Aus dem Lehrstuhl Anatomie III - Zellbiologie
Biomedizinisches Zentrum
Ludwig-Maximilians-Universität München
Vorstand: Prof. Dