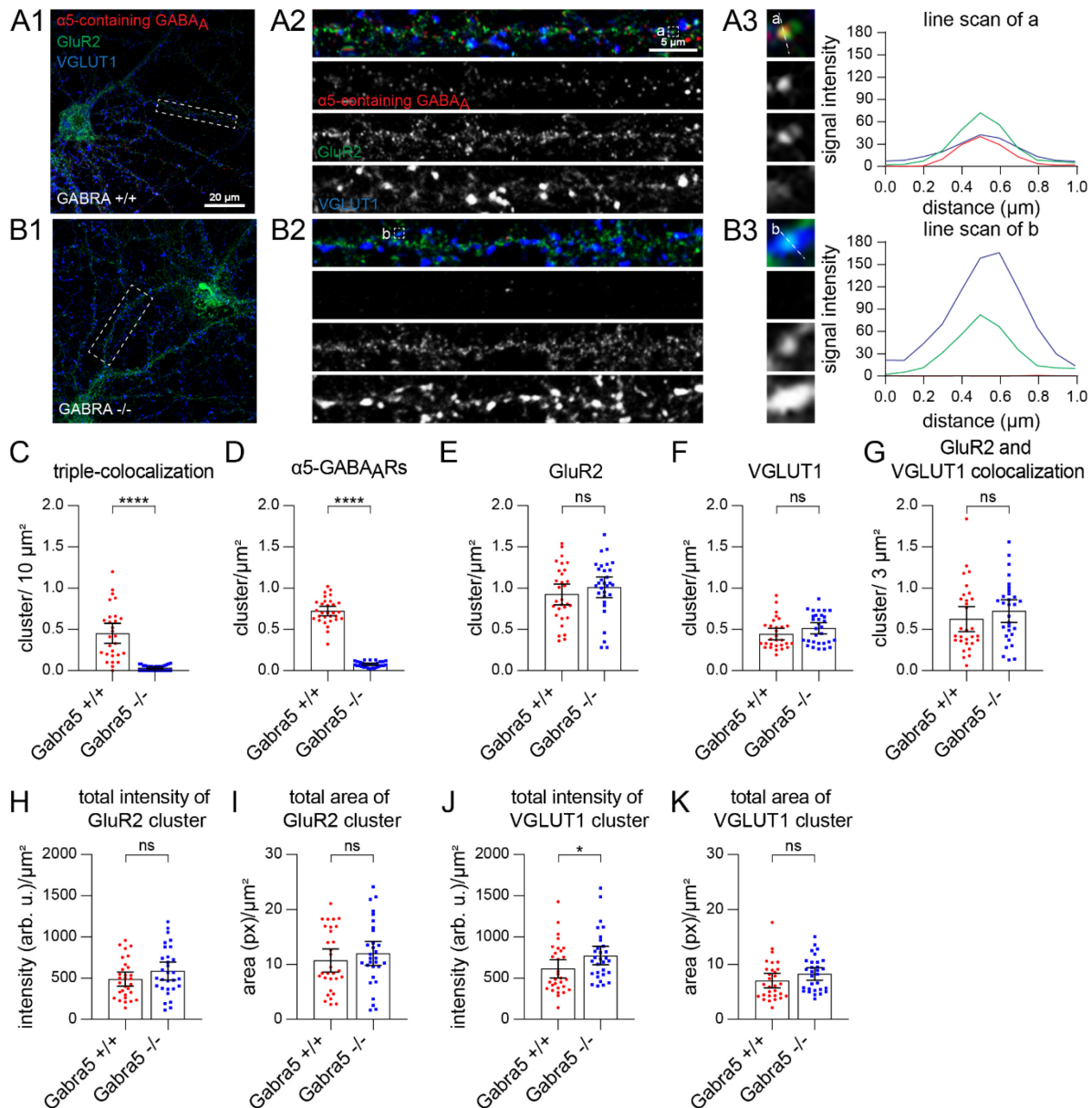
The analysis of $\alpha 5$ -GABA_ARs cluster density of the triple-colocalizations showed a significant difference in wildtype samples compared to *Gabra5* knockout-derived dendrites, again proving the specificity of the $\alpha 5$ -GABA_ARs antibody (Fig. 18 C, D). However, the previously observed increased cluster density numbers in *Gabra5* knockout samples analyzing synaptophysin and PSD95 were not complemented by probing GluR2 (Fig. 18 E) and VGLUT1 (Fig. 18 F)

Also, no significant differences between the two genotypes were observed regarding increased excitatory synapse numbers when using the protein marker for GluR2 and VGLUT1 (Fig. 18 G). A detailed analysis of the total intensity and total area of the GluR2 cluster did not show any significant changes in *Gabra5* knockout-derived dendrites compared to wildtype controls (Fig. 18 H, I). However, comparing the total intensity of the VGLUT1 puncta revealed a significant increase in the *Gabra5* knockout condition (Fig. 18 J) without significant differences regarding the total area (Fig. 18 K).

Calculating the percentage of the cluster density of $\alpha 5$ -GABA_ARs puncta in these triple-colocalizations relative to their total number showed that around 6 % are near excitatory synapses in *Gabra5* wildtype-derived dendrites (WT = mean $\pm$ SEM = 6.092% $\pm$ 0.7455%, n = 28). Approximately 23 % of excitatory synapses were detected to contain $\alpha 5$ -GABA_ARs (WT = mean $\pm$ SEM = 23.00% $\pm$ 2.070%, n = 28) (Fig. 18 L; Supplementary data Table 2). Additionally, the analysis revealed that approximately 40% of $\alpha 5$ -GABA_ARs puncta seem to colocalize with the GluR2 cluster (WT = mean $\pm$ SEM = 38.41% $\pm$ 2.386%, n = 30) (Supplementary data Table 2).

To further validate the triple-colocalization between $\alpha 5$ -GABA_ARs, GluR2, and VGLUT1, STED microscopy was performed. The images obtained confirm the previously observed results using confocal microscopy (Fig. 18) and show that $\alpha 5$ -GABA_AR puncta are in nanometer-scale proximity to GluR2 puncta in the presence of VGLUT1 (Fig. 19 D, E, F). Furthermore, two different line scans (a, b) revealed that the intensity level of $\alpha 5$ -GABA_ARs, GluR2, and VGLUT1 peak within the same area of approximately 0.1 μm (Fig. 10).

These results suggest that $\alpha 5$ -GABA_ARs not only seem to colocalize with PSD95 and synaptophysin but also with different specific excitatory pre- and postsynaptic proteins. Furthermore, the increased intensity of VGLUT1 clusters in presynaptic terminals of *Gabra5* knockout-derived dendrites could reinforce previous synaptophysin findings, suggesting a potential role for $\alpha 5$ -GABA_ARs in the development or maturation of presynaptic terminals. Since synaptophysin appears early in presynaptic terminal development (Liebau et al., 2007), further experiments should investigate when $\alpha 5$ -GABA_ARs begin to influence VGLUT1 protein levels.



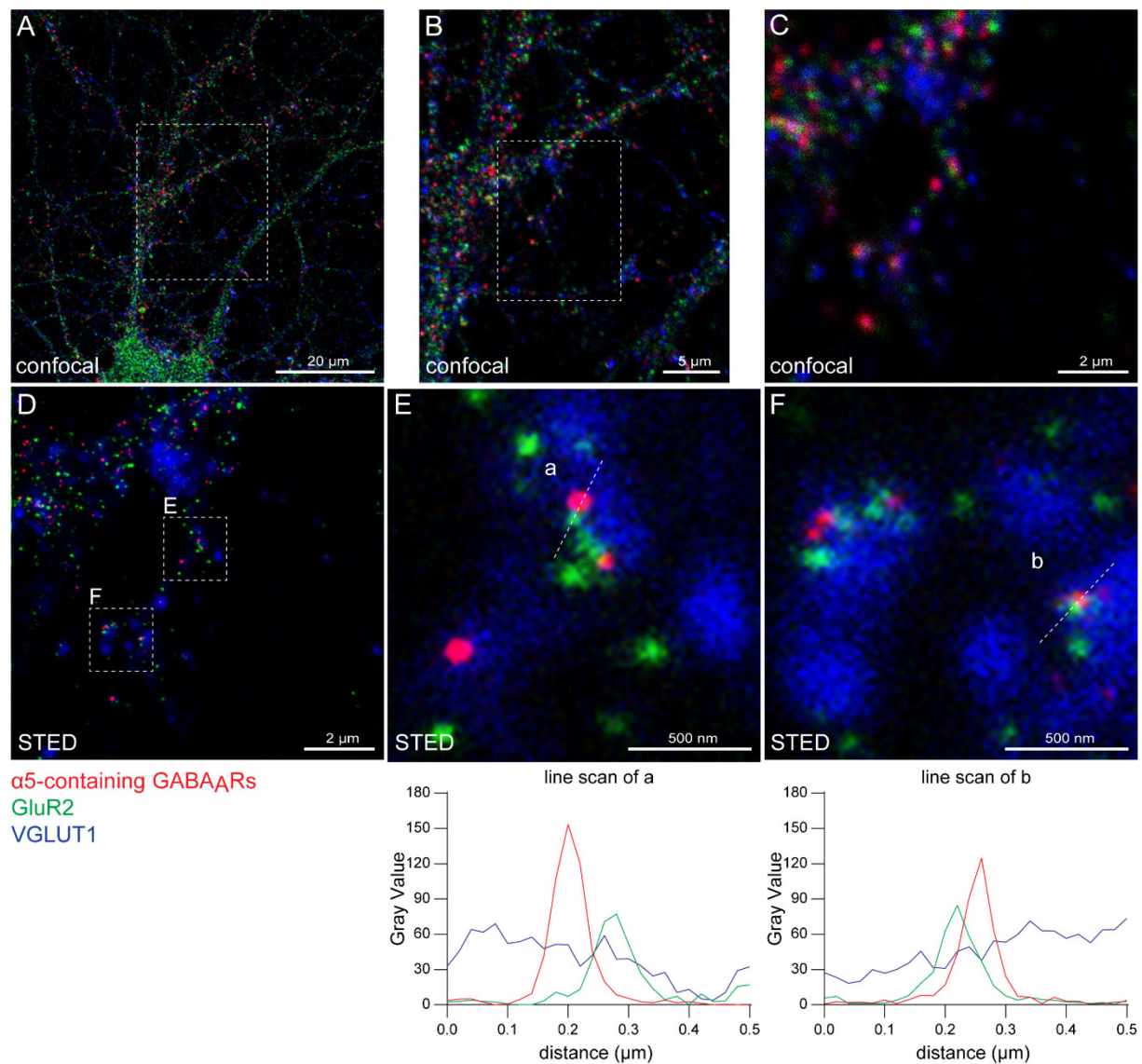


Figure 19: Representative Stimulated Emission Depletion (STED) image of immunofluorescence staining of $\alpha 5$ -GABA_A receptors and GluR2. **A-C:** Confocal image of live surface $\alpha 5$ -GABA_ARs immunostaining (red) in wildtype (+/+) hippocampal primary neurons (DIV19). GluR2 (green) and VGLUT1 (blue) were stained after fixation. **D-F:** The merged image of live $\alpha 5$ -GABA_ARs immunostaining and GluR2 was recorded with STED microscopy, and VGLUT1 was recorded with confocal microscopy. **B:** Enlargement of a dendrite section, highlighted by the white box in A. **C-D:** Enlargement of a dendrite section, highlighted by the white box in B. **E-F:** Enlargement of synaptic terminals, highlighted by the gray box in D. The gray values of exemplified triple-colocalization were plotted along the lines (a,b). 2 independent experiments

3.9 Increased VGLUT1 cluster density during neuronal development in *Gabra5* knockout-derived dendrites

An increased number of excitatory synapses, PSD95, and synaptophysin total area, as well as a significantly higher intensity of VGLUT1 puncta in *Gabra5* knockout-derived dendrites, were observed in the previous experiments. The potential influence of GABAergic signal cascade on the maturation of pre- and postsynapse was mentioned in a former publication (reviewed by (Huang, 2009)). Postsynaptic proteins such as PSD95 also seem to influence the stabilization process of pre- and postsynaptic terminals (Taft & Turrigiano, 2014).

These studies, the previous results of this thesis, and the fact that the abundance, as well as the location of $\alpha 5$ -GABA_ARs, showed to influence dendritic spine maturation (Brady & Jacob, 2015; Nuwer et al., 2021), raising new questions about the potential influence of $\alpha 5$ -GABA_ARs activity on the presynaptic terminal formation during different neurodevelopmental phases. Furthermore, the hypothesis emerged that the absence of $\alpha 5$ -GABA_ARs might alter the concentration of presynaptic proteins, leading to an increased abundance of presynaptic excitatory terminals.

To test this hypothesis, neurons at earlier developmental stages were analyzed. Previous experiments (not shown) suggest differences in VGLUT1 cluster density around DIV 10 to DIV 8 and DIV 12. Consequently, $\alpha 5$ -GABA_ARs and VGLUT1 clusters were analyzed and compared in DIV 10 and DIV 12 wildtype- and *Gabra5* knockout-derived dendritic regions.

Figure 20 exemplifies the co-immunostaining of dendrites derived from wildtype (Fig. 20 A-B) and *Gabra5* knockout (Fig. 20 C-D) neuronal cultures. In wildtype conditions, clusters of $\alpha 5$ -GABA_ARs are detectable in DIV 10 and DIV 12. No signals were detected for $\alpha 5$ -GABA_ARs in *Gabra5* knockout conditions.

There was no significant difference in cluster density of $\alpha 5$ -GABA_ARs detected in wildtype conditions between DIV 10 and DIV 12 (Fig. 20 E). Quantification of the cluster density of VGLUT1 shows no significant difference between wildtype and *Gabra5* knockout conditions at DIV 10 (Fig. 20 F). However, analysis of DIV 12 neurons reveals a significant increase in VGLUT1 cluster density in *Gabra5* knockout-derived dendritic regions as compared to wildtype controls (Fig. 20 G).

The analysis of total intensity and area shows no differences at DIV 10 for the two genotypes (Fig. 20 H, J). However, at DIV 12, VGLUT1 puncta significantly increases in total intensity and area in *Gabra5* knockout conditions (Fig. 20 I, K).

These initial data suggest that the absence of $\alpha 5$ -GABA_ARs could potentially affect the formation of excitatory presynaptic terminals during neuronal development.

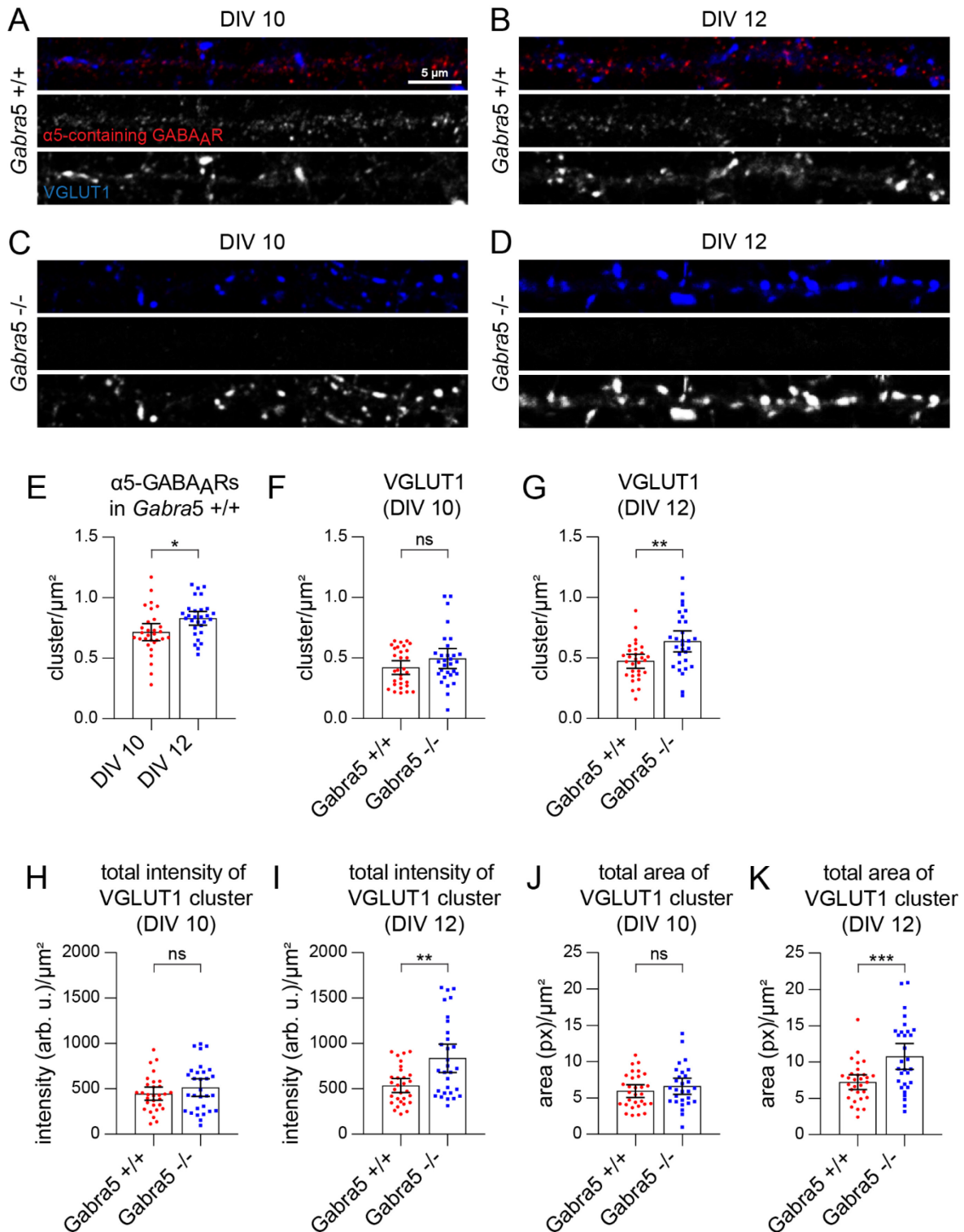


Figure 20: Immunocytochemical analysis of $\alpha 5$ -GABA_A receptors and VGLUT1 in dendritic regions at days-in-vitro (DIV) 10 and 12. A-D: Live surface immunostainings of $\alpha 5$ -GABA_ARs (red) were conducted in secondary dendrites of *Gabra5* wildtype ($+/+$) (A-B) and *Gabra5* knockout ($-/-$) (C-D) derived primary hippocampal neurons at DIV10 and DIV12. In addition, VGLUT1 (blue) was probed after fixation. E: Quantification of the normalized cluster density (cluster/ μ m²) of $\alpha 5$ -GABA_ARs in DIV10 ($n = 30$) and DIV12 ($n = 29$) in *Gabra5* $+/+$. F-K: Quantification of the normalized parameters in *Gabra5* $+/+$ ($n = 30$ (F, G, H, I, J, K)) and *Gabra5* $-/-$ ($n = 28$ (J), 30 (F, G, H, I, K)) conditions. F: Cluster density (cluster/ μ m²) of VGLUT1 at DIV10. G: Cluster density (cluster/ μ m²) of VGLUT1 at DIV12. H: Total intensity (arb. u./ μ m²) of VGLUT1 at DIV10. I: Total intensity (arb. u./ μ m²) of VGLUT1 at DIV12. J: Total area (px/ μ m²) of VGLUT1 at DIV10. K: Total area (px/ μ m²) of VGLUT1 at DIV12. E-K: Error bars represent the mean $\pm$ standard error of the mean (SEM). Unpaired t-test: E, G, H, J, K. ns $p > 0.05$, * p

≤ 0.05 , ** $p \leq 0.01$, *** $p \leq 0.001$. Mann-Whitney test: F, I. ns $p > 0.05$, ** $p \leq 0.01$. 3 independent experiments. Exact values for mean $\pm$ SEM are listed in the supplementary data table 2

3.10 Triple-colocalization of $\alpha 5$ -containing GABA_A receptors, VGAT, and VGLUT1

The previous experiments were conducted to understand better whether $\alpha 5$ -GABA_ARs are found near excitatory synapses. The results suggest a small fraction of $\alpha 5$ -GABA_ARs in nanometer-scale proximity of excitatory synaptic marker proteins, similar to DiSs.

It was shown that DiSs oppose a presynaptic excitatory and inhibitory terminal (Chen et al., 2012), and $\alpha 5$ -GABA_ARs are highly sensitive towards small concentrations of GABA (Caraiscos et al., 2004). Therefore, the activation of $\alpha 5$ -GABA_ARs on synaptic areas could be mediated by various mechanisms, including GABA diffusion from opposing presynaptic terminals, interactions with astrocytes (reviewed by (W. Koh et al., 2023; Yoon et al., 2012)), and spillover-related effects (Mortensen & Smart, 2006). In the past decade, several new theories have emerged to challenge the classical view that presynaptic terminals release only one type of neurotransmitter. These theories also explore the presence of presynapses containing both excitatory and inhibitory neurotransmitters (reviewed by (Tritsch et al., 2016)). The different ways how neurotransmitters might reach $\alpha 5$ -GABA_ARs raise questions about the activation of DiSs containing $\alpha 5$ -GABA_ARs.

To address these questions, additional immunostainings were performed to find the first indicators for the activation system of these $\alpha 5$ -GABA_ARs-containing DiSs. $\alpha 5$ -GABA_ARs, VGAT, an inhibitory presynaptic marker, and VGLUT1, an excitatory presynaptic marker, were analyzed for colocalizations in dendritic regions derived from wildtype hippocampal neuronal cells.

The immunostainings of $\alpha 5$ -GABA_ARs, VGAT, and VGLUT1 are exemplified in Figure 21 A-C. Enlargements of two triple-colocalizations are displayed with related line scan analysis (Fig. 21 B-C). Both line scans suggest a peak of intensity for all three proteins within the same area of around 0.2 μ m. The quantification of the different proteins and their colocalizations is displayed in Figure 21 D-E.

The distribution analysis revealed that approximately 3 % of $\alpha 5$ -GABA_ARs (WT = mean $\pm$ SEM = 3.406% $\pm$ 0.4824%, n = 30), around 8 % of VGAT (WT = mean $\pm$ SEM = 7.852% $\pm$ 1.061%, n = 30), and approximately 5 % of VGLUT1 puncta (WT = mean $\pm$ SEM = 5.005% $\pm$ 0.7081%, n = 30) relative to their total cluster number, are in triple-colocalizations with each other. Focusing on the inhibitory synapses marked by the colocalization of $\alpha 5$ -GABA_ARs and VGAT showed that around 15 % of $\alpha 5$ -GABA_ARs (WT = mean $\pm$ SEM = 14.72% $\pm$ 1.272%, n = 30) are in synaptic areas. Additionally, the data suggests that approximately 21 % of these $\alpha 5$ -containing inhibitory synapses can be found near VGLUT1 puncta (WT = mean $\pm$ SEM = 21.89% $\pm$ 2.549%, n = 30) (Fig.21 E, Supplementary data Table 2).

By comparing the different triple-colocalizations of the past experiments, such as colocalizations between $\alpha 5$ -GABA_ARs/PSD95/Synaptophysin, $\alpha 5$ -GABA_ARs/GluR2/VGLUT1, $\alpha 5$ -GABA_ARs/VGAT/VGLUT1, and $\alpha 2$ -GABA_ARs/PSD95/VGLUT1, similar cluster densities can be observed (Fig. 21 F).

To further understand if the triple-colocalization between $\alpha 5$ -GABA_ARs, VGLUT1, and VGAT is formed by two separate presynaptic terminals in the near proximity of a postsynaptic terminal or of one presynapse containing both neurotransmitters, STED microscopy was employed. The images indicate that some clusters from all three proteins can be found within a distance of approximately 200 nm (Fig. 22).

These results indicate a small population of VGLUT1 and VGAT puncta near $\alpha 5$ -GABA_ARs. Furthermore, the images acquired through STED microscopy seem to strengthen the hypothesis of two different presynaptic terminals binding to one postsynaptic side.

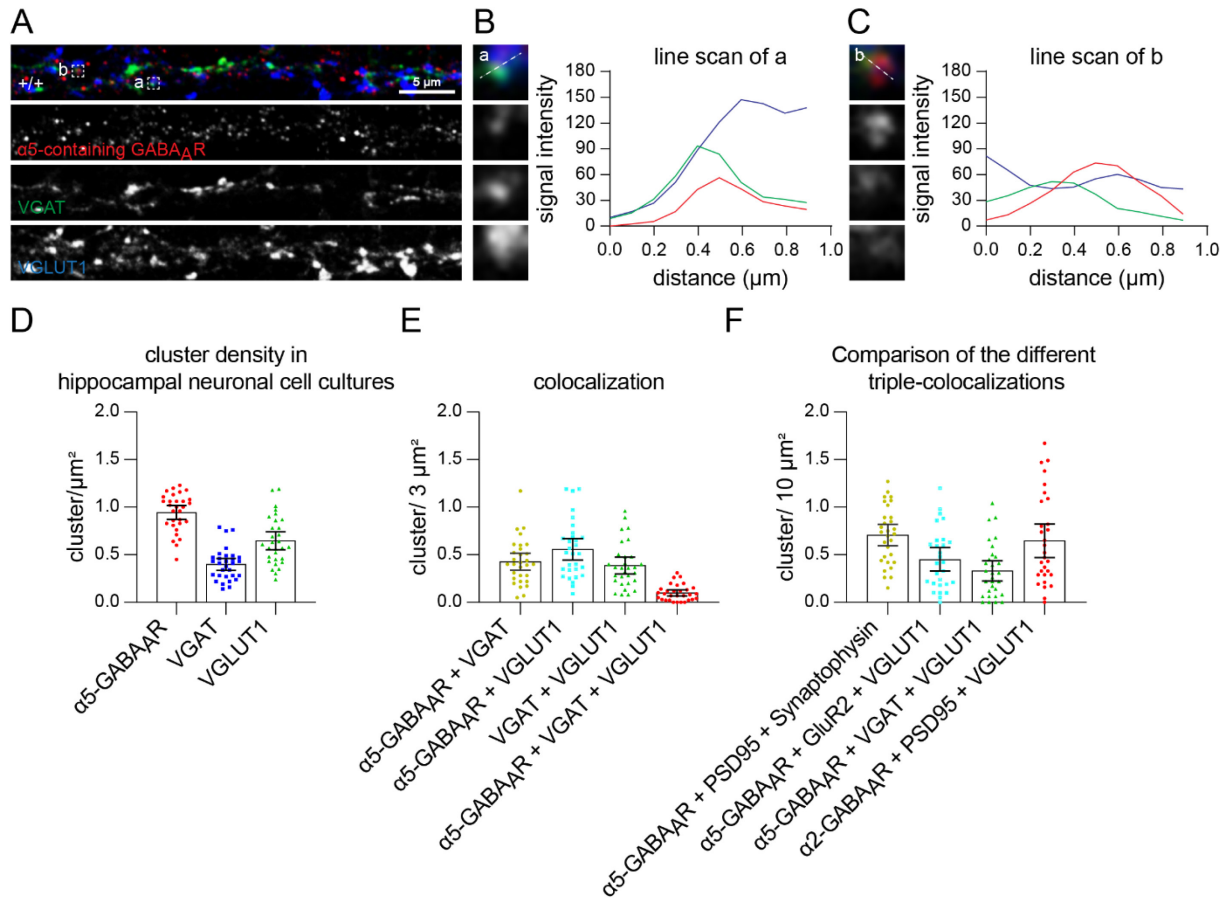


Figure 21: Immunocytochemical analysis of $\alpha 5$ -containing GABA_A receptors, VGAT, and VGLUT1 in dendritic regions. **A-C:** Live-immunostainings of $\alpha 5$ -GABA_ARs (red) were conducted in secondary dendrites of wildtype (+/+) derived primary hippocampal neurons (DIV18-20). In addition, VGAT (green) and VGLUT1 (blue) were probed after fixation. Enlargements of a 40 μm -long dendrite section are displayed in all three channels (A). Exemplified triple-colocalizations, highlighted by gray squares (a-b) in A, were enlarged, and signal intensities of individual channels were plotted along the lines (B-C). **D:** Quantification of the normalized cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs (n = 30), VGAT (n = 30), and VGLUT1 (n = 30). **E:** Quantification of the normalized cluster density (cluster/ μm^2) of colocalization between $\alpha 5$ -GABA_ARs and VGAT (n = 30), $\alpha 5$ -GABA_ARs and VGLUT1 (n = 30), VGAT and VGLUT1 (n = 30), and triple-colocalization ($\alpha 5$ -GABA_ARs/VGAT/VGLUT1) (n = 30). **F:** Quantification of the different triple-colocalization combinations tested: $\alpha 5$ -GABA_ARs/PSD95/synaptophysin (Fig. 11), $\alpha 5$ -GABA_ARs/GluR2/VGLUT1 (Fig. 18), $\alpha 5$ -GABA_ARs/VGAT/VGLUT1 (Fig. 21), and $\alpha 2$ -GABA_ARs/PSD95/VGLUT1 (Fig. 23). Error bars represent the mean $\pm$ standard error of the mean (SEM). 3 independent experiments. Exact p-values and values for mean $\pm$ SEM are listed in the supplementary data table 2

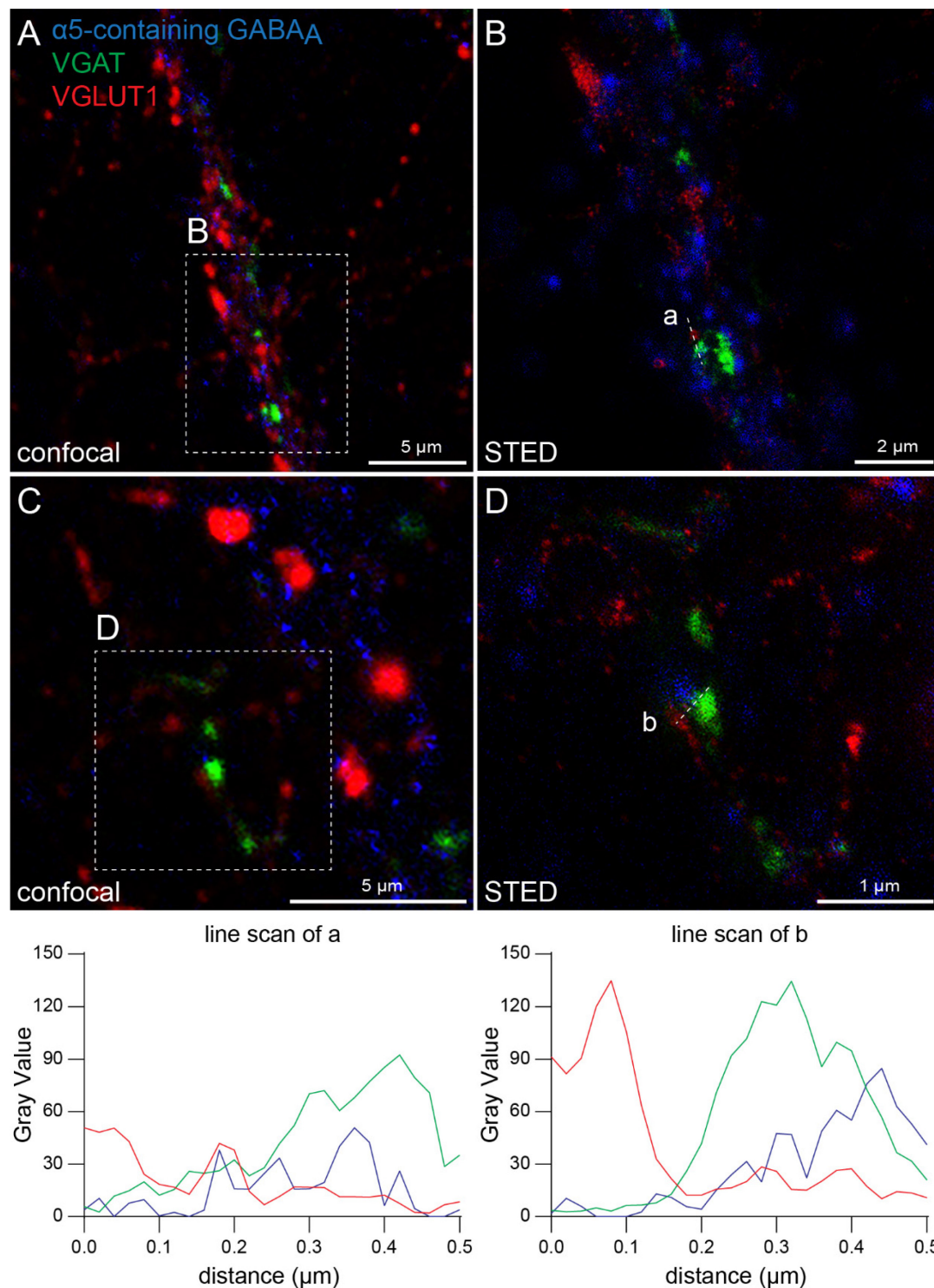


Figure 22: Representative Stimulated Emission Depletion (STED) image of immunofluorescence staining of VGLUT1 and VGAT in the proximity of $\alpha 5$ -GABA_ARs. **A, C:** Confocal image of live surface $\alpha 5$ -GABA_ARs immunostaining (blue) in wildtype (+/+) hippocampal primary neurons (DIV18). VGAT (green) and VGLUT1 (red) were stained after fixation. **B, D:** The merged image of VGLUT1 and VGAT was acquired with STED microscopy, and $\alpha 5$ -GABA_ARs immunostaining was imaged with confocal microscopy. The gray values of exemplified triple-colocalization (B, D) were plotted along the lines (a,b). 2 independent experiments

3.11 Triple-colocalization of $\alpha 2$ -containing GABA_A receptors, PSD95 and VGLUT1

Kleinjan et al. reported that approximately 75 % of DiS are associated with the $\gamma 2$ -containing GABA_A receptors ($\gamma 2$ -GABA_ARs) (Kleinjan et al., 2023). This γ -subunit frequently appears in GABA_A receptors containing the $\alpha 5$ -subunit (McKernan & Whiting, 1996). Approximately 20% of $\alpha 2$ -GABA_ARs were identified in heterologous $\alpha 2/\alpha 5$ -containing GABA_ARs in hippocampal neurons (Del Río et al., 2001). Since the previous data suggest that only a small fraction of $\alpha 5$ -GABA_ARs are located on spines, analyzing an α -subunit, which is found to be predominantly on synaptic sides, could provide a better perspective into the distribution of $\alpha 5$ -GABA_ARs. Synaptic $\alpha 2$ -GABA_ARs were also associated with the $\gamma 2$ -subunit (McKernan & Whiting, 1996). Therefore, the colocalization of $\alpha 2$ -GABA_ARs with PSD95, as an excitatory postsynaptic marker, and VGLUT1, as a presynaptic excitatory marker, were analyzed.

An example of a triple-immunostaining in dendritic regions derived from wildtype samples is shown in Figure 23 A-C. For better representation, an enlarged section of a dendritic region is also presented (Fig 23 B). Subsequent line-scan analysis indicates that the three proteins peak within the same region of approximately 0.4 μm (Fig. 23 C). For further calculations, cluster densities for each protein were counted (Fig. 23 D). In addition, the combinations of the colocalizations and the number of triple-colocalizations were analyzed (Fig. 23 E).

Analyzing the percentage of the different protein puncta in colocalization based on their total number shows that approximately 25 % of $\alpha 2$ -GABA_ARs colocalize with PSD95 ($25.08\% \pm 1.305\%$, $n = 30$). Further examination of triple-colocalization reveals that around 6 % of $\alpha 2$ -GABA_ARs ((WT = mean $\pm$ SEM = $6.485\% \pm 0.6227\%$, $n = 30$), 9% of PSD95 ((WT = mean $\pm$ SEM = $8.843\% \pm 1.071\%$, $n = 30$), and 10 % of VGLUT1 ((WT = mean $\pm$ SEM = $10.04\% \pm 1.098\%$, $n = 30$) are involved in triple-colocalizations. Additionally, the data suggest that about 31% of excitatory synapses (WT = mean $\pm$ SEM = PSD95 + VGLUT1 = $31.27\% \pm 3.404\%$, $n = 30$) are colocalized with $\alpha 2$ -GABA_ARs (Fig. 23 E, Supplementary data Table 2).

All these findings suggest that $\alpha 2$ -containing GABA_A receptors are also colocalized with excitatory synapses and that approximately one-third of excitatory synapses appear to be in close proximity to $\alpha 2$ -containing GABA_A receptors. Similar distributions were found for $\alpha 5$ -GABA_ARs, as previous data show.

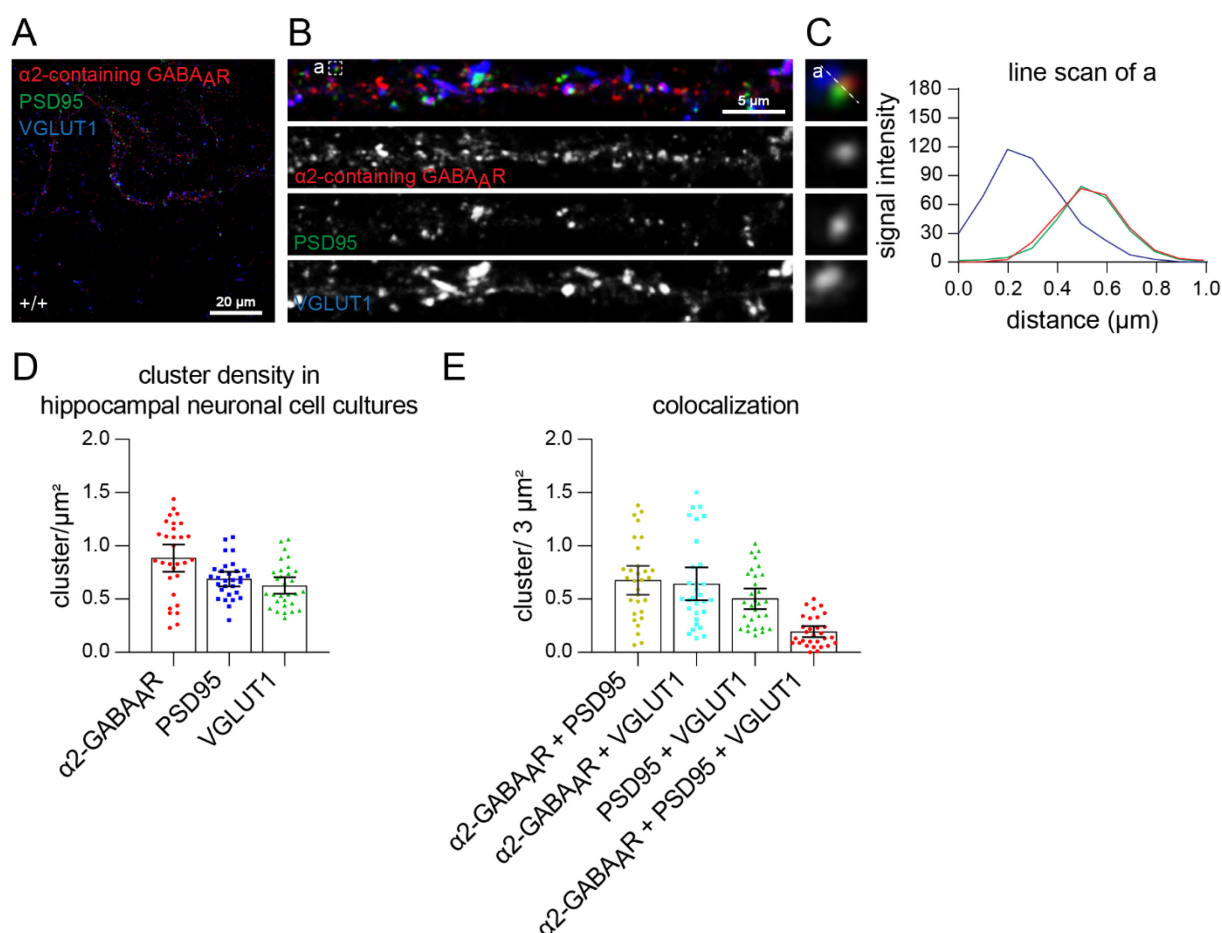


Figure 23: Immunocytochemical analysis of $\alpha 2$ -containing GABA_A receptors, PSD95, and VGLUT1 in dendritic regions. **A-C:** Surface-immunostainings of $\alpha 2$ -GABA_ARs (red) were conducted in secondary dendrites of wildtype ($+/+$) derived primary hippocampal neurons (DIV18). In addition, PSD95 (green) and VGLUT1 (blue) were probed after fixation. Enlargements of a 40 μ m-long dendrite section, highlighted by the gray box in A, are displayed in all three individual channels (B). Exemplified triple-colocalization, highlighted by the gray square (a) in B, was enlarged, and the signal intensity of individual channels was plotted along the line (C). **D:** Quantification of the normalized cluster density (cluster/ μ m²) of $\alpha 2$ -GABA_ARs ($n = 30$), PSD95 ($n = 30$), and VGLUT1 ($n = 30$). **E:** Quantification of the normalized cluster density (cluster/ μ m²) of colocalization between $\alpha 2$ -GABA_ARs and PSD95 ($n = 30$), $\alpha 2$ -GABA_ARs and VGLUT1 ($n = 30$), PSD95 and VGLUT1 ($n = 30$), and triple-colocalization ($n = 30$). D-E: Error bars represent the mean $\pm$ standard error of the mean (SEM). 3 independent experiments. Exact data for mean $\pm$ SEM are listed in supplementary data table 2

3.12 $\alpha 5$ -containing GABA_ARs cluster density changes in RDX knockout-derived dendrites but is not affected by chemical LTP

Research on $\alpha 5$ -GABA_ARs and their location at the synapse has also highlighted their interaction with scaffold proteins at the extrasynaptic site. Over the last years, different studies revealed a cascade in which radixin, an extrasynaptic scaffold protein of $\alpha 5$ -GABA_ARs, appears to release the receptor following NMDA-mediated LTP induction. This release allows $\alpha 5$ -GABA_ARs to diffuse to inhibitory synapses, where they are re-anchored by the scaffold protein gephyrin (Davenport et al., 2021; Hausrat et al., 2015; Loebrich et al., 2006). This mechanism may also influence the distribution of $\alpha 5$ -GABA_ARs towards spine synapses and the formation of DiSs.

To test whether the distribution of $\alpha 5$ -GABA_ARs towards spine synapses is modulated by increased activity, chemical LTP (cLTP) was induced to observe any potential changes in diffusion parameters inside and outside PSD95-positive regions. Therefore, neurons were treated with forskolin and rolipram, and $\alpha 5$ -GABA_ARs trajectories were analyzed. The data suggest that the induction of cLTP did not result in any differences in mean square displacement (Fig. 24 A, D), diffusion coefficient (Fig. 24 B, E), and confinement radius (Fig. 24 C, F) of $\alpha 5$ -GABA_ARs compared to basal conditions.

To investigate whether the depletion of radixin affects the cluster density of the discovered triple-colocalizations, $\alpha 5$ -GABA_ARs, PSD95, and synaptophysin were labeled in wildtype- and *RDX* knockout-derived primary hippocampal neurons. Subsequently, the triple- and colocalizations were analyzed using the ImageJ plugin ComDet.

The following figure exemplifies the triple-immunostaining in samples from the two genotypes (Fig. 25 A-D). In addition, an enlarged section of a dendritic region is shown for every condition (Fig. 25 B, D).

No differences in cluster density for the triple-colocalization between $\alpha 5$ -GABA_ARs, PSD95, and synaptophysin were detected (Fig. 25 E). However, a significant increase in *RDX* knockout-derived dendritic regions can be detected by quantifying the cluster density of $\alpha 5$ -GABA_ARs (Fig. 25 F) and PSD95 (Fig. 25 G). No significant differences were observed in cluster density of synaptophysin (Fig. 25 H). Analyzing the colocalization of $\alpha 5$ -GABA_ARs and PSD95 puncta indicates a significant increase in the *RDX* knockout condition (Fig. 25 I). However, no significant differences were seen in the colocalizations between $\alpha 5$ -GABA_ARs and synaptophysin (Fig. 25 J) and PSD95 and synaptophysin (Fig. 25 K).

The percentage of $\alpha 5$ -GABA_ARs, PSD95, and synaptophysin puncta in triple- and colocalizations was calculated relative to their total number detected in a 40 μ m dendrite section. In triple-colocalizations of both genotypes, approximately 8 to 9 % of $\alpha 5$ -GABA_ARs (WT vs. KO = mean $\pm$ SEM = 9.270 % $\pm$ 0.9444 % (n= 30), vs. 8.567 % $\pm$ 1.008 % (n= 30); t-test p = 0.6130), approximately 10 % of PSD95 (WT vs. KO = mean $\pm$ SEM = 10.84 % $\pm$ 0.8541 % (n= 30), vs. 10.24 % $\pm$ 1.009 % (n= 30); Mann-Whitney test p = 0.9443), and around 13 % for synaptophysin (WT vs. KO = mean $\pm$ SEM = 13.56% $\pm$ 1.245% (n= 30), vs. 13.07 % $\pm$ 1.047 % (n= 30); t-test p = 0.7653) was detected (Supplementary data Table 2).

Analyzing the percentage of $\alpha 5$ -GABA_ARs and PSD95 puncta in colocalization with each other showed no significant difference for $\alpha 5$ -GABA_ARs in wildtype and *RDX* knockout-derived neurons (WT vs. KO = mean $\pm$ SEM = 29.51% $\pm$ 1.770% (n= 30), vs. 29.20% $\pm$ 1.276% (n= 30); t-test p = 0.8878). Additionally, no significant differences were found for PSD95 clusters

in both genotypes (WT vs. KO = mean $\pm$ SEM = 35.68% $\pm$ 1.298% (n = 30), vs. 38.42% $\pm$ 1.509% (n = 30); t-test p = 0.1742) (Supplementary data Table 2).

RDX knockout-derived dendrites exhibit a higher total intensity of $\alpha 5$ -GABA_ARs puncta (Fig. 25 L). No difference regarding the total intensity for synaptophysin (Fig. 25 M) and PSD95 puncta (Fig. 25 N) was detected among the genotypes.

These results indicate that $\alpha 5$ -GABA_ARs in PSD95-positive regions are stable and do not seem to be influenced by changes in neuronal activity. However, a higher colocalization of $\alpha 5$ -GABA_ARs with PSD95 was observed in the absence of radixin. Analyzing the proportions of the proteins suggests that the distribution of $\alpha 5$ -GABA_ARs between extrasynaptic and synaptic sides has not changed. Furthermore, the increased number of $\alpha 5$ -GABA_ARs and PSD95 colocalizations might occur due to the increase in total $\alpha 5$ -GABA_ARs and PSD95 cluster numbers.

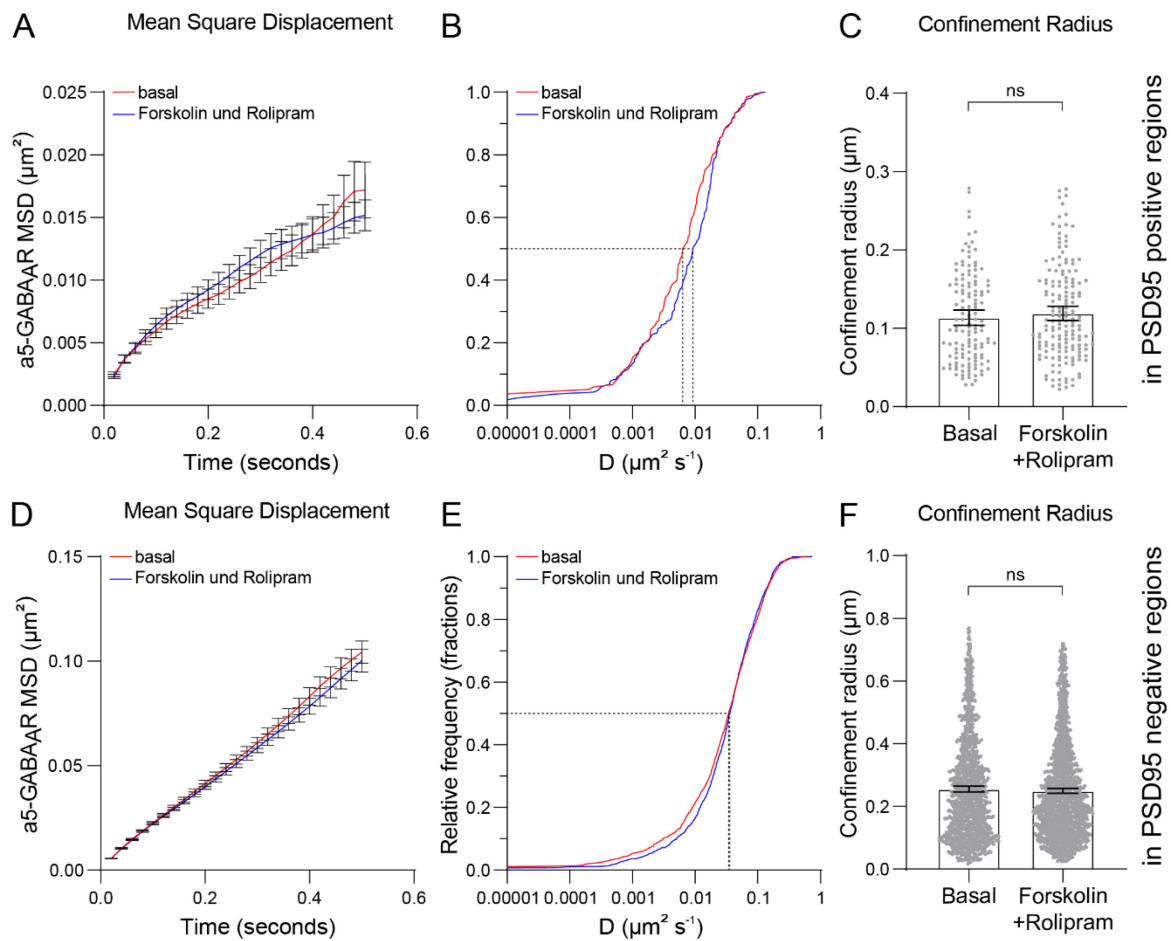


Figure 24: Comparison of the diffusion properties of $\alpha 5$ -GABA_ARs inside and outside of PSD95-positive regions in basal condition and after the induction of chemical LTP. A-F: $\alpha 5$ -GABA_ARs quantum dot (QD) trajectories inside PSD95-positive regions (A-C) and PSD95-negative regions (D-F) under basal conditions and after the induction of chemical LTP with Forskolin and Rolipram. **A:** Mean Square Displacement (MSD) versus time plot for $\alpha 5$ -GABA_ARs quantum dot trajectories. **B:** Cumulative probability plot for $\alpha 5$ -GABA_ARs quantum dot diffusion coefficients. **C:** Confinement radius for $\alpha 5$ -GABA_ARs quantum dots (Basal vs. Forskolin + Rolipram = 0.1135 $\pm$ 0.004951 (n = 133), vs. 0.1191 $\pm$ 0.004680 (n = 164)). **D:** Mean Square Displacement (MSD) versus time plot for $\alpha 5$ -GABA_ARs quantum dot trajectories. **E:** Cumulative probability plot for $\alpha 5$ -GABA_ARs quantum dot diffusion coefficients. **F:** Confinement radius for $\alpha 5$ -GABA_ARs quantum dots (Basal vs. Forskolin + Rolipram = 0.2554 $\pm$ 0.004702 (n = 1247), vs. 0.2493 $\pm$ 0.003944 (n = 1545)). Error bars represent the mean $\pm$ standard error of the mean (SEM). Kruskal-Wallis test: C, F. ns $p \geq 0.05$. 3 independent experiments

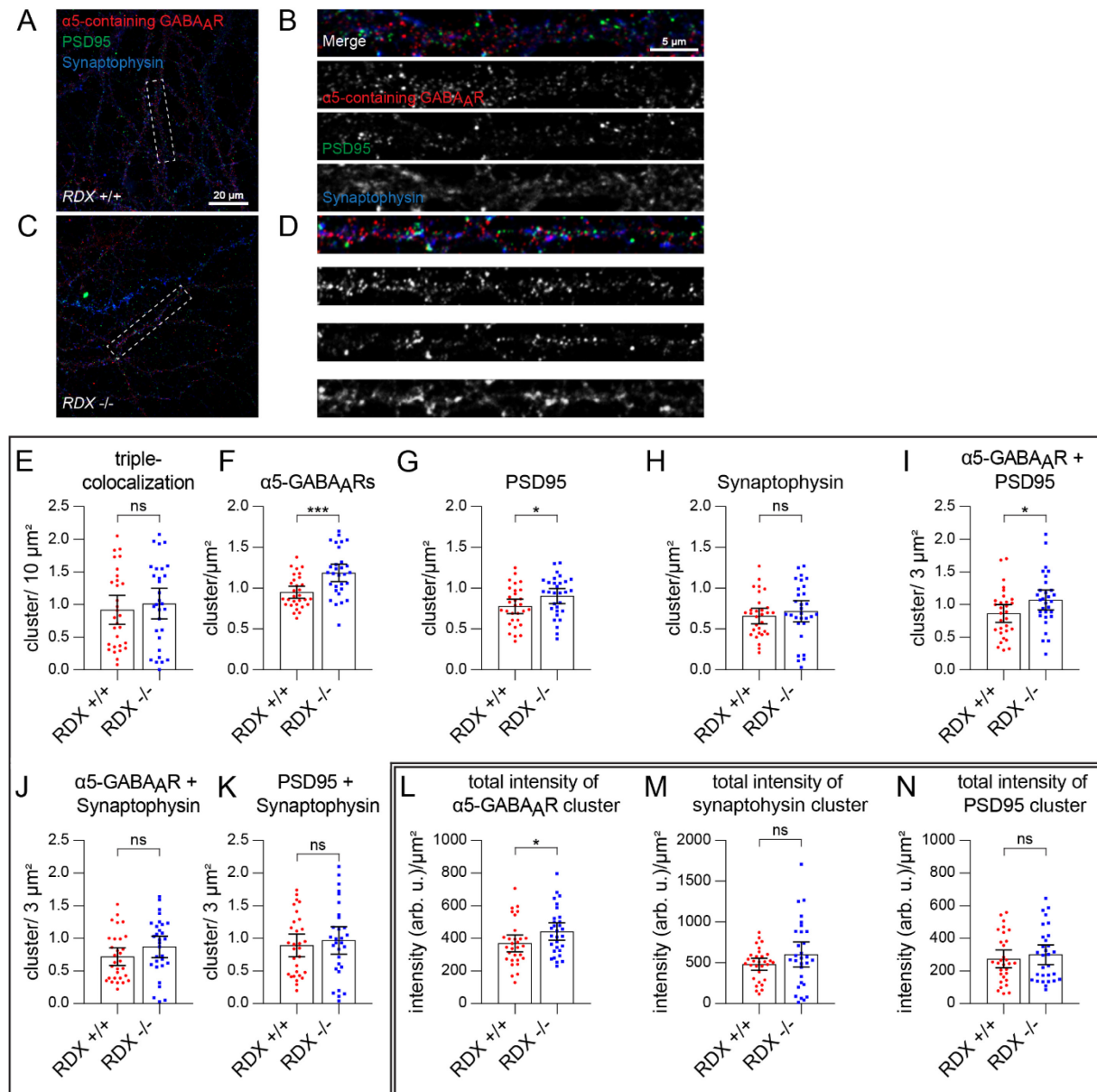


Figure 25: Immunocytochemical analysis of $\alpha 5$ -GABA_A receptors, PSD95, and synaptophysin in dendritic regions. **A-D:** Live surface immunostainings of $\alpha 5$ -GABA_ARs (red) were conducted in secondary dendrites of *RDX* wildtype (+/+) (A-B), and *RDX* knockout (-/-) (C-D) derived primary hippocampal neurons (DIV18). In addition, PSD95 (green) and synaptophysin (blue) were probed after fixation. Enlargements of a 40 μm -long dendrite section, highlighted by gray boxes in A and C, are displayed in all three individual channels (B, D). **E-N:** Quantification of the normalized parameter in *RDX* +/+ (n = 30) and *RDX* -/- (n = 30) conditions. **E:** Cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs in triple-colocalizations with PSD95 and synaptophysin. **F:** Cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs. **G:** Cluster density (cluster/ μm^2) of PSD95. **H:** Cluster density (cluster/ μm^2) of synaptophysin. **I:** Cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs in colocalizations with PSD95. **J:** Cluster density (cluster/ μm^2) of $\alpha 5$ -GABA_ARs in colocalizations with synaptophysin. **K:** Cluster density (cluster/ μm^2) of the colocalizations of PSD95 and synaptophysin. **L:** Total intensity (arb. u./ μm^2) of $\alpha 5$ -GABA_ARs puncta. **M:** Total intensity (arb. u./ μm^2) of synaptophysin puncta. **N:** Total intensity (arb. u./ μm^2) of PSD95 puncta. E-K: Error bars represent the mean $\pm$ standard error of the mean (SEM). Unpaired t-test: F, G, H, I, K, L, M. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001. Mann-Whitney test: E, J, N. ns p > 0.05, * p < 0.05. 3 independent experiments. Exact values for the mean $\pm$ SEM are listed in the supplementary data table 2.

4. Project 1: Discussion

Over the past decade, introducing dually innervated spines and their potential function in neuronal networks has sparked new questions (Chapman et al., 2022; J. L. Chen et al., 2012; Kleinjan et al., 2023; Villa et al., 2016). These emerging findings suggest that research on neurodevelopmental disorders and their associated receptors may risk overlooking novel phenotypes in sub-synaptic architecture. One protein of particular interest is $\alpha 5$ -GABA_ARs, which is linked to several disorders and syndromes, including Autism Spectrum disorder (Mesbah-Oskui et al., 2017), psychiatric diseases (Perez et al., 2023), and the appearance of memory impairment, both post-anesthesia (Zurek et al., 2014) and age-related (Prevot et al., 2020).

The first project focused on the appearance of $\alpha 5$ -GABA_ARs at spines in the nanometer-scale proximity to excitatory synapses. The colocalizations of $\alpha 5$ -GABA_ARs with excitatory pre- and postsynaptic markers such as PSD95, GluR2, and VGLUT1 were successfully observed using confocal and STED microscopy. Analyzing the trajectories of $\alpha 5$ -GABA_ARs through STORM microscopy revealed a slower diffusion pattern and decreased mean square displacement near PSD95-positive regions. Additionally, the results indicate new phenotypes in *Gabra5* knockout-derived neuronal cultures, which showed increased density of PSD95 and synaptophysin puncta, along with enhanced formation of excitatory synapses. Interestingly, further analysis in *RDX* knockout-derived dendrites also indicated an increase of $\alpha 5$ -GABA_ARs at PSD95-positive regions. These findings provide the first evidence of $\alpha 5$ -GABA_ARs on spines near excitatory synapses.

4.1 Technical limitation of the study and new ideas of standardization of analyzing $\alpha 5$ -containing GABA_ARs cluster

The study on $\alpha 5$ -GABA_ARs using the ComDet plugin introduced innovative, future-oriented methods of analyzing the protein colocalization in greater detail. However, limitations became apparent while measuring the cluster density. The three-pixel colocalization threshold between cluster centers, in this thesis, the equivalent to approximately 300 nm, close to the maximum resolution of the confocal microscope (200 nm) (reviewed in (Elliott, 2020; MacDonald et al., 2015)), likely caused several colocalizations to be missed, suggesting a significant number went undetected. For instance, data from the experiment, examining the colocalization of $\alpha 5$ -GABA_ARs with excitatory synapses, shows that approximately 9 % of $\alpha 5$ -GABA_ARs colocalizes with 10 % PSD95 and 10 % synaptophysin (Fig 11). However, analyzing only the colocalizations between two clusters shows that 26 % and 27 % of $\alpha 5$ -GABA_ARs colocalize with PSD95 or synaptophysin. Therefore, using three different puncta reduced the likelihood of detecting a colocalization, which could result in smaller numbers in the proportional analysis. To address this obstacle, focusing initially on the colocalization of two proteins may provide a clearer understanding of protein distribution and their proportional occurrence. However, further analysis and comparisons with the literature should consider this limitation.

Another limitation regarding the discussion of the results is the problematic comparison of rat and human samples. Previous studies on GABA_ARs have shown discrepancies in their distribution among different organisms. In general, mice samples seem to have a higher abundance of $\alpha 4$ and δ -subunit, whereas rat samples seem to have a generally higher abundance of $\alpha 1$, $\beta 1$, and $\gamma 2$ (Hörtnagl et al., 2013). Regarding the $\alpha 5$ -GABA_AR by comparing

mice and rat analyses, higher expression of the $\alpha 5$ - and δ -subunit was observed in mice dentate gyrus samples (Hörtnagl et al., 2013). Interestingly, the δ -subunit is mostly associated with the GABA_A receptors located in an extrasynaptic or perisynaptic area in different regions of the brain, such as the dentate gyrus (W. Wei et al., 2003) and GABA_A receptors containing the $\alpha 5$ and/or the δ -subunit are considered the primary GABA_A receptors associated with tonic inhibition in the hippocampus (Glykys et al., 2008). However, the δ -subunit seems primarily associated with the formation of the $\alpha 4$ and $\alpha 6$ -subunit (reviewed in (Zheleznova et al., 2009)). Therefore, these should be kept in mind during comparisons of different published studies.

The thesis also highlights the need to reconsider how protein density measurements involving $\alpha 5$ -GABA_AR are conducted. In past studies, $\alpha 5$ -GABA_ARs were divided into synaptic and extrasynaptic pools (Brünig et al., 2002; Hausrat et al., 2015). With new insights into $\alpha 5$ -GABA_ARs on spines and the formation of DiS, another factor must be implemented. The measurement of dendritic spine density in L2/3 pyramidal cells shows a two-fold increase in inhibitory spine synapse density in distal apical dendrites, 125 μ m or more from the soma (J. L. Chen et al., 2012). Thus, when studying synaptic $\alpha 5$ -GABA_ARs, the distance from the soma and the location on shafts or spines should also be contemplated. A new standardization method should be considered for dividing $\alpha 5$ -GABA_ARs into three pools: extrasynaptic pool, inhibitory shaft synapses, and $\alpha 5$ -containing DiS. Furthermore, differences between studies including $\alpha 5$ -GABA_ARs showed that the developmental stage of the brain or cell cultures (Brady & Jacob, 2015; Nuwer et al., 2021) and sex (Le et al., 2022) have to be taken more into account when discussing $\alpha 5$ -GABA_ARs and comparing the results of the various studies.

Another limitation of the study is the lack of data based on the immunogold staining method and its analysis with EM to confirm further the localization of $\alpha 5$ -GABA_ARs on spines near excitatory synapses. However, the analysis based on DAB immunostaining and EM analysis created first insights.

4.2 Small differences of $\alpha 5$ -containing GABA_ARs in *Gabra5* wildtype- and heterozygous-derived neurons

The study results demonstrate the colocalization of $\alpha 5$ -GABA_ARs with different excitatory pre- and postsynaptic marker proteins.

Live surface immunostaining of $\alpha 5$ -GABA_ARs by targeting their extrasynaptic tail enables a more accurate interpretation of colocalizations with the different proteins. Thus, false-positive colocalizations due to protein trafficking to or from the cell membrane during immunostaining are unlikely. Therefore, the observed colocalizations of $\alpha 5$ -GABA_ARs with PSD95 or GluR2 presumably occur at the plasma membrane, specifically on spines and near excitatory synapses. In a previous study, the presence of $\alpha 5$ -GABA_ARs on spines was mentioned before, though their intensity levels were lower compared to $\alpha 1$ - and $\alpha 2$ -GABA_AR immunostainings (Brünig et al., 2002).

The analysis of the triple-colocalizations ($\alpha 5$ -GABA_AR, PSD95, and synaptophysin) revealed no significant difference in the frequency of appearances between *Gabra5* wildtype- and heterozygous-derived dendrites. The total number of $\alpha 5$ -GABA_AR puncta was also similar, indicating that the density of $\alpha 5$ -GABA_ARs on the plasma membrane remains unchanged in heterozygous samples (Fig. 16 A1). However, the altered total intensity of $\alpha 5$ -GABA_ARs in triple-colocalizations in wildtype neurons (Fig. 11 K), when considering the unchanged overall total intensity between genotypes (Fig. 16 B1), suggests that under heterozygous conditions,

$\alpha 5$ -GABA_ARs puncta may have a lower protein concentration within these triple-colocalizations.

Notably, there is a trend towards the increased overall total intensity of $\alpha 5$ -GABA_ARs in *Gabra5* wildtype samples compared to heterozygous ones (WT vs. HET, $p = 0.0681$, Fig. S1 16 B1). The p -value indicating a trend could explain why the proportion of the total intensity of $\alpha 5$ -GABA_AR in triple-colocalizations relative to the overall total intensity does not differ between *Gabra5* wildtype- and heterozygous-derived neurons.

Further analysis of the total area of $\alpha 5$ -GABA_AR in triple-colocalizations reveals that $\alpha 5$ -GABA_AR puncta appear to be smaller in heterozygous conditions (Fig. 11 L), although the total area of $\alpha 5$ -GABA_AR in dendrites remains consistent between wildtype and heterozygous samples (Fig. 16 C1). Thus, the results suggest that the total area of $\alpha 5$ -GABA_AR in triple-colocalization with PSD95 and synaptophysin is affected in *Gabra5* heterozygous hippocampal neurons. According to the thesis data, despite the overall total protein concentration remaining unchanged, fewer $\alpha 5$ -GABA_ARs are detected in the triple-colocalization of heterozygous neurons. Therefore, it could be suggested that the size and protein concentration of the $\alpha 5$ -GABA_AR cluster seem to be altered in DiSs of *Gabra5* heterozygous-derived neurons. However, additional immunostainings are needed to determine whether the observed trend in total intensity becomes statistically significant.

4.3 Proportional differences of $\alpha 5$ -containing GABA_ARs in triple- and co-localizations with synaptic proteins

The analysis of $\alpha 5$ -GABA_ARs, PSD95, and synaptophysin using confocal microscopy showed that approximately 8-9 % of total $\alpha 5$ -GABA_ARs appear in triple-colocalizations within a 40 μ m dendrite section of wildtype neurons (Supplementary data table 1). Additionally, around 6 % of $\alpha 5$ -GABA_ARs are colocalized with both GluR2 and VGLUT1 in triple-colocalizations (Supplementary data table 2). Notably, approximately 26 % of $\alpha 5$ -GABA_ARs were found to colocalize with PSD95 puncta (Supplementary data table 1), an roughly 40% with GluR2 puncta (Supplementary data table 2). Moreover, 30% of PSD95 (Supplementary data table 1) and GluR2 clusters (Supplementary data table 2) appear to colocalize with $\alpha 5$ -GABA_ARs. The colocalization results contrast with earlier findings that reported less than 10 % of $\alpha 5$ -GABA_AR clusters contained PSD95 (Brünig et al., 2002). Brünig et al. also observed less than 10 % of $\alpha 5$ -GABA_AR clusters colocalized with GluR1, though GluR1 appeared less distinct than PSD95 puncta (Brünig et al., 2002). Discrepancies between the current findings and those of Brünig et al. may stem from differences in antibodies, immunostaining protocols, criteria for identifying $\alpha 5$ -GABA_AR clusters, or imaging techniques. A more recent study indicated that approximately two-thirds of $\alpha 5$ -GABA_ARs were considered extrasynaptic, with one-third being synaptic in the dorsal horn of the spinal cord (Perez-Sanchez et al., 2017), aligning with the observed densities in the colocalizations. Furthermore, 38 % of the total number of inhibitory synapses was found on DiS (Gemin et al., 2021).

The differences between triple- and colocalizations may partly arise from limitations in the plugin used. However, the thesis's results could also imply that not all spines containing excitatory postsynaptic proteins and $\alpha 5$ -GABA_ARs are opposed by a presynaptic bouton. Since around 30 % of $\alpha 5$ -GABA_ARs also colocalize with synaptophysin and around 17% with VGLUT1, this phenomenon may be related to limitations of the plugin or the imaging resolution.

4.4 How do $\alpha 5$ -containing GABA_ARs bind near excitatory synapses?

The reduction in mean square displacement, diffusion coefficient, and confinement radius of $\alpha 5$ -GABA_ARs within PSD95-positive regions compared to areas outside these regions supports the immunostaining data indicating that $\alpha 5$ -GABA_ARs can be localized near postsynaptic terminals. However, this data does not explain why $\alpha 5$ -GABA_ARs slow down and spend more time in PSD95-positive regions.

The presence of gephyrin in PSD95-positive spines (J. L. Chen et al., 2012) might account for the altered diffusion of $\alpha 5$ -GABA_ARs, as gephyrin anchors $\alpha 1$ - or $\alpha 2$ -GABA_A receptors and regulates their stability at inhibitory synapses (Kneussel et al., 1999). Confocal microscopy analysis of DiS distribution in the CA1 region of the hippocampus revealed that at least 13 % of spines displayed both PSD95 and gephyrin signals (Kleinjan et al., 2023). Furthermore, spines containing gephyrin clusters showed higher uncaging-evoked inhibitory postsynaptic currents than spines lacking gephyrin, suggesting that spines with gephyrin clusters contain functional GABAergic receptors (Villa et al., 2016). Notably, the loss of the gephyrin cluster did not affect spine persistence; however, larger clusters appeared to be associated with a higher stabilization of spines (van Versendaal et al., 2012). Therefore, spines positive for GFP-tagged gephyrin seemed more stable than those lacking gephyrin-GFP (Isshiki et al., 2014).

Previous trajectory and immunostaining analyses confirmed the association of $\alpha 5$ -GABA_ARs with radixin (Hausrat et al., 2015; Loebrich et al., 2006). However, new findings of radixin's co-occurrence with $\alpha 5$ -GABA_ARs on inhibitory synapses (Magnin et al., 2019) may offer additional potential explanations regarding $\alpha 5$ -GABA_ARs on DiSs. Next to radixin and gephyrin, another possibility is the involvement of novel scaffold proteins, such as MPP2 (Schmerl et al., 2022), which was shown to bind $\alpha 1$ -GABA_ARs in subregions near excitatory synapses.

Alternatively, $\alpha 5$ -GABA_ARs might be anchored indirectly by an unidentified protein functioning similarly to scaffold proteins such as PSD95. While the scaffold protein binds directly to NMDARs in the PSD (Kornau et al., 1995), it indirectly binds AMPARs over the TARPs (Tomita et al., 2003). Such an indirect binding complex could also be attributed to $\alpha 5$ -GABA_ARs at DiSs.

4.5 The connection between $\alpha 2$ -containing GABA_ARs and $\alpha 5$ -containing GABA_ARs near excitatory synapses

As previously noted, 26 % of $\alpha 5$ -GABA_ARs colocalize with PSD95 puncta (Supplementary data table 1.). Interestingly, a similar proportion, 26 %, of $\alpha 2$ -GABA_ARs also colocalize with PSD95 (Supplementary data table 2.). However, these findings contrast with Brünig et al.'s semiquantitative analysis, which showed that clusters of $\alpha 1$ - and $\alpha 2$ -GABA_ARs contain less than 3% of PSD95 puncta (Brünig et al., 2002).

Additionally, the thesis's data reveal that approximately 25 % of $\alpha 2$ -GABA_AR appear to be opposed by VGLUT1-positive presynaptic terminals (Supplementary data table 2.). Other studies indicated that around 72 % of $\alpha 2$ -GABA_ARs are opposed by synapsin I-positive terminals (Brünig et al., 2002) and that between 67% and 74% of $\alpha 2$ -GABA_ARs are found at synaptic sites (Hausrat et al., 2015).

A recent study reported that about 20 % of homer1-positive spines contain $\alpha 1$ -GABA_ARs and one of their new associated scaffold proteins MPP2 (Schmerl et al., 2022). Notably, $\alpha 2$ -GABA_ARs and $\beta 3$ -containing GABA_ARs are also associated with MPP2 (Schmerl et al., 2022). Since GABA_A receptors are pentameric complexes, it has been shown that $\alpha 1$ -, $\alpha 2$ -, and $\beta 3$ -

subunits can co-occur with $\alpha 5$ -subunits (McKernan & Whiting, 1996). Araujo et al. demonstrated that $\alpha 1/\alpha 5$ -containing GABA_ARs represent about 9 % of the total $\alpha 1$ -GABA_ARs and roughly 20 % of total $\alpha 5$ -GABA_ARs (Araujo et al., 1998). In hippocampal neurons, approximately 20 % of $\alpha 2$ -GABA_ARs were identified within $\alpha 2/\alpha 5$ -containing GABA_ARs (Del Río et al., 2001). Interestingly, a study by Brady & Jacob suggests a direct interaction between $\alpha 5$ -GABA_ARs and gephyrin and that $\alpha 5$ -GABA_ARs do not fully rely on the binding capabilities of the $\alpha 1$ - or $\alpha 2$ -subunits. However, their findings indicate that GABA_A receptor with mixed subunits ($\alpha 1/\alpha 2$ with $\alpha 5$) have a higher affinity to bind to gephyrin than homologous $\alpha 5$ -containing GABA_ARs (Brady & Jacob, 2015).

Although the $\alpha 2$ - and $\alpha 5$ -GABA_AR subtypes display different levels of synaptic localization, their similarity of around 20 to 30 % in co-assembly and/or presence in spines is noteworthy. Therefore, the thesis results may suggest that the observed $\alpha 5$ subunits appear as heterodimers in combination with the $\alpha 2$ subunit near excitatory synapses or on DiSs.

4.6 $\alpha 5$ -containing GABA_ARs and their connection to the presynaptic terminal

The thesis's data show that only 3% of $\alpha 5$ -GABA_ARs colocalize in a triple-colocalization with VGLUT1 and VGAT. The centers of the VGAT and VGLUT1 cluster, presumed to be distinct presynaptic terminals, might often be too far apart (more than 3 pixels) to be counted as colocalizations. Therefore, the small number could be again a limitation of the analysis plugin ComDet or the imaging resolution. Therefore, the discussion focuses primarily on the colocalizations. The results indicate that approximately 15 % of $\alpha 5$ -GABA_ARs and 34 % of VGAT clusters colocalize with each other (Supplementary data table 2). In a recent study, nearly 100 % of identified DiS had opposing VGAT-positive presynapses (Kleinjan et al., 2023). Therefore, 35 % of the VGAT clusters could resemble the PSD95 and GluR2 colocalization percentages with $\alpha 5$ -GABA_ARs. Interestingly, around 28 % of the VGLUT1 clusters (Supplementary data table 2) appear to colocalize with approximately 19 % of $\alpha 5$ -GABA_ARs (Supplementary data table 2), also aligning with the approximately 26% of $\alpha 5$ -GABA_ARs found near PSD95 (Supplementary data table 1). Furthermore, 31 % of spines exhibiting GFP-tagged gephyrin were shown before colocalizing with VGLUT1, while 67 % colocalizing with VGLUT2 (Isshiki et al., 2014).

Besides their association with VGAT and VGLUT1, approximately 27 % of $\alpha 5$ -GABA_ARs appear to colocalize with synaptophysin clusters in *Gabra5* wildtype-derived neurons (Supplementary data table 1). Remarkably, Brünig et al. reported in 2002 that around 24 % of $\alpha 5$ -GABA_ARs colocalize with synapsin-I-positive boutons (Brünig et al., 2002). Double-label immunofluorescence analysis of synapsin-I and synaptophysin in hippocampal neuronal cultures indicates a similar distribution pattern throughout neuronal development (Fletcher et al., 1991).

However, analyzing *Gabra5* knockout-derived hippocampal neurons revealed a higher density of PSD95 and synaptophysin puncta, along with an elevated density of excitatory synapses. Proportion analyses suggest that the increased formation may result from synaptophysin puncta's higher appearance, which colocalizes with PSD95. Furthermore, VGLUT1 clusters exhibit higher intensities in *Gabra5* knockout conditions. This phenomenon raises questions about how the absence of $\alpha 5$ -GABA_ARs could affect the formation of excitatory synapses. Initial experiments indicate that a potential phenotype might already be detectable during early

neurodevelopmental stages (between DIV10 and 12). The number of VGLUT1 clusters increased in *Gabra5* knockout conditions, whereas in wildtype samples, VGLUT1 cluster density remained stable (Supplementary data table 2). This suggests that the absence of $\alpha 5$ -GABA_ARs might lead to an elevated density of presynaptic terminals.

The knockout of the NMDA subunit NR2B made it possible to observe an increase in GluR1 and GluR2 levels. Interestingly, the increased expression of AMPA receptors resulted in elevated VGLUT1 and VGLUT2 protein levels (Hall et al., 2007). The positive influence of AMPARs on presynaptic terminal stability was later demonstrated by showing that AMPA receptor overexpression increases VGLUT1-positive terminal number (Ripley et al., 2011). The inhibition of $\alpha 5$ -GABA_ARs at DIV 21 increased the NMDA subunit NR2A (Nuwer et al., 2021), which is known for its positive influence on AMPA receptor recruitment (Myung et al., 2005). Therefore, the thesis's data might suggest that $\alpha 5$ -GABA_ARs at DIV 12 hyperpolarize the membrane. The absence of $\alpha 5$ -GABA_ARs, along with the consequent NR2A upregulation may drive increased AMPAR expression at the synapse, impacting presynaptic terminals and resulting in a higher VGLUT1 cluster count. One discrepancy between the thesis's results and other studies is the age of the cells. Nuwer et al. argued that DIV 14 resembles postnatal day 14, during which GABA_A receptors depolarize (Nuwer et al., 2021). However, neuronal development and maturation phases can vary between cultures (reviewed in (Biffi et al., 2013)) and research groups. This may also apply to the onset of the so-called GABA shift (Peerboom & Wierenga, 2021), which coincides with increased levels of the Cl⁻-extruding K⁺/Cl⁻ co-transporter, KCC2 (Rivera et al., 1999). Further experiments should analyze the GABA shift in the culture setup used for the thesis, potentially by measuring KCC2 levels. Additionally, immunostaining of AMPA receptors and PSD95 would further support the hypothesis.

To the authors' knowledge, the influence of $\alpha 5$ -GABA_ARs on presynaptic proteins, particularly synaptophysin, has not been previously demonstrated. However, interesting similarities can be found in the pain research. *Gabra5* knockout mice, lacking the receptor in the spinal cord dorsal horn, may experience increased duration and sensitivity of chronic pain (Perez-Sanchez et al., 2017). Interestingly, overexpression of synaptophysin in the spinal cord horn is also associated with heightened sensitivity to head-induced pain (H. H. Zhang et al., 2012). In the $\alpha 5$ (H105R) mice model (Crestani et al., 2002), the reduction in heightened pain sensitivity following diazepam administration was mildly diminished (Knabl et al., 2008).

Conversely, different studies show that the increase of $\alpha 5$ -GABA_ARs seems to affect pain response. Formalin-induced pain responses increased mRNA levels of $\alpha 5$ -GABA_ARs and increased intensity in the dorsal spinal cord and dorsal root ganglia. Treatment with $\alpha 5$ -NAM reversed some of these effects (Bravo-Hernández et al., 2016). Furthermore, the inhibition of $\alpha 5$ -GABA_ARs has been shown to decrease pain thresholds in mice (Xue et al., 2017).

Thus, whether the decrease or increase of $\alpha 5$ -GABA_ARs regulated pain intensity remains unclear. However, an increase of synaptophysin levels and a decrease of $\alpha 5$ -GABA_ARs may contribute to the maintenance of chronic pain.

4.7 Are $\alpha 5$ -containing GABA_ARs on spines radixin-dependent?

RDX knockout-derived neurons show an overall increase in $\alpha 5$ -GABA_ARs density (Fig 25 F.). This increase may result from the enhanced diffusion rate of $\alpha 5$ -GABA_ARs, leading to the appearance of more puncta. The interaction between radixin and $\alpha 5$ -GABA_ARs, particularly regarding receptor diffusion from the extrasynaptic pool to the postsynaptic terminal, has been extensively studied (Davenport et al., 2021; Hausrat et al., 2015).

No increase in synaptophysin levels was observed (Fig 25 H.), consistent with earlier findings indicating that a higher presence of synaptic $\alpha 5$ -GABA_ARs does not alter the presynaptic density (Hausrat et al., 2015).

The increased diffusion and clustering density of $\alpha 5$ -GABA_ARs may also account for their higher colocalization with PSD95. Interestingly, despite the absence of radixin, $\alpha 5$ -GABA_ARs are still found near an excitatory postsynaptic marker. This suggests that radixin may not be the primary scaffold protein of $\alpha 5$ -GABA_ARs at DiS. Instead, radixin at synapses may be predominantly associated with interneuron-interneuron connections (Magnin et al., 2019), possibly limited to these types of neurons.

Interestingly, cLTP did not alter the speed or travel distance of $\alpha 5$ -GABA_ARs inside or outside PSD95-positive regions (Fig 24). Given that NMDAR activity drives $\alpha 5$ -GABA_ARs toward synapses (Davenport et al., 2021), this finding suggests that the diffusion of $\alpha 5$ -GABA_ARs towards PSD95-positive spines is not dependent on the regulating role of neuronal activity, at least for the used protocol. In exploring the parameters necessary for DiS formation in hippocampal cells, researchers found that a higher percentage of spines containing gephyrin clusters had inactive presynaptic terminals, indicating that excitatory activity is not required for the formation or maintenance of DiS (Kleinjan et al., 2023). Therefore, a future experiment could focus on the activity of $\alpha 5$ -GABA_ARs near PSD95 in *RDX* knockout-derived dendrites to determine whether the increased density of $\alpha 5$ -GABA_ARs is solely due to diffusion or serves a functional purpose. The increased occurrences of slowly decaying mIPSCs suggest an enhanced contribution of $\alpha 5$ -GABA_ARs to phasic currents at the synapse (Davenport et al., 2021; Hausrat et al., 2015).

A potential function of radixin in connection with $\alpha 5$ -GABA_ARs was explored by Hausrat et al., who demonstrated its role in regulating pre-pulse inhibition (PPI) (Hausrat et al., 2022). In $\alpha 5$ -H105R mice, a reduction in PPI was shown to be associated with the loss of $\alpha 5$ -GABA_ARs (Hauser et al., 2004). The histidine-to-arginine point mutation led to a 15 to 25 % reduction of $\alpha 5$ -GABA_ARs in the mutant mice brain (Crestani et al., 2002). In the same year, Collinson et al. showed that the absence of $\alpha 5$ -GABA_ARs in *Gabra5* knockout mice reduced tonic and phasic currents (Collinson et al., 2002). Subsequent experiments identified $\alpha 5$ -GABA_ARs' role in reduced phasic inhibition in $\alpha 5$ -H105R (Zarnowska et al., 2009). Hypothesizing that reduced radixin levels might increase $\alpha 5$ -GABA_ARs diffusion toward the postsynaptic terminal, thus influencing PPI, Hausrat et al. observed a PPI increase in heterozygous *RDX* knockout mice compared to wildtype (Hausrat et al., 2022). Therefore, the diffusion of $\alpha 5$ -GABA_ARs in spines could play a role in the discussed cascade. These findings may indicate a potential role for radixin and DiS containing $\alpha 5$ -GABA_ARs in the impaired sensorimotor gating ability seen in young patients with autism spectrum disorder (C. H. Cheng et al., 2018).

4.8 Potential functions of $\alpha 5$ -containing GABA_ARs near excitatory synapses

One function of $\alpha 5$ -GABA_ARs near excitatory synapses may be their efficient modulation of synaptic currents. The precise role of $\alpha 5$ -GABA_ARs at synapses remains not fully understood. Under quiescent neuronal conditions, $\alpha 5$ -GABA_ARs can influence extrasynaptic NMDAR-mediated excitatory plasticity but do not affect synaptic LTP (Davenport et al., 2021). $\alpha 5$ -GABA_ARs on synaptic terminals appear to function as a "contra-inductance on demand"-system. Computational analyses indicate that synaptic $\alpha 5$ -GABA_ARs, which are highly outward-rectifying, counterbalance NMDAR activity on demand and minimally affect small

AMPA-mediated postsynaptic currents. This limited impact on AMPAR-mediated currents is due to the slower gating kinetics of $\alpha 5$ -GABA_ARs, emphasizing their essential role alongside non- $\alpha 5$ -GABA_ARs with faster gating kinetics (Schulz et al., 2018).

Overexpression of $\alpha 1$ - and $\alpha 5$ -GABA_ARs in cortical neurons revealed that IPSCs mediated by $\alpha 5$ -GABA_ARs have rise and decay times two to four times slower than those mediated by $\alpha 1$ -GABA_ARs (Davenport et al., 2021). As a result, $\alpha 5$ -GABA_ARs produce small, energy-efficient PSPs during minor EPSPs but can increase conductance fourfold during stronger depolarization events, thereby acting as a counterbalance on demand (Schulz et al., 2018). The role of $\alpha 5$ -GABA_ARs in providing inhibitory modulation on demand is further demonstrated by their activation, which saturates newly occurring LTP without affecting pre-existing LTP (Davenport et al., 2021). The distinct roles of α - subunits at synapses become clearer when comparing $\alpha 1$ -GABA_ARs and $\alpha 5$ -GABA_ARs in their effect on cumulative NMDA-mediated LTP; specifically, $\alpha 1$ -GABA_ARs did not affect LTP accumulation following repeated theta-burst stimulation (Davenport et al., 2021). Additionally, IPSCs originating from synapses formed by somatostatin-expressing interneurons and nitric oxide synthase-expressing neurogliaform cells, targeting distal CA1 pyramidal dendrites, rely on at least 50 % $\alpha 5$ -GABA_ARs recruitment (Schulz et al., 2018). Blockade of synaptic $\alpha 5$ -GABA_ARs led to increased NMDAR-mediated currents, while increased $\alpha 5$ -GABA_ARs activity reduced NMDAR-mediated dendritic depolarization (Schulz et al., 2018). These findings indicate that dendrite-targeting interneurons can modulate NMDA-mediated plasticity in CA1 pyramidal cell dendrites through $\alpha 5$ -GABA_ARs activation. Thus, $\alpha 5$ -GABA_ARs on spines may regulate NMDA-mediated LTP, potentially preventing excitatory current overshoot and excitotoxicity.

Another function could be related to neurodevelopment and spine maturation. The higher percentage of approximately 14 % of $\alpha 5$ -GABA_ARs colocalized with GLuR2 compared to PSD95 could be attributed to differences in the maturation level of synapses or spines. Given that around 75-80% of mature, stable spines in adult neurons contain PSD95 clusters (Cane et al., 2014; Prange & Murphy, 2001; Villa et al., 2016), a similar result for GluR2 colocalization might have been anticipated. These findings suggest that, for this specific population of spines or DiS, maturation levels may also correlate with $\alpha 5$ -GABA_AR colocalization. The preferential binding of $\alpha 5$ -GABA_ARs to spines or near excitatory synapses with higher maturation could indicate a maintenance role, where $\alpha 5$ -GABA_ARs inhibit further spine and synaptic maturation to stabilize the excitatory synapse. However, how $\alpha 5$ -GABA_ARs function in a controlling cascade, knowing that the expression seems to peak in early development stages (Liu et al., 1998; Ramos et al., 2004), is uncertain. Additionally, a decrease in cortical $\alpha 5$ -GABA_ARs expression has been observed in aged rats (24 months) compared to younger rats (3 months) (Schmidt et al., 2010).

During early development, rat hippocampal neurons exhibit high concentrations of SAP-102 alongside high levels of the NMDA receptor subunit NR2B (Sans et al., 2000). Increased NR2B-NMDARs facilitate dendritogenesis in immature neurons by recruiting specific proteins, such as calmodulin-dependent kinase II (CaMKII) (Bustos et al., 2014, 2017). As neurons mature and the number of dendritic spines grows, protein levels start to shift. The synaptic protein density of PSD95 and the NMDA receptor subunit NR2A increases while SAP-102 and NR2B protein levels decline (Sans et al., 2000). Higher levels of PSD95 are associated with reduced dendritogenesis rates (Bustos et al., 2014). This switch appears to be activity-dependent, as increased neuronal activity leads to larger, more stable spines and synapses through PSD95 recruitment (De Roo et al., 2008; El-Husseini et al., 2000; Meyer et al., 2014). Notably, only spines with a consistently stable enlargement exhibit an increase in PSD95, while spines without this enlargement did not show this in PSD95 (Meyer et al., 2014). Further,

PSD95 knockdown in rat hippocampal cultures has shown that spine growth after LTP is reduced compared to cells with PSD95 expression intact (Ehrlich et al., 2007).

Localization of $\alpha 5$ -GABA_ARs at the synapse appears to reduce dendritic outgrowth during development, whereas their presence on extrasynaptic sites seems to promote dendritic outgrowth but reduces the number of mature mushroom spines (Brady & Jacob, 2015). When $\alpha 5$ -GABA_ARs are activated and potentially depolarize the plasma membrane, treatment with L-655,708, which reduces $\alpha 5$ -GABA_ARs activity, delays spine maturation and the transition from NR2B- to NR2A-dominant synapses (Nuwer et al., 2021). L-655,708 is described in the literature as an inverse-agonist (Xue et al., 2017) or as an $\alpha 5$ -NAM (Nuwer et al., 2021). Impairments of this shift could impact AMPAR recruitment, which is crucial for the stability of excitatory synapses. Notably, GluR2 has been linked to synapse stabilization (Ripley et al., 2011) and synaptic scaling (Gainey et al., 2009). Blocking NMDA receptors with APV led to increased AMPA-mediated miniature excitatory postsynaptic currents (Kato et al., 2007). An increase in NR2A level can be blocked by APV in young cells (Yashiro & Philpot, 2008). Furthermore, AMPAR subunit levels, including GluR1 and GluR2, increased in the absence of NR2B (Ferreira et al., 2015; Hall et al., 2007; C. C. Wang et al., 2011). One possible explanation is that NR2B-NMDAR activity affects TARP expression, suppressing AMPAR localization at synapses (Hall et al., 2007). Thus, NR2B-NMDARs may regulate AMPA receptor expression and synapse maturation (Gray et al., 2011). These findings suggest that active, depolarizing $\alpha 5$ -GABA_ARs may be required for the NR2B-to-NR2A shift and subsequent spine maturation.

Interestingly, in more mature hippocampal neurons, where $\alpha 5$ -GABA_ARs contribute to hyperpolarization, treatment with L-655,708 and the resulting decrease in $\alpha 5$ -GABA_AR activity, was found to increase synaptic NR2A-NMDAR density while reducing NR2B-NMDAR level (Nuwer et al., 2021). This contrasting behavior suggests that in mature spines and synapses, $\alpha 5$ -GABA_AR activity may inhibit further growth of spines and synapses. Additionally, inhibiting cumulative LTP due to $\alpha 5$ -GABA_AR activity (Davenport et al., 2021) could also limit additional PSD95 recruitment.

These cascades suggest two distinct functions of $\alpha 5$ -GABA_ARs on spines near excitatory synapses. During early development, depolarizing $\alpha 5$ -GABA_ARs facilitate the NR2Bto-NR2A switch, indirectly promoting AMPAR recruitment and enhancing synaptic stability. Later, once spines and synapses reach a certain level of maturation, $\alpha 5$ -GABA_ARs activity may reduce AMPA receptor recruitment by stabilizing NR2B levels. Supporting this theory is the observation that $\alpha 5$ -GABA_ARs seem to relocate and alter their activity patterns throughout the development of granule cells in the dentate gyrus. In young cells, higher synaptic $\alpha 5$ -GABA_ARs activity is observed; however, as maturation progresses and cells integrate into the hippocampal network, $\alpha 5$ -GABA_ARs activity shifts more toward tonic inhibition. In adult neurons, LTP appears to promote the relocation of $\alpha 5$ -GABA_ARs to synapses and the reduction in tonic inhibition (Davenport et al., 2021). Notably, NMDA-induced LTP has been shown to increase spine survivability (Hill & Zito, 2013), whereas LTD has the opposite effect, reducing spine numbers (Zhou et al., 2004). The potential for activity-dependent modification in spine formation and elimination mostly diminishes with age (Nägerl et al., 2004).

Given the specificity of these cascades and the relatively small proportion of $\alpha 5$ -GABA_ARs in synaptic areas, this organization might belong only to certain types of spines with synapses crucial for maintaining key neuronal circuits. The role of stabilizing particular spines and/or synapses would align with previous data showing that stable synapses on apical dendrites do not strengthen further after early developmental stages, exhibiting a stable conformation due

to reduced NMDAR expression (Yaeger et al., 2024). Additionally, Yeager et al. observed that synapses on apical dendrites selectively receive thalamic input (Yaeger et al., 2024). Notably, studies have shown that DiSs of neocortical pyramidal neurons receive more thalamocortical afferents containing VGLUT2 than VGLUT1. Spines in the presence of VGLUT2-carrying boutons were also larger and more frequently formed than those associated with VGLUT1-rich terminals (Kubota et al., 2007). Moreover, researchers observed that spines with gephyrin clusters were more commonly located in the proximity of VGLUT2-positive boutons, suggesting that spines with inhibitory synapses may receive more input from the thalamus (van Versendaal et al., 2012).

However, several findings may contradict these theories. In rat hippocampal neuronal cultures, approximately 60 % of the colocalization between $\alpha 5$ -GABA_ARs and gephyrin remained stable across developmental stages (Brady & Jacob, 2015). Noteworthy, in CA1 pyramidal neurons of mice, 43 % to 69 % of gephyrin puncta were located in DiS, with no distribution differences observed between P15 and P45, as both age points displayed similar densities (Kleinjan et al., 2023). Furthermore, the increased location of $\beta 3$ -GABA_ARs on postsynaptic terminals reduced the number of mature spines and decreased PSD95 density at excitatory synapses (Jacob et al., 2009). A previous study has shown that these $\alpha 5$ - and $\beta 3$ -subtypes are strongly linked (McKernan & Whiting, 1996).

In old male mice (22 months), chronic treatment with $\alpha 5$ -PAM increased the total dendritic length, total spine count, and spine density in CA1 pyramidal neurons (Prevot et al., 2020). Furthermore, the first data may suggest that GABA_BRs, not GABA_ARs, influence DiSs (Kleinjan et al., 2023; Lur & Higley, 2015). However, if the role of GABA_BRs toward DiS strengthens, it is notable that $\alpha 5$ -GABA_ARs enrichment on the synapses was observed indirectly after changes in GABA_BR activity (Gerrow & Triller, 2014).

4.9 Conclusion and Future Directions

The results of this thesis offer novel insights into the spatial organization and distribution of $\alpha 5$ -GABA_ARs in hippocampal neurons. To the author's knowledge, this is the first study to focus in detail on the localization of $\alpha 5$ -GABA_ARs near excitatory synapses, suggesting a promising area for future research on the function of $\alpha 5$ -GABA_ARs. A new phenotype of increased pre-synaptic terminal density was also observed in *Gabra5* knockout-derived neurons.

The finding that $\alpha 5$ -GABA_ARs can be located near PSD95 indicates the need for additional morphological studies. Since the STED and EM data already reveal detailed protein organization on the spine, an additional experiment using immunogold staining coupled with EM analysis would further strengthen the findings.

Analyzing specific cluster populations based on intensity and size to determine whether they are localized near spines could clarify the appearance of different distribution systems of $\alpha 5$ -GABA_ARs. However, the roles of the potential different populations are still not understood. Previous studies suggest that $\alpha 5$ -GABA_ARs may modulate excitatory synaptic activity on demand at DiS and could play a role in controlling neuronal circuits. Additionally, they may contribute to spine and synaptic maturation, as suggested by the observation that $\alpha 5$ -GABA_ARs knockdown results in an increased density of presynaptic terminals and excitatory synapses. Future experiments are required to gain deeper insights.

The colocalization of $\alpha 5$ -GABA_ARs with PSD95 and GLuR2 might suggest different developmental stages of spines, but it would be valuable to investigate whether $\alpha 5$ -GABA_ARs also colocalize with SAP102, which appear in spines earlier than PSD95. This could help pinpoint the timing of $\alpha 5$ -GABA_ARs recruitment to spines. Furthermore, an experimental setup examining the protein density of PSD95, synaptophysin, VGLUT1, and GluR2 in colocalization with $\alpha 5$ -GABA_ARs in *Gabra5* knockout-derived neurons at various developmental stages could enhance our understanding of $\alpha 5$ -GABA_ARs' influence on spine maturation. Analyzing spine morphology would also help determine whether $\alpha 5$ -GABA_ARs bind already to filopodia or only to mature mushroom spines.

Another future research goal should be identifying the scaffold proteins associated with $\alpha 5$ -GABA_ARs on spines. Immunostaining analysis of gephyrin and newer scaffold proteins such as MPP2 will contribute to this question. Additionally, behavioral studies combined with morphological analysis (IHC) could illuminate the role of $\alpha 5$ -GABA_ARs near excitatory synapses. Learning and memory tests, followed by spine morphology analysis and $\alpha 5$ -GABA_ARs localization, could reveal whether learning affects the number of $\alpha 5$ -GABA_ARs-containing dendritic spines (DiS).

Finally, these findings on $\alpha 5$ -GABA_ARs localization may have implications for future research on various diseases, neuronal circuitry development, memory formation, and related impairments. For example, the increased colocalization of $\alpha 5$ -GABA_ARs and PSD95 in the absence of radixin could be relevant for ASD research, as radixin mutations have been observed in an ASD patient (Zurek et al., 2016). However, the specific effects of these mutations remain unclear. Changes in $\alpha 5$ -GABA_ARs binding, potentially leading to reduced extrasynaptic localization, could alter the balance between excitatory and inhibitory synaptic development. Additionally, it would be interesting to examine whether a higher number of $\alpha 5$ -GABA_ARs appear near the PSD95 cluster in some of these mutations. Based on the neurodevelopmental timepoint, this colocalization could increase or inhibit spinogenesis, which could be the origin of specific ASD-like phenotypes.

5. Project 2: Results

5.1 Validation of the plasma membrane enriched tissue fraction and the radixin antibody specificity in wildtype- and *RDX* knockout-derived hippocampal lysate

The antibody used to pull down radixin was first validated in wildtype and *RDX* knockout-derived lysates to ensure the reliability of the data obtained by mass spectrometry. With this in mind, before starting the mass spectrometry analysis, a validation of the radixin antibody and the successful enrichment of the cellular components in the tissue lysate was confirmed.

Differential centrifugation was employed to fractionate radixin wildtype- (*RDX* +/+) and knockout-derived (*RDX* -/-) adult hippocampal lysates into distinct cell components. This process yielded three distinct lysate fractions: S1, containing whole cells and cytoskeletons; S2, enriched with cell organelles such as mitochondria; and P2, designed to exhibit isolated plasma membranes and associated proteins (reviewed in (Stillwell, 2016)). P2 lysates were used for the Mass Spectrometry analysis, which aligned with the project's goal of identifying new binding partners of radixin at the plasma membrane.

Figure 26 A shows the schematic overview of the centrifugation steps involved in obtaining the lysates.

To confirm the successful differentiation of the cellular components, the lysates were analyzed for specific proteins such as actin, GAPDH, γ -adaptin, N-Cadherin, and $\alpha 5$ -GABA_ARs, using SDS-Page and Western Blot (Fig. 26 B). The latter two proteins were mainly instrumental in demonstrating the enrichment of the plasma membrane and its associated proteins (P2 tissue fraction).

The Western Blot results showed that the differential centrifugation succeeded, indicative of enrichment in P2 lysates and a simultaneous loss in S2 lysates for N-Cadherin, and the enrichment of $\alpha 5$ -GABA_ARs in P2 lysates, as shown by higher intensity signals at the respective molecular weight. Furthermore, two radixin antibodies were knockout-validated in the experiment (Fig. 26 B).

After successfully validating the plasma membrane enrichment in the P2 tissue fraction and the radixin antibody, a protocol for immunoprecipitating radixin from the P2 lysates was developed. The Western Blot analysis demonstrates that radixin was successfully precipitated from the wildtype lysates with a concurrent clean IgG control (Fig. 26 C). Furthermore, no signal in the IP column was detected in P2 knockout-derived lysates, again proving the antibody's specificity.

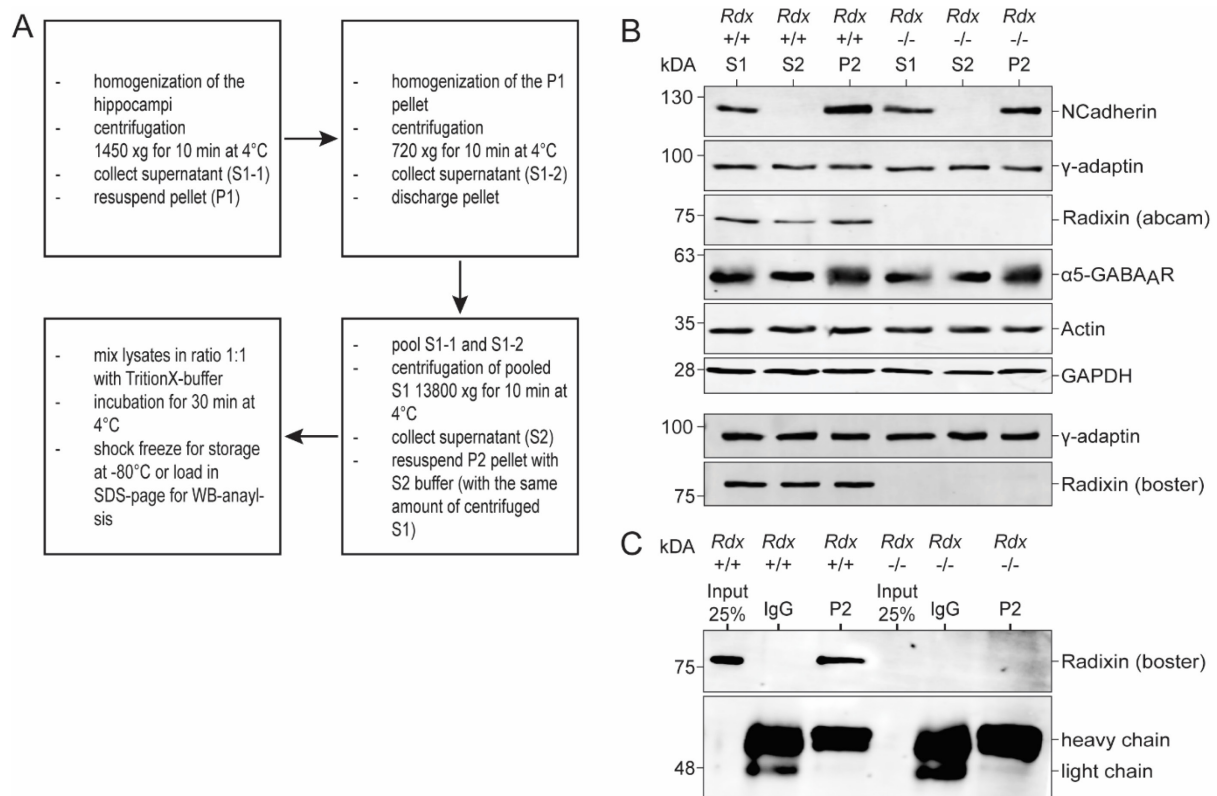


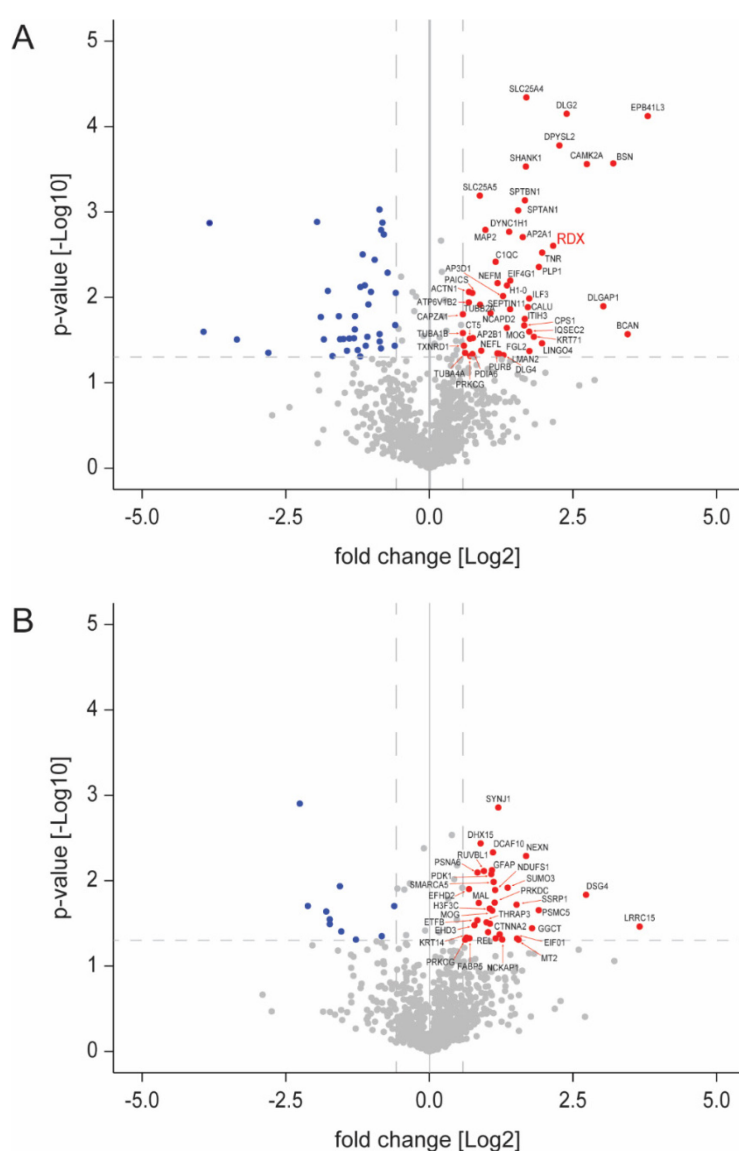
Figure 26: Validation of the differential centrifugation and the radixin antibodies **A:** Scheme of the various steps for the differential centrifugation of the radixin wildtype (+/+) and radixin knockout (-/-) adult hippocampal lysates. **B:** Western Blot analysis of the different lysates (S1, S2, and P2) using antibodies for the proteins N-Cadherin, radixin, α5-GABA_ARs, actin, GAPDH, and γ-adaptin, **C:** Immunoprecipitation of radixin from P2 wildtype (+/+) and knockout (-/-) derived adult hippocampal lysates. 3 independent experiments.

5.2 Mass Spectrometry analysis from immunoprecipitated samples of wildtype- and *RDX* knockout-derived hippocampal P2 lysates

After successfully validating the different fractions of the lysates and confirming the efficiency of the anti-radixin antibody, a new protocol for the immunoprecipitation of radixin was established.

On two separate experimental days, two precipitated samples from wildtype and *RDX* knockout sources (WT-IP and KO-IP), along with their corresponding controls, antibodies from the type immunoglobulin-G (WT-IgG and KO-IgG), were prepared from adult hippocampal P2 lysate (Fig. 26 C).

These four pairs of IP and IgG samples were subsequently analyzed by Mass Spectrometry at the University Medical Center Hamburg-Eppendorf (UKE), Institute of Clinical Chemistry and Laboratory Medicine, in the group of Mass Spectrometric Proteomics of Prof. Dr. Hartmut Schlüter.



(see supplementary data table 3).

The analysis identified 927 differentially abundant proteins across the pooled mass spectrometry trials. The final table was compiled by comparing WT-IPs with KO-IPs. By comparing the data between WT-IgG and KO-IgG, potential false positives due to nonspecific binding to the IgGs were excluded (Fig. 27 B). Proteins with a significantly higher abundance (p-value ≤ 0.05, foldchange difference > 1.5) were later excluded.

The final compiled volcano plot comprises 45 differential proteins that exhibited significantly higher abundance (p-value ≤ 0.05, foldchange difference > 1.5) in WT-IP samples compared to their knockout counterparts (Fig. 27 A). The resulting table (Tbl. 8) shows that radixin has the sixth-highest fold change of the detected proteins with a p-value ≤ 0.05. In addition to Table 1, an alternative table with the functions and the location of these 45 proteins was created based on the data provided by UniProt (<http://www.uniprot.org>)

Figure 27: Volcano plots of the proteins found in the mass spectrometry analysis following RDX immunoprecipitation. A-B: Radixin wildtype- and knockout-derived hippocampi (31-40 weeks age) mice brain lysates enriched with the plasma membrane components (P2) were used for the IPs. Anti-radixin antibody (**A**) (WT-IP/KO-IP) and an unspecific rabbit antibody from the type immunoglobulin-G (**B**) (WT-IgG/KO-IgG) were used. For the final volcano plot (**A**), all significantly higher abundant proteins (red dots, p -value ≤ 0.05 , foldchange difference > 1.5) from **B** were subtracted. **A:** Volcano plot shows 893 differentially abundant proteins generated by comparison of WT-IP vs. KO-IP. 45 significantly higher abundant proteins (red dots, p -value ≤ 0.05 , foldchange difference > 1.5) were detected in wild-type conditions. **B:** The volcano plot shows 34 significantly higher abundant proteins (red dots, p -value ≤ 0.05 , foldchange difference > 1.5) generated by comparison of WT-IgG vs. KO-IgG. Four different radixin wild-type and knockout mice were used for this experiment (N4).

Table 8: 45 differential proteins identified in precipitates for the protein radixin conducted from RDX wildtype- and knockout-derived hippocampal lysates. Radixin wildtype- and knockout-derived hippocampi (31-40 weeks age) mice brain lysates enriched with the plasma membrane components (P2) were used for the IPs. The, by mass spectrometry, identified proteins were later sorted according to the highest to lowest fold change. P -value ≤ 0.05 . Fold change > 1.5 . The identification number is based on UniProt (<http://www.uniprot.org>).

Nr.	Gene	Identificaiton number (mus musculus)	P-value	log2 fold change (fc)
1	BCAN	Q61361	0.0270047	3.45245
2	BSN	O88737	0.00026976	3.20226
3	DLGAP1	Q9D415	0.0127046	3.0291
4	CAMK2A	P11798	0.00027512	2.74156
5	DPYSL2	O08553	0.00016644	2.26531
6	RDX	P26043	0.00249313	2.15598
7	TNR	Q8BY19	0.00299653	1.96402
8	LINGO4	Q149C3	0.0345595	1.96133
9	PLP1	P60202	0.00440788	1.9072
10	KRT71	Q9R0H5	0.028969	1.81911
11	LMAN2	Q9DBH5	0.0425625	1.74171
12	IQSEC2	Q5DU25	0.0252441	1.7391
13	ILF3	Q9Z1X4	0.0103177	1.73905
14	CALU	O35887	0.0130284	1.715
15	SHANK1	D3YZU1	0.00029392	1.68022
16	SPTBN1	Q62261	0.00073053	1.66384
17	ITIH3	Q61704	0.0179139	1.66167
18	CPS1	Q8C196	0.0213361	1.65134
19	AP2A1	P17426	0.00197176	1.62656
20	SPTAN1	P16546	0.00095815	1.54664
21	EIF4G1	Q6NZJ6	0.00637778	1.40874
22	SEPTIN11	Q8C1B7	0.0137936	1.40584
23	DYNC1H1	Q9JHU4	0.0017095	1.38859
24	H1-0	P10922	0.00725826	1.35192
25	DLG4	Q62108	0.0474783	1.28954
26	AP3D1	O54774	0.00965965	1.28298
27	FGL2	P12804	0.0457011	1.21509
28	NEFM	P08553	0.00680914	1.18825
29	PURB	O35295	0.0453902	1.18529
30	C1QC	Q02105	0.0038332	1.15374
31	NCAPD2	Q8K2Z4	0.015324	1.06485
32	MAP2	P20357	0.00162114	0.975201
33	NEFL	P08551	0.0420971	0.903387
34	TUBB2A	Q7TMM9	0.0122146	0.883191
35	SLC25A5	P51881	0.00064418	0.878709
36	AP2B1	Q9DBG3	0.0299546	0.759308
37	PDIA6	Q922R8	0.0462158	0.749413
38	PAICS	Q9DCL9	0.00888842	0.748465
39	CCT5	P80316	0.030509	0.705892
40	ACTN1	Q7TPR4	0.00863708	0.690312
41	ATP6V1B2	P62814	0.0114551	0.687375
42	TUBA4A	P68368	0.0447456	0.622991
43	TXNRD1	Q9JMH6	0.0368824	0.602689
44	TUBA1B	P05213	0.0262454	0.583753
45	CAPZA1	P47753	0.0157876	0.583499

To identify potentially associated biological pathways, the gene names of these 45 proteins were entered into the web-based database EnrichR (<https://maayanlab.cloud/Enrichr/>). The data examination yielded 278 pathways in the databank REACTOME 2022. Only pathways with an overlap of three or more proteins and a p -value ≤ 0.05 were included to narrow down the potential pathways based on their significance. Based on this, 62 of the 278 pathways were elaborated in depth (Tbl 9). The 10th most significant pathways, for instance "L1CAM interactions", "Sensory processing of sound by inner hair cells of cochlea," "Sensory processing of sound," and "Recycling pathway of L1," are represented in Fig. 28 A.

Furthermore, filtering the table for pathways with the most overlapping proteins from the list in percentage is illustrated in a different graph (Fig. 28 B). Of the 62 pathways, eight directly involved radixin. Four pathways of the ranked top ten include radixin when the table was sorted by significance. The protein overlap graph includes only three of the eight pathways exhibiting radixin. However, novel potential pathways involving related cascades, such as the excitatory synapse, the Golgi apparatus, cellular responses to stress, and the immune system, could suggest a new role for radixin.

Table 9: Protein pathways based on the databank REACTOME 2022. Viewed biological pathways include three or more of the 45 identified proteins by Mass Spectrometry. Only pathways with an overlap of three or more proteins and a p -value ≤ 0.05 were included. Pathways, including radixin, are highlighted with RDX (red). Table sorted based on p -values. The data were obtained by the website EnrichR (<https://maayanlab.cloud/Enrichr/>)

Name	R-HSA-Nr.	Overlap	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes of the 45 identified proteins
L1CAM Interactions	R-HSA-373760	6 / 99	8.79E-08	2.44E-05	32.85690653	533.8177054	DPYSL2; RDX ; AP2A1; AP2B1; SPTAN1; SPTBN1
Formation of Tubulin Folding Intermediates by CCT/TriC	R-HSA-389960	4 / 26	3.22E-07	3.06E-05	88.39467849	1321.29499	TUBA1B; TUBB2A; CCT5; TUBA4A
Sensory Processing of Sound by Inner Hair Cells of Cochlea	R-HSA-9662360	5 / 67	3.99E-07	3.06E-05	40.10685484	590.9310643	CAPZA1; RDX ; BSN; SPTAN1; SPTBN1
Recycling Pathway of L1	R-HSA-437239	4 / 28	4.40E-07	3.06E-05	81.0203252	1185.850699	DPYSL2; RDX ; AP2A1; AP2B1
Sensory Processing of Sound	R-HSA-9659379	5 / 75	7.04E-07	3.91E-05	35.50892857	503.0424146	CAPZA1; RDX ; BSN; SPTAN1; SPTBN1
Cooperation of Prefoldin And TriC/CCT In Actin and Tubulin Folding	R-HSA-389958	4 / 33	8.72E-07	4.04E-05	67.03448276	935.2810847	TUBA1B; TUBB2A; CCT5; TUBA4A
ER To Golgi Anterograde Transport	R-HSA-199977	5 / 133	1.19E-05	4.73E-04	19.36230469	219.5489675	DYNC1H1; LMAN2; CAPZA1; SPTAN1; SPTBN1
Nephrin Family Interactions	R-HSA-373753	3 / 22	1.59E-05	5.53E-04	74.94736842	828.0722665	ACTN1; SPTAN1; SPTBN1
Post-chaperonin Tubulin Folding Pathway	R-HSA-389977	3 / 23	1.83E-05	5.64E-04	71.19642857	776.7906402	TUBA1B; TUBB2A; TUBA4A
COPI-mediated Anterograde Transport	R-HSA-6807878	4 / 80	3.12E-05	7.77E-04	25.51861361	264.7475538	DYNC1H1; CAPZA1; SPTAN1; SPTBN1
Transport to Golgi And Subsequent Modification	R-HSA-948021	5 / 164	3.27E-05	7.77E-04	15.56289308	160.7422373	DYNC1H1; LMAN2; CAPZA1; SPTAN1; SPTBN1
Prefoldin Mediated Transfer of Substrate To CCT/TriC	R-HSA-389957	3 / 28	3.35E-05	7.77E-04	56.94285714	586.7001545	TUBB2A; CCT5; TUBA4A
Glutamate Binding, Activation of AMPA Receptors and Synaptic Plasticity	R-HSA-399721	3 / 31	4.58E-05	9.09E-04	50.83418367	507.9194132	CAMK2A; AP2A1; AP2B1
Sealing of Nuclear Envelope (NE) By ESCRT-III	R-HSA-9668328	3 / 31	4.58E-05	9.09E-04	50.83418367	507.9194132	TUBA1B; TUBB2A; TUBA4A
Chaperonin-mediated Protein Folding	R-HSA-390466	4 / 90	4.96E-05	9.19E-04	22.53998866	223.4018711	TUBA1B; TUBB2A; CCT5; TUBA4A
Protein Folding	R-HSA-391251	4 / 96	6.39E-05	0.001109641	21.06362672	203.4483355	TUBA1B; TUBB2A; CCT5; TUBA4A
MHC Class II Antigen Presentation	R-HSA-2132295	4 / 104	8.72E-05	0.001426766	19.37073171	181.0534346	DYNC1H1; CAPZA1; AP2A1; AP2B1
Axon Guidance	R-HSA-422475	7 / 519	1.47E-04	0.002041687	6.99532278	61.73979213	DPYSL2; RDX ; AP2A1; AP2B1; SPTAN1; SPTBN1; EIF4G1
Platelet Degranulation	R-HSA-114608	4 / 125	1.78E-04	0.002352148	15.99193711	138.0987737	ITIH3; ACTN1; CALU; TUBA4A
Nervous System Development	R-HSA-9675108	7 / 545	1.98E-04	0.002482631	6.648356486	56.67966301	DPYSL2; RDX ; AP2A1; AP2B1; SPTAN1; SPTBN1; EIF4G1

Project 2: Radixin

Results

Response to Elevated Platelet Cytosolic Ca ²⁺	R-HSA-76005	4 / 130	2.07E-04	0.002482631	15.35346496	130.2732205	ITIH3; ACTN1; CALU; TUBA4A
Neuronal System	R-HSA-112316	6 / 386	2.14E-04	0.002482631	7.925101215	66.95128339	CAMK2A; NEFL; AP2A1; DLGAP1; AP2B1; SHANK1
Sensory Processing of Sound by Outer Hair Cells of Cochlea	R-HSA-9662361	3 / 53	2.30E-04	0.002562827	28.43571429	238.1601815	RDX ; SPTAN1; SPTBN1
Membrane Trafficking	R-HSA-199991	7 / 599	3.52E-04	0.003626889	6.025115576	47.90668354	DYNC1H1; LMAN2; CAPZA1; AP2A1; AP2B1; SPTAN1; SPTBN1
Asparagine N-linked Glycosylation	R-HSA-446203	5 / 282	4.14E-04	0.004113074	8.879963899	69.16605042	DYNC1H1; LMAN2; CAPZA1; SPTAN1; SPTBN1
Vesicle-mediated Transport	R-HSA-5653656	7 / 637	5.09E-04	0.004575043	5.650584795	42.84449175	DYNC1H1; LMAN2; CAPZA1; AP2A1; AP2B1; SPTAN1; SPTBN1
Nuclear Envelope (NE) Reassembly	R-HSA-2995410	3 / 75	6.42E-04	0.005387468	19.72519841	145.0053135	TUBA1B; TUBB2A; TUBA4A
Metabolism of Proteins	R-HSA-392499	12 / 1890	7.29E-04	0.005561515	3.500242037	25.28334878	DYNC1H1; TUBA1B; TUBB2A; LMAN2; CAPZA1; CALU; SPTAN1; PDIA6; SPTBN1; CCT5; TUBA4A; EIF4G1
Neurotransmitter Receptors and Postsynaptic Signal Transmission	R-HSA-112314	4 / 182	7.39E-04	0.005561515	10.8396821	78.16096653	CAMK2A; NEFL; AP2A1; AP2B1
Cellular Responses to Stress	R-HSA-2262752	7 / 722	0.001065	0.007051706	4.956937799	33.92744953	DYNC1H1; TXNRD1; CAPZA1; CAMK2A; ATP6V1B2; PDIA6; H1-O
Cellular Responses to Stimuli	R-HSA-8953897	7 / 736	0.001191	0.00767831	4.858205184	32.71019921	DYNC1H1; TXNRD1; CAPZA1; CAMK2A; ATP6V1B2; PDIA6; H1-O
M Phase	R-HSA-68886	5 / 380	0.001578	0.00953772	6.526666667	42.10666287	DYNC1H1; TUBA1B; TUBB2A; NCAPD2; TUBA4A
Mitotic Anaphase	R-HSA-68882	4 / 232	0.001811	0.010226128	8.441163885	53.29830055	DYNC1H1; TUBA1B; TUBB2A; TUBA4A
Mitotic Metaphase and Anaphase	R-HSA-2555396	4 / 233	0.001839	0.010226128	8.403876877	52.93103984	DYNC1H1; TUBA1B; TUBB2A; TUBA4A
Transmission Across Chemical Synapses	R-HSA-112315	4 / 246	0.002241	0.011978187	7.947188067	48.48615562	CAMK2A; NEFL; AP2A1; AP2B1
Developmental Biology	R-HSA-1266738	8 / 1073	0.002449	0.012714617	3.835046314	23.0561044	KRT71; DPYSL2; RDX ; AP2A1; AP2B1; SPTAN1; SPTBN1; EIF4G1
Cell-Cell Communication	R-HSA-1500931	3 / 120	0.002488	0.012714617	12.11111111	72.62386841	ACTN1; SPTAN1; SPTBN1
Platelet Activation, Signaling and Aggregation	R-HSA-76002	4 / 254	0.002515	0.012714617	7.689756098	46.02542746	ITIH3; ACTN1; CALU; TUBA4A
Host Interactions of HIV Factors	R-HSA-162909	3 / 130	0.00312	0.01471245	11.15185602	64.34644503	AP2A1; AP2B1; SLC25A5
RAF/MAP Kinase Cascade	R-HSA-5673001	4 / 271	0.003175	0.01471245	7.193934411	41.38194032	CAMK2A; NEFL; SPTAN1; SPTBN1
Post-translational Protein Modification	R-HSA-597592	9 / 1383	0.003232	0.014728403	3.380822416	19.38808597	DYNC1H1; TUBA1B; TUBB2A; LMAN2; CAPZA1; CALU; SPTAN1; PDIA6; SPTBN1
MAPK1/MAPK3 Signaling	R-HSA-5684996	4 / 277	0.003434	0.015111632	7.033681765	39.90928364	CAMK2A; NEFL; SPTAN1; SPTBN1
Beta-catenin Independent WNT Signaling	R-HSA-3858494	3 / 142	0.003998	0.016840875	10.18293936	56.22930196	CAMK2A; AP2A1; AP2B1
MAPK Family Signaling Cascades	R-HSA-5683057	4 / 318	0.005596	0.022876805	6.102532236	31.64617704	CAMK2A; NEFL; SPTAN1; SPTBN1
Cell Cycle, Mitotic	R-HSA-69278	5 / 523	0.006191	0.024587154	4.690395753	23.84905377	DYNC1H1; TUBA1B; TUBB2A; NCAPD2; TUBA4A
Cilium Assembly	R-HSA-5617833	3 / 186	0.008432	0.030844353	7.717408275	36.85594457	DYNC1H1; CCT5; TUBA4A
Mitotic Prometaphase	R-HSA-68877	3 / 186	0.008432	0.030844353	7.717408275	36.85594457	DYNC1H1; NCAPD2; TUBA4A
Hemostasis	R-HSA-109582	5 / 576	0.009204	0.033229947	4.243432574	19.89371895	ITIH3; ACTN1; CAPZA1; CALU; TUBA4A
Immune System	R-HSA-168256	10 / 1943	0.009834	0.035048178	2.663809031	12.31197598	DYNC1H1; CAPZA1; CAMK2A; FGL2; ATP6V1B2; AP2A1; AP2B1; SPTAN1; C1QC; EIF4G1
Sensory Perception	R-HSA-9709957	5 / 616	0.012064	0.040409833	3.957446809	17.48217738	CAPZA1; RDX ; BSN; SPTAN1; SPTBN1
Signaling by Rho GTPases	R-HSA-194315	5 / 644	0.014394	0.045473379	3.77856025	16.02454338	DYNC1H1; TUBA1B; ACTN1; SPTAN1; SPTBN1
HIV Infection	R-HSA-162906	3 / 229	0.014765	0.046118978	6.235461441	26.28568423	AP2A1; AP2B1; SLC25A5
Cell Cycle	R-HSA-1640170	5 / 654	0.015296	0.047247157	3.718412943	15.54361402	DYNC1H1; TUBA1B; TUBB2A; NCAPD2; TUBA4A
Signaling by Rho GTPases, Miro GTPases And RHOBTB3	R-HSA-9716542	5 / 660	0.015855	0.048434892	3.683206107	15.26429793	DYNC1H1; TUBA1B; ACTN1; SPTAN1; SPTBN1
RHO GTPase Cycle	R-HSA-9012999	4 / 441	0.017046	0.049655929	4.357425908	17.74270639	TUBA1B; ACTN1; SPTAN1; SPTBN1

Signal Transduction	R-HSA-162582	11 / 2465	0.018422	0.050705528	2.307289419	9.215823712	DYNC1H1; TUBA1B; ACTN1; CAMK2A; ATP6V1B2; NEFL; AP2A1; AP2B1; SPTAN1; SPTBN1; EIF4G1
Transport of Small Molecules	R-HSA-382551	5 / 706	0.0206	0.054540766	3.433309558	13.32971321	CAMK2A; ATP6V1B2; AP2A1; AP2B1; SLC25A5
Organelle Biogenesis and Maintenance	R-HSA-1852241	3 / 275	0.023864	0.059234253	5.168855042	19.3076251	DYNC1H1; CCT5; TUBA4A
Innate Immune System	R-HSA-168249	6 / 1035	0.02751	0.064484642	2.829632952	10.16743385	DYNC1H1; CAPZA1; FGL2; ATP6V1B2; SPTAN1; C1QC
Extracellular Matrix Organization	R-HSA-1474244	3 / 291	0.027603	0.064484642	4.877728175	17.51019447	BCAN; ACTN1; TNFR
Signaling by WNT	R-HSA-195721	3 / 294	0.028337	0.065243494	4.82670594	17.20035098	CAMK2A; AP2A1; AP2B1
Disease	R-HSA-1643685	8 / 1736	0.037935	0.080504342	2.280655656	7.462004149	DYNC1H1; BCAN; TXNRD1; CAMK2A; AP2A1; AP2B1; SLC25A5; SPTBN1

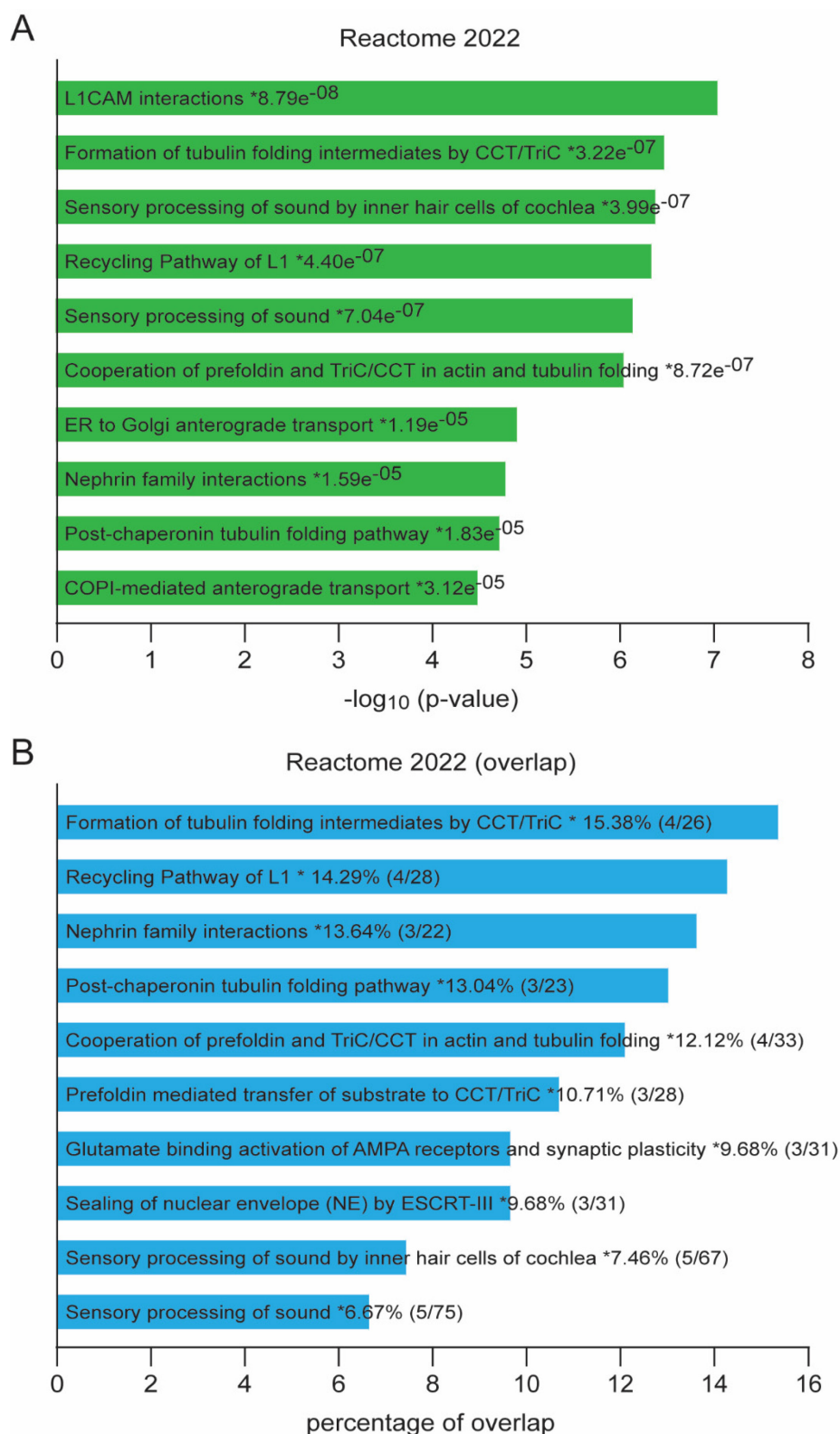


Figure 28: Summary of the top ten ranked pathways based on the databank REACTOME 2022. The graphs display the top ten pathways exhibiting three or more of the identified 45 proteins of the Mass Spectrometry analysis. The pathways are sorted by significance (A) and protein overlap (B). The data were obtained by the web-based database EnrichR (<https://maayanlab.cloud/Enrichr/>)

Another approach to analyze potential interactions among the identified proteins is through STRING analysis (Fig. 29). Gene names were entered into the STRING database (<https://string-db.org/>, Version: 12.0) to examine and illustrate potential interaction sources. STRING organizes connections/interactions (edges) based on evidence from multiple sources. The most substantial evidence comes from curated databases or experimentally determined interactions. Other sources of evidence include gene neighborhood, gene fusion, and gene co-occurrence. The weakest evidence is derived from text-mining associations, which reflect mentions of both genes in the same publication. The interaction/evidence score increases when both names appear in the same paragraph or sentence. Co-expression data summarizes findings of target gene co-expression and may include potential co-expression of putative homologs. The final evidence source, protein homology, incorporates experimental/biochemical data from the target genes/proteins and their homologs in other organisms.

The interactions' evidence is scored on a confidence scale from 0 to 1, with 1 being the highest confidence. For this STRING analysis, the minimum required interaction score was set to medium confidence (0.400), meaning that interactions between two genes had a combined score of at least 0.400.

The analysis shows that RDX (radixin) is directly connected to Dlg4, Sptbn1, Sptan1, Actn1, and Capza1. To illustrate the scoring system, evidence for two proteins connected to RDX is illustrated in further detail.

One example is the interactions between Rdx and Sptan1. Experimental/biochemical data (protein homology) support this interaction, with studies suggesting potential protein-protein interactions between Rdx and Sptan1, as demonstrated in co-immunoprecipitation assays with samples of *Mus musculus* (J. Li et al., 2017). In other organisms, protein-protein interactions have been observed; for instance, the radixin homolog moesin was shown to interact with Sptan1 in co-immunoprecipitation assays (Lee et al., 2019). The combined biochemical evidence score for this interaction is 0.076. Text-mining produced a score of 0.135, with Rdx and Sptan1 co-mentioned in abstracts and publications (e.g. (Menon & Gupton, 2016; Nelson & Jenkins, 2017)), along with mentions of radixin homologs like ezrin (Chin et al., 2014; Shestakova et al., 1998). The highest score (0.500) derives from curated database associations, which include pathways gathered from studies with *Mus musculus* related to outer and inner hair cell stereocilium cytoskeletons (Reactome Pathways, August 2022). Similar interactions are noted in *Homo sapiens*-related databases.

Another example involves the interactions between Rdx and Dlg4. The "co-expression" evidence scored 0.054, based on findings in *Caenorhabditis elegans* (F07C6.4-FERM domain-containing protein and Dlg1) and *Oryctolagus cuniculus* (FRMD4A- FERM domain containing 4A and DLG5). The "homology" evidence scored 0.093, indicating potential protein-protein interactions between the homologs Dlg1 and ezrin (Bonilha & Rodriguez-Boulan, 2001; Go et al., 2021). Text-mining analysis scored 0.696, with mentions of radixin and Dlg4/PSD95 in publications regarding *Mus musculus* (Bauler et al., 2008; Pan et al., 2006) and mentions of homologs in other organisms (Kawaguchi & Asano, 2022; Mairot et al., 2021).

STRING analysis provides a comprehensive overview of these proteins' potential connections and supporting evidence. It also highlights that while some interaction evidence is robust, independent validation of these potential interactions is essential.

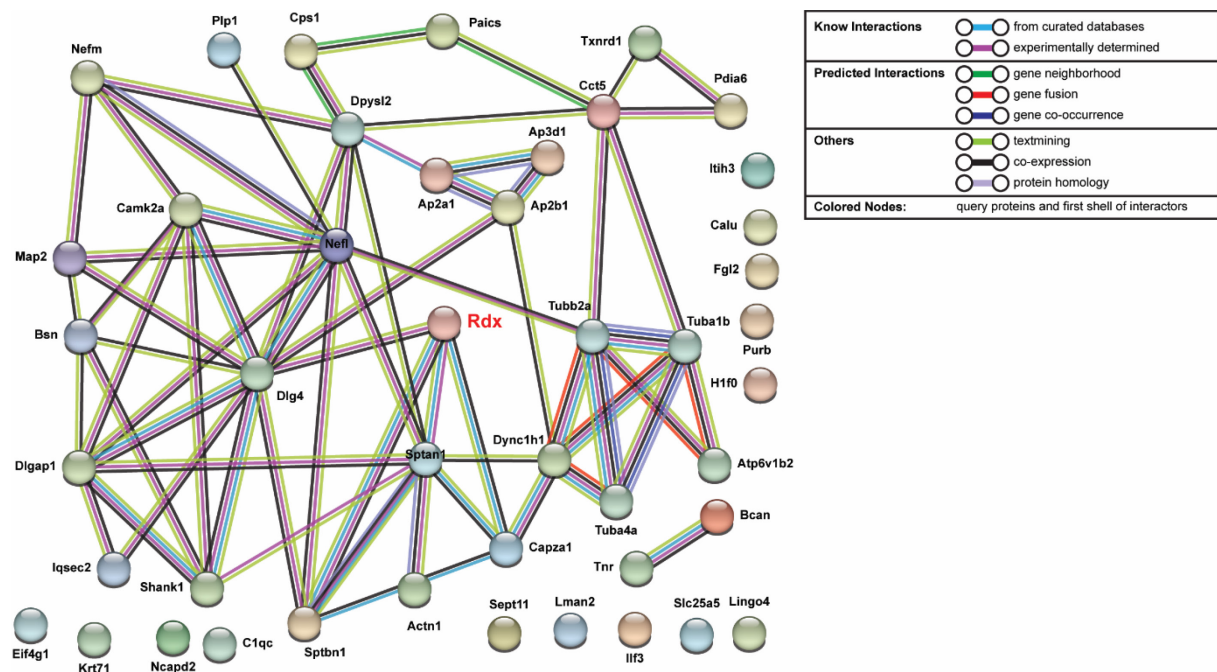


Figure 29: STRING analysis of the 45 proteins with the connection between the proteins. (<https://string-db.org/>; Version: 12.0). The different colored interactions (edges) illustrate the origin of the potential connections: from curated databases (turquoise), experimentally determined (purple), gene neighborhood (green), gene fusion (red), gene co-occurrence (blue), textmining (lime green), co-expression (black), and protein homology (heather purple).

5.3 Validation of different identified proteins

The previous figures comprise the proteins found using mass spectrometry in immunoprecipitants for radixin, their function, occurrence in different biological pathways, and their connections. However, as the database research revealed, a complete validation of the potential interactions between radixin and these proteins cannot be entirely conducted. Furthermore, based on the information gathered through databases and STRING analysis, most of the mentioned proteins do not directly interact with radixin.

Therefore, some proteins found in the screening approach were validated regarding their interaction with radixin using complement experiments. The proteins of interest were chosen based on information from the previous analyses and their appearance in pathways related to sound sensory processing by inner hair cells or cochlea, excitatory synapses, and axon guidance.

Myo18a is a known interactor with ezrin (Matsui et al., 2011) and, therefore, can be used as an indirect control protein for the experiment efficiency. As a negative control, the protein synapsin 1/2 was chosen, as it is a known synaptic marker involved in neurotransmitter release at the presynaptic terminal and the regulation of neurogenesis (reviewed by (Greengard et al., 1993; Hilfiker et al., 1999)) and is not connected to radixin.

In addition, the protein DLG4, known as PSD95, was chosen based on the results gathered about $\alpha 5$ -GABA_ARs and potential bindings on spines. Since PSD95 was chosen as a protein of interest, GKAP, with its high binding affinity to PSD95 and its clustering with NMDA receptors (Kim et al., 1997), would be an interesting new binding partner of radixin.

AP2B1, part of the AP2-complex that regulates receptor-mediated endocytosis, could strengthen new roles for radixin in the internalization of vesicles and related diseases such as schizophrenia and depression (reviewed by (Nakatsu & Ohno, 2003; Zúñiga Mouret et al., 2024)).

The last protein of interest is bassoon. The mass spectrometry data detected one of the highest abundances for the protein. Radixin plays an essential role in the extrasynaptic area by anchoring the $\alpha 5$ -GABA_AR.

Since radixin can exist in either a closed or open conformation, conducting a Co-IP with a changed ratio of radixin favoring its open conformation could result in a higher protein binding efficiency and detection by Western Blot. Therefore, Co-IPs were not performed using hippocampal lysates. Instead, primary cortical cultures from mice were transduced with an AAV carrying the T564D-mutant radixin gene to ensure most radixin proteins remain in their open conformation. After several days of incubation, the cells were harvested, and the lysate was used for Co-IP. Since the T564D-mutant is tagged with GFP, the Co-IP was performed with an anti-gephyrin antibody (GFP-RDX).

Thus, PSD95, Myo18a, and GKAP signals were found in the Co-IP samples (Fig. 30 A). However, technical difficulties regarding the size of the bassoon protein made detecting it in Western Blot impossible. Another method to validate the potential binding affinity of radixin to the selected proteins was ICC. Therefore, a positive colocalization of radixin with PSD95 (Fig. 30 B), AP2B1 (Fig. 30 C), and bassoon (Fig. 30 D) was detected.

The results of the Co-IP and/or immunocytochemistry experiments suggest that PSD95, Myo18a, GKAP, AP2B1, and bassoon do bind directly or indirectly to radixin and, therefore, validate the results of the mass spectrometry analysis.

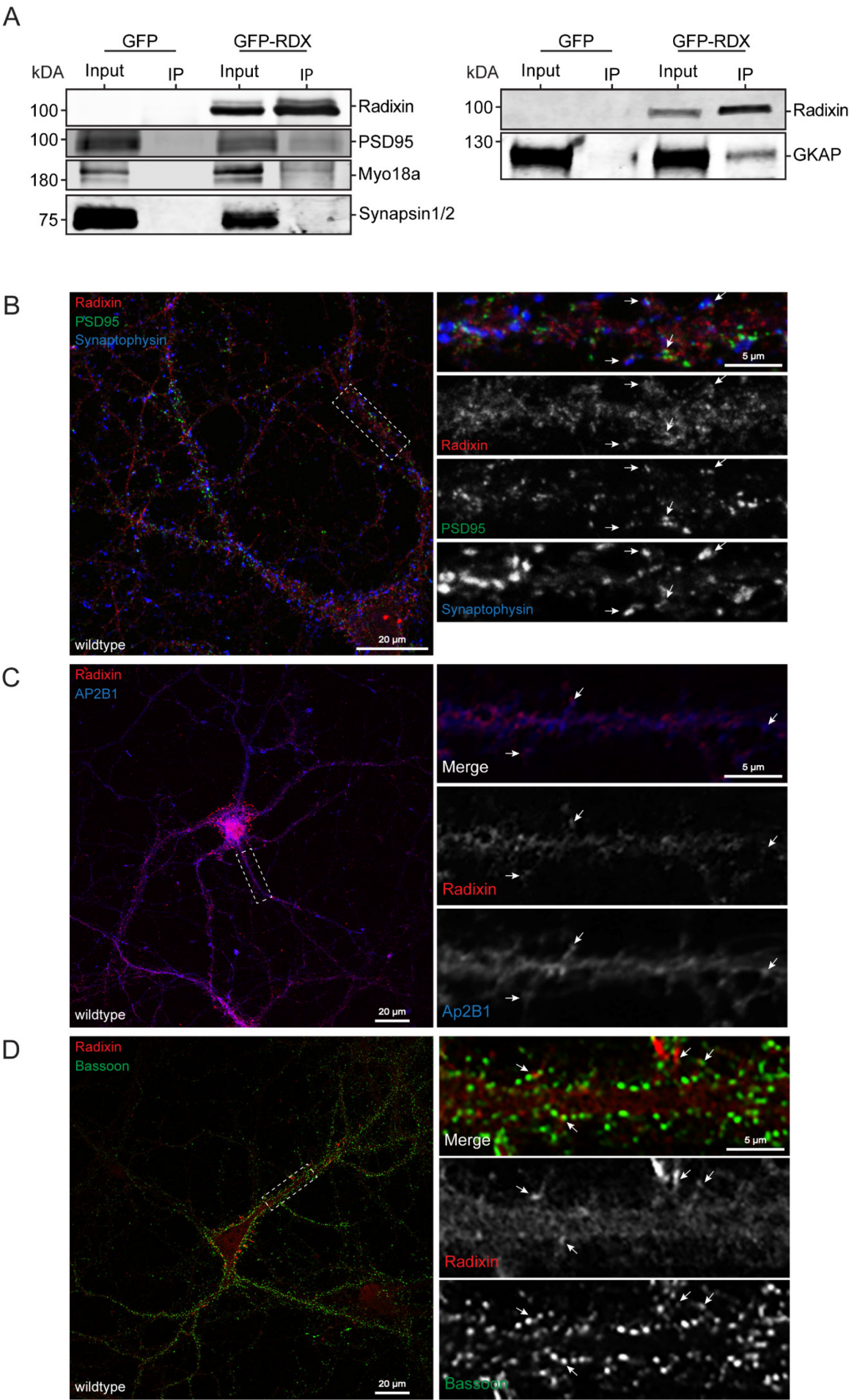


Figure 30: Validation of proteins identified in precipitates for the protein radixin and analyzed by Mass Spectrometry. A: Cortex lysates were treated with the radixin TD mutant to ensure the open confirmation of radixin. Lysates were created from DIV20 neuronal cell cultures, and immunoprecipitations were performed for radixin. PSD95 (DLG4), Myo18a, Synapsin1/2, and GKAP were analyzed using SDS-Page and Western Blot. The harvesting of the neuronal cells, the creation of the lysates, and the performing of the IPs, SDS-page, and Western Blots were conducted by Christoph Janiesch. **B-D:** Immunostainings were conducted in RDX wildtype-derived primary hippocampal neurons (DIV16-20). Enlargements of a dendrite section, highlighted by gray boxes in B, C, and D, are displayed in all two to three individual channels. White arrows mark exemplified colocalizations. They exemplified Immunostainings of radixin (red) with PSD95 (green) and synaptophysin (blue) (B), with Ap2B1 (blue) (C) with bassoon (green) (D). Cell culture preparation, immunostaining, and imaging of experiments in C and D were performed by Yvonne Pechmann. A: 3 independent experiments. B-D: 2-3 independent experiments

6. Project 2: Discussion

The second project was dedicated to the scaffold protein of $\alpha 5$ -GABA_ARs, radixin. The investigation for potential new binding partners aimed to provide novel insight into plasma membrane organization. Immunoprecipitation of radixin from *RDX* wildtype- and knockout-derived hippocampal lysates was followed by mass spectrometry analysis, identifying various proteins. The analysis of the lysates and the subsequent validation of the proteins by various methods revealed potential binding partners such as DLG4 (PSD95), bassoon, AP2B1, and GKAP. These findings introduce new hypotheses regarding radixin and its role on the plasma membrane.

6.1 Technical limitations

The table of identified proteins does not include known radixin-binding partners. This could be attributed to the conformation of radixin. A previous study has shown that radixin's homolog ezrin binding affinity to various proteins depends on its conformational state (Viswanatha et al., 2013), which could also be the case for radixin in this study.

Additionally, the binding site of the anti-radixin antibody used for the pulldown may overlap with the binding sites of potential interaction partners. Another factor may be the incubation duration of the lysate with the antibodies during the immunoprecipitation. The protocol specifies a two-hour incubation at 4 °C, but longer incubation times could increase the binding probability of proteins. The number of available binding sites might also influence the amount of protein binding. Different antibody concentrations were tested during the IP protocol development, although these samples were never assessed by mass spectrometry and only tested for radixin.

6.2 New potential binding partners of radixin

A review of publications on ERM proteins revealed a limited number of proteomics-based studies, especially on radixin. Consequently, this discussion incorporates proteomic studies and research involving the inhibition or knockout of ERM proteins. Additionally, studies using human and rat samples were included in the comparison.

Bayés et al. analysis of the postsynaptic density of the human neocortex identified 1,461 proteins (Bayés et al., 2010). A comparison revealed that 32 of the 45 proteins identified in this thesis were also found in the postsynaptic density of the human neocortex. Interestingly, in addition to *RDX*, the genes *MSN*, *EZR*, *GABRA1*, and *GABRA4* were also identified (Bayés et al., 2010). These findings reinforce the newly identified potential binding partners in this thesis's mass spectrometry analysis.

Furthermore, using human brain samples, Wingo et al. conducted a whole-brain proteomic analysis and a proteome-wide association study of cognitive trajectory and protein co-expression network analysis (Wingo et al., 2019). They also examined these networks' so-called "hub" nodes, or hub-proteins. Hub-proteins play essential roles in protein-protein interaction networks, exhibiting high levels of connections with diverse proteins. The inhibition or absence of these proteins can lead to dysfunction or dysregulation in processes and pathways, often observed in various diseases and disorders. Hub proteins are thus crucial for the stability and functionality of networks, biochemical processes, and cascades (reviewed in (He & Zhang, 2006; Hu et al., 2017; Vallabhajosyula et al., 2009)). The study identified 38

proteins associated with cognitive trajectory independently of AD pathologies, such as β -amyloid plaques and neurofibrillary tangles (Wingo et al., 2019), highlighting their general importance for cognitive stability. Six identified proteins (DLG4, AP2B1, SEPT11, BSN, AP2A2, and NEFL) overlapped with proteins identified in the thesis's radixin study. In the study of Wingo et al., DLG4, AP2B1, SEPT11, BSN, and AP2A2 showed higher abundance in cognitive stability, while no changes were observed for NEFL. This overlap suggests that radixin may also play a role in cognitive stability.

Another study also found different alterations in protein levels in AD samples. Specifically, Zhang et al. observed changes in moesin expression levels (Q. Zhang et al., 2018), which is notable given moesin's approximately 80 % similarity to radixin (Sato et al., 1992). This similarity may support the observed protein changes, such as altered ILF2 expression (Q. Zhang et al., 2018), while ILF3 was identified in the thesis's mass spectrometry analysis (Tbl. 8). Further changes in expression levels were observed for CALU, PDIA6, PURB, and TXNRD1 (Q. Zhang et al., 2018), all of which were also detected in the thesis's analysis (Tbl. 8). Additional comparisons revealed that ATP6V1G2 expression was altered in AD samples (Q. Zhang et al., 2018), while the radixin results showed an interaction with ATP2V1B2. Both are members of the ATP6V1 family and are associated with cancer development (X. Li et al., 2020). Altered SLC25A3 expression in AD samples (Q. Zhang et al., 2018) is also noteworthy, as SLC25A5 exhibits potential interactions with radixin (Tbl. 8). Both proteins belong to the solute carrier family 25, are located in the mitochondrial membrane, and are linked to various diseases, including cancer and endoplasmic reticulum stress (reviewed in (G. Zhang et al., 2024)).

The inhibition of radixin in rat brains' subventricular zone led to altered expression of various proteins associated with cellular morphology, cell-to-cell signaling, interaction, and organization (Persson et al., 2013). Comparative analysis revealed shared or related proteins between the study and the thesis's results. For instance, the tubulin alpha-1B chain (Tbl. 8) was found in both studies (Persson et al., 2013). Another potential connection is the altered expression of dynactin subunit 2 after radixin inhibition (Persson et al., 2013). The thesis's data indicate a potential interaction with the F-actin-capping protein subunit alpha1 (Tbl. 8), which plays, based on UniProt, a role in stabilizing the dynactin structure (supplementary data table 3). In this thesis, a potential connection of radixin to the V-type proton ATPase subunit B was preserved (Tbl. 8). Notably, Persson et al. found altered expression levels of the V-type proton ATPase subunit F following radixin inhibition (Persson et al., 2013). Another protein identified among the 45 proteins in this thesis's radixin study was dihydropyrimidinase-related protein 2 (Tbl. 8). Interestingly, radixin inhibition led to altered expression levels of dihydropyrimidinase-related protein 1 (Persson et al., 2013).

Additionally, altered expression of reticulon-4/Nogo A protein was noted (Persson et al., 2013). Nogo A is associated with neuronal development and the structural and functional maintenance of the central nervous system. It is recognized as a significant myelin-associated inhibitor of axonal regeneration and is thus a target protein in multiple sclerosis (MS) research (reviewed in (Kalafatakis et al., 2023; J. Wang et al., 2021)). One of the 45 proteins identified in this thesis is the leucine-rich repeat and immunoglobulin-like domain-containing NOGO receptor-interacting protein 4 (LINGO4) (Tbl. 8). While research on LINGO4 is limited, it is known to form heterocomplexes with its homolog, LINGO1, the neurite outgrowth inhibitor receptor-interacting protein (Guillemain et al., 2020). LINGO1 is also a major target in MS research due to its role as an inhibitor of axonal regeneration and is associated with diseases such as AD-like conditions, traumatic brain injury, spinal cord injury, Parkinson's disease,

tremor, epilepsy, and psychiatric disorders (reviewed in (Andrews & Fernandez-Enright, 2015; Kalafatakis et al., 2023; Mi et al., 2004; Park et al., 2005)). Interestingly, in addition to LINGO4, PLP1 was also identified in the thesis's data (Tbl. 8), a protein also associated with MS (Cloake et al., 2018). Thus, the connection of radixin with LINGO4 could suggest potential links to various pathologies in neuronal diseases.

Another novel potential interactor with radixin is the eukaryotic translation initiation factor 4 gamma 1 (eIF4G1) (Tbl. 8). eIF4G1 is associated with cancer (Jaiswal et al., 2018) and autism spectrum disorder (Gonatopoulos-Pournatzis et al., 2020) and has been identified in the proteomic analysis of early diagnostic markers for type 2 diabetes mellitus (Nimer et al., 2023). Potential connections between diabetes and ERM proteins have been noted in previous studies (Lopez et al., 2010; McRobert et al., 2003).

The protein Myo18a is classified as an unconventional myosin, as several studies showed it is not associated with ATPase motor activity (Guzik-Lendrum et al., 2013; M. H. Taft et al., 2013). However, it has been observed to act as a cross-linker between the cytoskeleton and the Golgi apparatus by binding to F-actin and GOLPH3 (M. H. Taft et al., 2013). Additionally, it appears to play a role in trafficking from the Golgi to the plasma membrane (Dippold et al., 2009; Rahajeng et al., 2019). Its strong connection to the cytoskeleton has led to investigations of the potential interactions with ERM proteins. In mass spectrometry-based proteomic analysis, Myo18a was found to be associated with ezrin (Matsui et al., 2011). Although the potential binding of other ERM-proteins was discussed, no further experiments were conducted due to the absence of radixin in B cells. Interestingly, Myo18A can be localized on the intracellular side of the plasma membrane (Matsui et al., 2011; Mori et al., 2005). The interaction between radixin and Myo18a could be seen as an indirect control for the IP efficiency, considering its close homologous similarities with ezrin.

The β 2-adaptin protein (AP2b1) was identified through mass spectrometry analysis, and colocalization with radixin was observed in hippocampal neurons using confocal microscopy. The protein is involved in clathrin-dependent endocytosis, and its disruption, for example, in homozygous mice mutants, leads to cleft palate and early lethality (W. Li et al., 2010). Pathway analysis connects AP2b1 and radixin with axon guidance, nervous system development, L1CAM interactions, the recycling pathway of L1, and developmental biology. The association with neuronal development is further supported by findings that the knockdown of AP2b1 reduces dendrites and GluA2 levels (Koscielny et al., 2017). Additionally, radixin has been shown to interact with the plasticity-related gene 2 (PRG-2), which is necessary for the guidance of thalamocortical axons (J. Cheng et al., 2016) and the generation of axon filopodia (Brosig et al., 2019). Ezrin is also associated with axon outgrowth (Antoine-Bertrand et al., 2011). Therefore, the interaction with the β 2-adaptin protein could suggest another connection between radixin and neuronal development.

Bassoon, a presynaptic scaffold protein involved in neurotransmitter release (Richter et al., 1999), and radixin were associated with the pathways "Sensory Processing of Sound by Inner Hair Cells of Cochlea", "Sensory Processing of Sound" and "Sensory Perception".

The presence of radixin in these pathways aligns with previous studies linking radixin (Hausrat et al., 2022; Kitajiri et al., 2004; Prasad et al., 2020) and its family member, the band 4.1-like protein I (Yeo et al., 2011), to the auditory system and potential complications such as deafness. Bassoon promotes vesicular replenishment in the endbulb synapses of auditory nerve fibers, which is associated with transmitting auditory information (Mendoza Schulz et al., 2014). However, the unusual and unpredictable bassoon identification challenges the notion of radixin's exclusive presence in the extrasynaptic area.

In addition to its known interactions with various proteins among the 45 identified proteins, such as Ap2a1, Slc25a5, NEFL, Tuba1a, Tuba2b, and Camk2a. Shank1 (Fernández et al., 2009), disc large MAGUK scaffold protein 4 (DLG4), also known as PSD95, is not included in any found pathway. While radixin and DLG4 have not been previously associated, earlier studies showed that homologs of DLG4 and radixin, namely disc large MAGUK scaffold protein 1 (DLG1) and ezrin, interact with one another (Lasserre et al., 2010; Lue et al., 1996). Furthermore, EBP50/NHERF1, a known binding partner of ERM-proteins (Finnerty et al., 2004; Reczek et al., 1997; Terawaki et al., 2006), was also shown to bind to DLG1 (Bonilha & Rodriguez-Boulán, 2001). The guanylate kinase-associated protein (GKAP), also known as DLG-associated protein 1 (DLGAP1), was identified in the mass spectrometry results of this thesis. The disc-large associated protein family functions as scaffold proteins at excitatory synapses and is part of the synaptic scaling cascade (reviewed in (Rasmussen et al., 2017)). Moreover, GKAP is associated with the binding to a Shank-Homer complex and PSD95 (Hayashi et al., 2009). It is implicated in several diseases and disorders, including attention deficit hyperactivity disorder (Fan et al., 2018) and autism spectrum disorder (Coba et al., 2018; Hassani Nia et al., 2022). Potential interactions among radixin, GKAP, and PSD95 may provide new targets for the study of neurodevelopmental diseases. These novel interactions would also strengthen the thesis's data, suggesting that $\alpha 5$ -GABA_ARs are near excitatory synapses.

6.3 Conclusion

The mass spectrometry analysis of potential new binding partners of radixin revealed several novel interactions related to pre- and postsynaptic organization and structures. These findings suggest new roles for radixin in excitatory synapses, axonal guidance, and neurodevelopment. Future research on these data should aim to validate these identified proteins and interactions, exploring their functions, especially regarding associated neurodevelopmental diseases and disorders.

8. Acknowledgements

Already ten years ago, I decided to pursue a scientific career with the goal of completing a PhD study. I grew professionally and personally during my time in Hamburg and at the Center for Molecular Neurobiology. I had the privilege of meeting many incredible people and scientists to whom I want to express my gratitude.

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To my dear friend Yuhao, thank you for the much-needed breaks from the lab. They kept me grounded and helped me recharge!

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Ich danke euch für eure ganze Unterstützung all die Jahre, die mich zu diesem Punkt in meinem Leben geführt haben. Egal, wie viel Arbeit vor mir lag oder welche privaten Herausforderungen ich zu bewältigen hatte, ihr wart immer da für mich. Worte können nicht beschreiben, wie glücklich ich bin, euch an meiner Seite zu wissen!

9. Supplementary data

Table 1: Exact p-values and values for mean $\pm$ SEM for Figures 11, 16 and 17

Fig. 11	cluster density (cluster/ 10 μm ²): triple-colocalizations	total intensity (arb. u./μm ²): α5-containing GABA _A in triple-colocalizations	total area (px/μm ²): α5-containing GABA _A in triple-colocalizations
Gabra5 +/+	mean ± SEM = 0.7070 ± 0.05515	mean ± SEM = 39.01 ± 4.239	mean ± SEM = 0.7897 ± 0.07032
Gabra5 -/+	mean ± SEM = 0.6553 ± 0.08378	mean ± SEM = 25.51 ± 3.354	mean ± SEM = 0.4870 ± 0.05712
Gabra5 -/-	mean ± SEM = 0.02931 ± 0.008877		
Gabra5 +/+ vs Gabra5 -/+	p = >0.999	p = 0.0149	p = 0.0015
Gabra5 +/+ vs Gabra5 -/-	p = <0.0001		
Gabra5 -/+ vs Gabra5 -/-	p = <0.0001		

Fig. 16	cluster density (cluster/μm ²): α5-containing GABA _A	total intensity (arb. u./μm ²): α5-containing GABA _A	total area (px/μm ²): α5-containing GABA _A
Gabra5 +/+	mean ± SEM = 0.8147 ± 0.02800	mean ± SEM = 352.6 ± 22.52	mean ± SEM = 8.125 ± 0.4292
Gabra5 -/+	mean ± SEM = 0.7310 ± 0.04715	mean ± SEM = 289.7 ± 24.56	mean ± SEM = 7.195 ± 0.5891
Gabra5 -/-	mean ± SEM = 0.04600± 0.004761	mean ± SEM = 5.955 ± 0.6416	mean ± SEM = 0.3350 ± 0.03945
Gabra5 +/+ vs Gabra5 -/+	p = 0.1853	p = 0.0681	p = 0.3241
Gabra5 +/+ vs Gabra5 -/-	p = <0.0001	p = <0.0001	p = <0.0001
Gabra5 -/+ vs Gabra5 -/-	p = <0.0001	p = <0.0001	p = <0.0001
	cluster density (cluster/μm ²): PSD95	total intensity (arb. u./μm ²): PSD95	total area (px/μm ²): PSD95
Gabra5 +/+	mean ± SEM = 0.6803 ± 0.01814	mean ± SEM = 312.0 ± 18.46	mean ± SEM = 7.241 ± 0.2825
Gabra5 -/+	mean ± SEM = 0.7273 ± 0.03283	mean ± SEM = 346.7 ± 29.86	mean ± SEM = 8.065 ± 0.5185
Gabra5 -/-	mean ± SEM = 0.9300 ± 0.02512	mean ± SEM = 375.9 ± 24.82	mean ± SEM = 10.12 ± 0.4519
Gabra5 +/+ vs Gabra5 -/+	p = 0.4987	p = 0.6933	p = 0.4453
Gabra5 +/+ vs Gabra5 -/-	p = <0.0001	p = 0.2019	p = <0.0001
Gabra5 -/+ vs Gabra5 -/-	p = <0.0001	p = 0.7935	p = 0.0032
	cluster density (cluster/μm ²): Synaptophysin	total intensity (arb. u./μm ²): Synaptophysin	total area (px/μm ²): Synaptophysin
Gabra5 +/+	mean ± SEM = 0.7237 ± 0.03914	mean ± SEM = 548.1 ± 56.31	mean ± SEM = 9.626 ± 0.8555
Gabra5 -/+	mean ± SEM = 0.7957 ± 0.04520	mean ± SEM = 674.6 ± 51.67	mean ± SEM = 11.48 ± 0.8957
Gabra5 -/-	mean ± SEM = 0.8923 ± 0.04863	mean ± SEM = 652.6 ± 40.8	mean ± SEM = 12.81 ± 0.8340
Gabra5 +/+ vs Gabra5 -/+	p = 0.5876	p = 0.0874	p = 0.3441
Gabra5 +/+ vs Gabra5 -/-	p = 0.0261	p = 0.1576	p = 0.0317
Gabra5 -/+ vs Gabra5 -/-	p = 0.3372	p = >0.9999	p = 0.6277

Fig. 17	cluster density (cluster/ 3 μm ²): colocalization of PSD95 and synaptophysin	cluster density (cluster/ 3 μm ²): colocalization of α5-containing GABA _A and PSD95	cluster density (cluster/ 3 μm ²): colocalization of α5-containing GABA _A and synaptophysin	
Gabra5 +/+	mean ± SEM = 0.7873 ± 0.04213	mean ± SEM = 0.6503 ± 0.03899	mean ± SEM = 0.6653 ± 0.04538	
Gabra5 -/+	mean ± SEM = 0.8703 ± 0.06948	mean ± SEM = 0.6210 ± 0.04997	mean ± SEM = 0.6247 ± 0.06204	
Gabra5 -/-	mean ± SEM = 1.061 ± 0.06168			
Gabra5 +/+ vs Gabra5 -/+	p = 0.6880	p = 0.6452	p = 0.5988	
Gabra5 +/+ vs Gabra5 -/-	p = 0.0043			
Gabra5 -/+ vs Gabra5 -/-	p = 0.0710			
	total intensity (arb. u./μm ²): PSD95 cluster in colocalization with synaptophysin	total intensity (arb. u./μm ²): Synaptophysin cluster in colocalization with PSD95	total area (px/μm ²): PSD95 cluster in colocalization with synaptophysin	total area (px/μm ²): Synaptophysin cluster in colocalization with PSD95
Gabra5 +/+	mean ± SEM = 176.4 ± 14.03	mean ± SEM = 266.2 ± 27.01	mean ± SEM = 3.252 ± 0.1942	mean ± SEM = 3.847 ± 0.3270
Gabra5 -/+	mean ± SEM = 196.7 ± 24.44	mean ± SEM = 306.1 ± 27.20	mean ± SEM = 3.733 ± 0.3803	mean ± SEM = 4.620 ± 0.4556
Gabra5 -/-	mean ± SEM = 200.8 ± 17.35	mean ± SEM = 307.6 ± 16.51	mean ± SEM = 4.434 ± 0.3074	mean ± SEM = 5.481 ± 0.3731
Gabra5 +/+ vs Gabra5 -/+	p = >0.9999	p = 0.5225	p = 0.6045	p = 0.4141
Gabra5 +/+ vs Gabra5 -/-	p = >0.9999	p = 0.1442	p = 0.0214	p = 0.0114
Gabra5 -/+ vs Gabra5 -/-	p = >0.9999	p = >0.9999	p = 0.2856	p = 0.3210

Table 2: Exact p-values and values for mean $\pm$ SEM for Figures 18, 20, 21, 23, and 25

Fig. 18	cluster density (cluster/ 10 μm²): triple-colocalization	cluster density (cluster/μm²): α5-containing GABA _A R	cluster density (cluster/μm²): GluR2	cluster density (cluster/μm²): VGLUT1	cluster density (cluster/ 3 μm²): colocalization of GluR2 and VGLUT1
<i>Gabra5</i> +/+	mean ± SEM = 0.4514 ± 0.06024	mean ± SEM = 0.7220 ± 0.02839	mean ± SEM = 0.9240 ± 0.06186	mean ± SEM = 0.4428 ± 0.03381	mean ± SEM = 0.6250 ± 0.07408
<i>Gabra5</i> -/-	mean ± SEM = 0.03345 ± 0.006043	mean ± SEM = 0.07567 ± 0.006153	mean ± SEM = 1.011 ± 0.06150	mean ± SEM = 0.5120 ± 0.03344	mean ± SEM = 0.7230 ± 0.06744
<i>Gabra5</i> +/+ vs <i>Gabra5</i> -/-	p = <0.0001	p = <0.0001	p = 0.3246	p = 0.1105	p = 0.2118
	total intensity (arb. u./μm²): GluR2	total area (px/μm²): GluR2	total intensity (arb. u./μm²): VGLUT1	total area (px/μm²): VGLUT1	cluster density (cluster/ 3 μm²): colocalization of α5-containing GABA _A Rs and GluR2
<i>Gabra5</i> +/+	mean ± SEM = 488.5 ± 42.33	mean ± SEM = 10.73 ± 1.023	mean ± SEM = 614.6 ± 54.49	mean ± SEM = 7.055 ± 0.6336	mean ± SEM = 0.8573 ± 0.07052
<i>Gabra5</i> -/-	mean ± SEM = 587.5 ± 52.78	mean ± SEM = 12.00 ± 1.079	mean ± SEM = 773.0 ± 54.71	mean ± SEM = 8.272 ± 0.5607	
<i>Gabra5</i> +/+ vs <i>Gabra5</i> -/-	p = 0.1489	p = 0.3918	p = 0.0296	p = 0.0654	

Fig. 20	cluster density (cluster/μm²): α5-containing GABA _A R (<i>Gabra5</i> +/+)	Fig. 20	cluster density (cluster/μm²): VGLUT1 (DIV10)	cluster density (cluster/μm²): VGLUT1 (DIV 12)
DIV 10	mean ± SEM = 0.7143 ± 0.03426	<i>Gabra5</i> +/+	mean ± SEM = 0.4207 ± 0.02814	mean ± SEM = 0.4727 ± 0.02815
DIV 12	mean ± SEM = 0.8293 ± 0.02796	<i>Gabra5</i> -/-	mean ± SEM = 0.4953 ± 0.04030	mean ± SEM = 0.6370 ± 0.04261
DIV 10 vs DIV 12	p = 0.0122	<i>Gabra5</i> +/+ vs <i>Gabra5</i> -/-	p = 0.2809	p = 0.0021
	total intensity (arb. u./μm²): VGLUT1 (DIV 10)		total area (px/μm²): VGLUT1 (DIV 10)	total area (px/μm²): VGLUT1 (DIV 12)
<i>Gabra5</i> +/+	mean ± SEM = 445.9 ± 35.50		mean ± SEM = 5.951 ± 0.4278	mean ± SEM = 7.233 ± 0.4989
<i>Gabra5</i> -/-	mean ± SEM = 5184 ± 481.5		mean ± SEM = 6.607 ± 0.5449	mean ± SEM = 10.76 ± 0.8729
<i>Gabra5</i> +/+ vs <i>Gabra5</i> -/-	p = 2609		p = 0.3445	p = 0.0009

Fig. 21	cluster density (cluster/μm²): α5-containing GABA _A R	cluster density (cluster/μm²): VGAT	cluster density (cluster/μm²): VGLUT1
+/+	mean ± SEM = 0.9450 ± 0.03630	mean ± SEM = 0.3983 ± 0.03069	mean ± SEM = 0.6473 ± 0.04632
	cluster density (cluster/ 3 μm²): colocalization of α5-containing GABA _A R and VGAT	cluster density (cluster/ 3 μm²): colocalization of α5-containing GABA _A R and VGLUT1	cluster density (cluster/ 3 μm²): colocalization of VGAT and VGLUT1
+/+	mean ± SEM = 0.4277 ± 0.04362	mean ± SEM = 0.5573 ± 0.05541	mean ± SEM = 0.3880 ± 0.04339
	cluster density (cluster/ 10 μm²): colocalization of α5-containing GABA _A R, PSD95, and synaptophysin	cluster density (cluster/ 10 μm²): colocalization of α5-containing GABA _A R, GluR2, and VGLUT1	cluster density (cluster/ 10 μm²): colocalization of α5-containing GABA _A R, VGAT, and VGLUT1
+/+	mean ± SEM = 0.7070 ± 0.05515	mean ± SEM = 0.4514 ± 0.06024	mean ± SEM = 0.3310 ± 0.05203

Fig. 23	cluster density (cluster/μm²): α2-containing GABA _A R	cluster density (cluster/μm²): PSD95	cluster density (cluster/μm²): VGLUT1
+/+	mean ± SEM = 0.8847 ± 0.06310	mean ± SEM = 0.6880 ± 0.03266	mean ± SEM = 0.6263 ± 0.03793
	cluster density (cluster/ 3 μm²): colocalization of α2-containing GABA _A R and PSD95	cluster density (cluster/ 3 μm²): colocalization of α2-containing GABA _A R and VGLUT1	cluster density (cluster/ 3 μm²): colocalization of PSD95 and VGLUT1
+/+	mean ± SEM = 0.7113 ± 0.07138	mean ± SEM = 0.7183 ± 0.08017	mean ± SEM = 0.6120 ± 0.04566

Fig. 25	cluster density (cluster/ 10 μm²): triple-colocalization of α5-containing GABA _A R, PSD95, and synaptophysin	cluster density (cluster/μm²): α5-containing GABA _A R	cluster density (cluster/μm²): PSD95	cluster density (cluster/μm²): Synaptophysin	cluster density (cluster/ 3 μm²): colocalization of α5-containing GABA _A R and PSD95						
<i>RDX</i> +/+	mean ± SEM = 0.9180 ± 0.1074	mean ± SEM = 0.9497 ± 0.03570	mean ± SEM = 0.7780 ± 0.04352	mean ± SEM = 0.6593 ± 0.04728	mean ± SEM = 0.8547 ± 0.06713						
<i>RDX</i> -/-	mean ± SEM = 1.013 ± 0.1155	mean ± SEM = 1.187 ± 0.05236	mean ± SEM = 0.9037 ± 0.04450	mean ± SEM = 0.7173 ± 0.06328	mean ± SEM = 1.060 ± 0.07430						
<i>RDX</i> +/+ vs <i>RDX</i> -/-	p = 0.5594	p = 0.0004	p = 0.0481	p = 0.4657	p = 0.0445						
	cluster density (cluster/ 3 μm²): colocalization of α5-containing GABA _A R and synaptophysin	cluster density (cluster/ 3 μm²): colocalization of PSD95 and Synaptophysin	total intensity (arb. u./μm²): α5-containing GABA _A R	Fig. 25 α5-GABA_AR + PSD95 <table><tr><th>Genotype</th><th>Cluster Density (cluster/ 3 μm²)</th></tr><tr><td><i>RDX</i> +/+</td><td>0.8547 ± 0.06713</td></tr><tr><td><i>RDX</i> -/-</td><td>1.060 ± 0.07430</td></tr></table>		Genotype	Cluster Density (cluster/ 3 μm²)	<i>RDX</i> +/+	0.8547 ± 0.06713	<i>RDX</i> -/-	1.060 ± 0.07430
Genotype	Cluster Density (cluster/ 3 μm²)										
<i>RDX</i> +/+	0.8547 ± 0.06713										
<i>RDX</i> -/-	1.060 ± 0.07430										
<i>RDX</i> +/+	mean ± SEM = 0.7187 ± 0.06649	mean ± SEM = 0.8913 ± 0.08407	mean ± SEM = 368.9 ± 25.14								
<i>RDX</i> -/-	mean ± SEM = 0.8700 ± 0.08032	mean ± SEM = 0.9690 ± 0.1026	mean ± SEM = 441.7 ± 26.15								
<i>RDX</i> +/+ vs <i>RDX</i> -/-	p = 0.1390	p = 0.5605	p = 0.0495								
	total intensity (arb. u./μm²): PSD95	total intensity (arb. u./μm²): Synaptophysin									
<i>RDX</i> +/+	mean ± SEM = 274.0 ± 26.85	mean ± SEM = 481.4 ± 35.95									
<i>RDX</i> -/-	mean ± SEM = 299.5 ± 29.43	mean ± SEM = 601.4 ± 74.94									
<i>RDX</i> +/+ vs <i>RDX</i> -/-	p = 0.6228	p = 0.1542									

Table 3: Functions and locations of the 45 differential proteins identified by Mass Spectrometry. Radixin wildtype- and knockout-derived hippocampi (31–40 weeks age) mice brain lysates enriched with the plasma membrane components (P2) were used for the IPs. The proteins are sorted according to the highest to lowest fold change in Table 1. The number for kDa, functions, and location are based on UniProt (<http://www.uniprot.org>).

Nr.	Protein name	kDa	Function	location
1	Brevican core protein	96	May play a role in the terminally differentiating and the adult nervous system during postnatal development. Could stabilize interactions between hyaluronan (HA) and brain proteoglycans.	Cell membrane
2	Protein bassoon	419	Scaffold protein of the presynaptic cytomatrix at the active zone (CAZ) (...); (...) participates in the formation of Golgi-derived membranous organelles termed Piccolo-Bassoon transport vesicles (PTVs) that are transported along axons to sites of nascent synaptic contacts; At the presynaptic active zone, regulates the spatial organization of synaptic vesicle cluster, the protein complexes that execute membrane fusion and compensatory endocytosis; Functions also in processes other than assembly such as the regulation of specific presynaptic protein ubiquitination (...); Mediates also synapse to nucleus communication (...)	Cell membrane
3	Disks large-associated protein 1	110	Part of the postsynaptic scaffold in neuronal cells.	Cell membrane
4	Calcium/calmodulin-dependent protein kinase type II subunit alpha	54	Calcium/calmodulin-dependent protein kinase that functions autonomously after Ca^{2+} /calmodulin-binding and autophosphorylation, and is involved in various processes, such as synaptic plasticity, neurotransmitter release and long-term potentiation (By similarity); Member of the NMDAR signaling complex in excitatory synapses, it regulates NMDAR-dependent potentiation of the AMPAR and therefore excitatory synaptic transmission (By similarity); Regulates dendritic spine development (By similarity). ; Also regulates the migration of developing neurons (By similarity). ; (...)	Golgi apparatus membrane, Cytoplasm
5	Dihydropyrimidinase-related protein 2	62	Plays a role in neuronal development and polarity, as well as in axon growth and guidance, neuronal growth cone collapse and cell migration. Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. May play a role in endocytosis.	Cell membrane, Melanosome, Cytoplasm, synaptic vesicle membrane, clathrin-coated vesicle membrane
6	Radixin	69	Probably plays a crucial role in the binding of the barbed end of actin filaments to the plasma membrane.	extracellular matrix, Protein located outside the cell membrane
7	Tenascin-R	150	Neural extracellular matrix (ECM) protein involved in interactions with different cells and matrix components. These interactions can influence cellular behavior by either evoking a stable adhesion and differentiation, or repulsion and inhibition of neurite growth. (...)	Cytoplasm, presynaptic active zone, cytoskeleton, synaptic vesicle membrane
8	Leucine-rich repeat and immunoglobulin-like domain containing-NOGO receptor-interacting protein 4	64	no function mentioned on uniprot	Protein located outside the cell membrane
9	Myelin proteolipid protein	30	This is the major myelin protein from the central nervous system. It plays an important role in the formation or maintenance of the multilamellar structure of myelin.	Endoplasmic reticulum membrane, Golgi apparatus, Protein located outside the cell membrane, Melanosome, Sarcoplasmic reticulum lumen
10	Keratin, type II cytoskeletal 71	57	Plays a central role in hair formation. Essential component of keratin intermediate filaments in the inner root sheath (IRS) of the hair follicle.	Synapse, postsynaptic density, dendritic spine, dendrite, cytoplasm
11	Vesicular integral-membrane protein VIP36	40	Plays a role as an intracellular lectin in the early secretory pathway. Interacts with N-acetyl-D-galactosamine and high-mannose type glycans and may also bind to O-linked glycans. Involved in the transport and sorting of glycoproteins carrying high mannose-type glycans (By similarity).	Cytoskeleton
12	IQ motif and SEC7 domain-containing protein 2	162	Is a guanine nucleotide exchange factor for the ARF GTP-binding proteins.	Cytoskeleton, centrosome
13	Interleukin enhancer-binding factor 3	96	RNA-binding protein that plays an essential role in the biogenesis of circular RNAs (circRNAs) (...). As a consequence, participates in a wide range of transcriptional and post-transcriptional processes. Binds to poly-U elements and AU-rich elements (AREs) in the 3'-UTR of target mRNAs (By similarity).; Upon viral infection, ILF3 accumulates in the cytoplasm and participates in the innate antiviral response. (...)	Mitochondrion, nucleus, cell membrane
14	Calumenin	37	Involved in regulation of vitamin K-dependent carboxylation of multiple N-terminal glutamate residues. Seems to inhibit gamma-carboxylase GGCX. Binds 7 calcium ions with a low affinity (By similarity).	Cell membrane, postsynaptic density, cytoplasm, axon, dendritic spine, dendrite
15	SH3 and multiple ankyrin repeat domains protein 1	226	Seems to be an adapter protein in the postsynaptic density (PSD) of excitatory synapses that interconnects receptors of the postsynaptic membrane including NMDA-type and metabotropic glutamate receptors, and the actin-based cytoskeleton. Plays a role in the structural and functional organization of the dendritic spine and synaptic junction. Overexpression promotes maturation of dendritic spines and the enlargement of spine heads via its ability to recruit Homer to postsynaptic sites, and enhances presynaptic function (By similarity).	cell membrane, postsynaptic density, synapse
16	Spectrin beta chain, non-erythrocytic 1	274	Fodrin, which seems to be involved in secretion, interacts with calmodulin in a calcium-dependent manner and is thus candidate for the calcium-dependent movement of the cytoskeleton at the membrane. Plays a critical role in central nervous system development and function.	Cytosol, cytoskeleton, cell membrane
17	Inter-alpha-trypsin inhibitor heavy chain H3	99	May act as a carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix protein, including those on cell surfaces in tissues to regulate the localization, synthesis and degradation of hyaluronan which are essential to cells undergoing biological processes.	Cytoskeleton
18	Carbamoyl-phosphate synthase [ammonia], mitochondrial	165	Involved in the urea cycle of ureotelic animals where the enzyme plays an important role in removing excess ammonia from the cell.	Cytoplasm, Nucleus, Stress granule
19	AP-2 complex subunit alpha-1	108	Component of the adaptor protein complex 2 (AP-2) (...); AP-2 is involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin-coated vesicles, CCVs) which are destined for fusion with the early endosome. (...); AP-2 also serves as a cargo receptor to selectively sort the membrane proteins involved in receptor-mediated	Protein located outside the cell membrane

			endocytosis. ; AP-2 seems to play a role in the recycling of synaptic vesicle membranes from the presynaptic surface. (...); AP-2 may also play a role in maintaining normal post-endocytic trafficking through the ARF6-regulated, non-clathrin pathway. (...); AP-2 is responsible for the endocytosis of ADAM10.; The AP-2 alpha subunit acts via its C-terminal appendage domain as a scaffolding platform for endocytic accessory proteins. (...);	
20	Spectrin alpha chain, non-erythrocytic 1	285	Fodrin, which seems to be involved in secretion, interacts with calmodulin in a calcium-dependent manner and is thus candidate for the calcium-dependent movement of the cytoskeleton at the membrane.	Nucleus

Nr.	Protein name	kDa	Function	location
21	Eukaryotic translation initiation factor 4 gamma 1	176	Component of the protein complex eIF4F, which is involved in the recognition of the mRNA cap, ATP-dependent unwinding of 5'-terminal secondary structure and recruitment of mRNA to the ribosome. (...)	Nucleus, nucleolus , cytoplasm
22	Septin-11	50	Filament-forming cytoskeletal GTPase. May play a role in cytokinesis (Potential). May play a role in the cytoarchitecture of neurons, including dendritic arborization and dendritic spines, and in GABAergic synaptic connectivity (By similarity).	Cytoplasm
23	Cytoplasmic dynein 1 heavy chain 1	532	Cytoplasmic dynein 1 acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules. Dynein has ATPase activity; the force-producing power stroke is thought to occur on release of ADP. Plays a role in mitotic spindle assembly and metaphase plate congression.	Protein located outside the cell membrane
24	Histone H1.0	21	Histones H1 are necessary for the condensation of nucleosome chains into higher-order structures. The histones H1.0 are found in cells that are in terminal stages of differentiation or that have low rates of cell division.	Cytoskeleton
25	Disks large homolog 4	80	Postsynaptic scaffolding protein that plays a critical role in synaptogenesis and synaptic plasticity by providing a platform for the postsynaptic clustering of crucial synaptic proteins (...); Interacts with the cytoplasmic tail of NMDA receptor subunits and shaker-type potassium channels. Required for synaptic plasticity associated with NMDA receptor signaling. Overexpression or depletion of DLG4 changes the ratio of excitatory to inhibitory synapses in hippocampal neurons. ; May reduce the amplitude of ASIC3 acid-evoked currents by retaining the channel intracellularly. ; May regulate the intracellular trafficking of ADR1B. ; Also regulates AMPA-type glutamate receptor (AMPA) immobilization at postsynaptic density keeping the channels in an activated state in the presence of glutamate and preventing synaptic depression (Probable). ; Under basal conditions, cooperates with FYN to stabilize palmitoyltransferase ZDHHC5 at the synaptic membrane through FYN-mediated phosphorylation of ZDHHC5 and its subsequent inhibition of association with endocytic proteins (By similarity).	Cell membrane
26	AP-3 complex subunit delta-1	135	Part of the AP-3 complex, an adaptor-related complex which is not clathrin-associated. The complex is associated with the Golgi region as well as more peripheral structures. It facilitates the budding of vesicles from the Golgi membrane and may be directly involved in trafficking to lysosomes (By similarity); Involved in process of CD8+ T-cell and NK cell degranulation (By similarity); In concert with the BLOC-1 complex, AP-3 is required to target cargos into vesicles assembled at cell bodies for delivery into neurites and nerve terminals (...).	Golgi apparatus membrane
27	Fibroleukin	49	Converts prothrombin to thrombin.	Cytoskeleton, dendrite
28	Neurofilament medium polypeptide	96	Neurofilaments usually contain three intermediate filament proteins: NEFL, NEFM, and NEFH which are involved in the maintenance of neuronal caliber. May additionally cooperate with the neuronal intermediate filament proteins PRPH and INA to form neuronal filamentous networks (...).	Nucleus, cytoplasm
29	Transcriptional activator protein Pur-beta	34	Transcriptional regulator which can act as an activator or a repressor (...); Represses the transcription of ACTA2 in fibroblasts and smooth muscle cells via its ability to interact with the purine-rich strand of a MCAT-containing element in the 5' flanking region of the gene (...); Represses the transcription of MYOCD, capable of repressing all isoforms of MYOCD but the magnitude of the repressive effects is most notable for the SMC-specific isoforms (...); Promotes hepatic glucose production by activating the transcription of ADCY6, leading to cAMP accumulation, increased PKA activity, CREB activation, and increased transcription of PCK1 and G6PC genes (...); Has capacity to bind repeated elements in single-stranded DNA such as the purine-rich single strand of the PUR element located upstream of the MYC gene (By similarity); Participates in transcriptional and translational regulation of alpha-MHC expression in cardiac myocytes by binding to the purine-rich negative regulatory (PNR) element (By similarity); Modulates constitutive liver galectin-3 gene transcription by binding to its promoter (By similarity); May play a role in the dendritic transport of a subset of mRNAs (By similarity).	Axon, Cytoskeleton
30	Complement C1q subcomponent subunit C	26	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system. (...)	Axon, Cytoskeleton
31	Condensin complex subunit 1	156	Regulatory subunit of the condensin complex, a complex required for conversion of interphase chromatin into mitotic-like condense chromosomes. (...); May target the condensin complex to DNA via its C-terminal domain; May promote the resolution of double-strand DNA catenanes (intertwines) between sister chromatids (...); Early in neurogenesis, may play an essential role to ensure accurate mitotic chromosome condensation in neuron stem cells, ultimately affecting neuron pool and cortex size.	Cytoplasm
32	Microtubule-associated protein 2	199	The exact function of MAP2 is unknown but MAPs may stabilize the microtubules against depolymerization. They also seem to have a stiffening effect on microtubules.	Endoplasmic reticulum lumen, cell membrane, melanosome
33	NEFL protein	62	Neurofilaments usually contain three intermediate filament proteins: NEFL, NEFM, and NEFH which are involved in the maintenance of neuronal caliber. May additionally cooperate with the neuronal intermediate filament proteins PRPH and INA to form neuronal filamentous networks (...).	Cell membrane, myelin membrane
34	Tubulin beta-2A chain	50	Tubulin is the major constituent of microtubules, a cylinder consisting of laterally associated linear protofilaments composed of alpha- and beta-tubulin heterodimers. Microtubules grow by the addition of GTP-tubulin dimers to the microtubule end, where a stabilizing cap forms. Below the cap, tubulin dimers are in GDP-bound state, owing to GTPase activity of alpha-tubulin.	Nucleus
35	ADP/ATP translocase 2	33	ADP:ATP antiporter that mediates import of ADP into the mitochondrial matrix for ATP synthesis, and export of ATP out to fuel the cell (...). Cycles between the cytoplasmic-open state (c-state) and the matrix-open state (m-state): (...). In addition to its ADP:ATP antiporter activity, also involved in mitochondrial uncoupling and mitochondrial permeability transition pore (mPTP) activity (...). Plays a role in mitochondrial uncoupling by acting as a proton transporter: (...). Proton transporter activity is inhibited by ADP:ATP antiporter activity, suggesting that SLC25A5/ANT2 acts as a master regulator of mitochondrial energy output by maintaining a delicate balance between ATP	cell membrane, cytoskeleton, microvillus

			production (ADP:ATP antiporter activity) and thermogenesis (proton transporter activity (...)). Probably mediates mitochondrial uncoupling in tissues that do not express UCP1 (...). Also plays a key role in mPTP opening, (...). It is however unclear if SLC25A5/ANT2 constitutes a pore-forming component of mPTP or regulates it (...). Acts as a regulator of mitophagy independently of ADP:ATP antiporter activity: (...).; As part of the mitotic spindle-associated MMXD complex it may play a role in chromosome segregation (By similarity).	
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Nr.	Protein name	kDa	Function	location
36	AP-2 complex subunit beta	105	Component of the adaptor protein complex 2 (AP-2). (...);AP-2 is involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin-coated vesicles, CCVs) which are destined for fusion with the early endosome. (...); AP-2 also serves as a cargo receptor to selectively sort the membrane proteins involved in receptor-mediated endocytosis. (...); AP-2 may also play a role in maintaining normal post-endocytic trafficking through the ARF6-regulated, non-clathrin pathway.; During long-term potentiation in hippocampal neurons, AP-2 is responsible for the endocytosis of ADAM10 (...); The AP-2 beta subunit acts via its C-terminal appendage domain as a scaffolding platform for endocytic accessory proteins; (...); The AP-2 beta subunit binds to clathrin heavy chain, promoting clathrin lattice assembly; clathrin displaces at least some CLASPs from AP2B1 which probably then can be positioned for further coat assembly (By similarity).	Cytoplasm, cytoskeleton, synapse, dendritic spine, axon
37	Protein disulfide-isomerase A6	48	May function as a chaperone that inhibits aggregation of misfolded proteins (...); Negatively regulates the unfolded protein response (UPR) through binding to UPR sensors such as ERN1, which in turn inactivates ERN1 signaling (By similarity).; May also regulate the UPR via the EIF2AK3 UPR sensor (By similarity).; Plays a role in platelet aggregation and activation by agonists such as convulxin, collagen and thrombin (By similarity).	Cytoplasm, synapse, postsynaptic density
38	Bifunctional phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase	47	Bifunctional phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazole succinocarboxamide synthetase catalyzing two reactions of the de novo purine biosynthetic pathway.	Mitochondrion inner membrane, may localize to non-mitochondrial membranes
39	T-complex protein 1 subunit epsilon	60	Component of the chaperonin-containing T-complex (TRiC), a molecular chaperone complex that assists the folding of proteins upon ATP hydrolysis.; The TRiC complex mediates the folding of WRAP53/TCAB1, thereby regulating telomere maintenance.; As part of the TRiC complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia.; The TRiC complex plays a role in the folding of actin and tubulin.	Cytoskeleton, cell cortex
40	Alpha-actinin-1	103	F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures. This is a bundling protein (By similarity)	Cytoskeleton, myofibril, cytosol, cell membrane
41	V-type proton ATPase subunit B, brain isoform	57	Non-catalytic subunit of the V1 complex of vacuolar(H ⁺)-ATPase (V-ATPase), a multisubunit enzyme composed of a peripheral complex (V1) that hydrolyzes ATP and a membrane integral complex (V0) that translocates protons (...); V-ATPase is responsible for acidifying and maintaining the pH of intracellular compartments and in some cell types, is targeted to the plasma membrane, where it is responsible for acidifying the extracellular environment (By similarity).; In renal intercalated cells, can partially compensate the lack of ATP6V1B1 and mediate secretion of protons (H ⁺) into the urine under base-line conditions but not in conditions of acid load (...);	Extracellular matrix
42	Tubulin alpha-4A chain	50	Tubulin is the major constituent of microtubules, a cylinder consisting of laterally associated linear protofilaments composed of alpha- and beta-tubulin heterodimers. Microtubules grow by the addition of GTP-tubulin dimers to the microtubule end, where a stabilizing cap forms. Below the cap, tubulin dimers are in GDP-bound state, owing to GTPase activity of alpha-tubulin.	Cytoskeleton
43	Thioredoxin reductase 1, cytoplasmic	67	Reduces disulfideprotein thioredoxin (Trx) to its dithiol-containing form. Homodimeric flavoprotein involved in the regulation of cellular redox reactions, growth and differentiation. Contains a selenocysteine residue at the C-terminal active site that is essential for catalysis. Also has reductase activity on hydrogen peroxide (H2O2).	Cytoskeleton
44	Tubulin alpha-1B chain	50	Tubulin is the major constituent of microtubules, a cylinder consisting of laterally associated linear protofilaments composed of alpha- and beta-tubulin heterodimers. Microtubules grow by the addition of GTP-tubulin dimers to the microtubule end, where a stabilizing cap forms. Below the cap, tubulin dimers are in GDP-bound state, owing to GTPase activity of alpha-tubulin	Cytoskeleton
45	F-actin-capping protein subunit alpha-1	33	F-actin-capping proteins bind in a Ca ²⁺ -independent manner to the fast growing ends of actin filaments (barbed end) thereby blocking the exchange of subunits at these ends. Unlike other capping proteins (such as gelsolin and severin), these proteins do not sever actin filaments. May play a role in the formation of epithelial cell junctions (By similarity).; Forms, with CAPZB, the barbed end of the fast growing ends of actin filaments in the dynactin complex and stabilizes dynactin structure. The dynactin multiprotein complex activates the molecular motor dynein for ultra-processive transport along microtubules (By similarity).	Cytoplasm

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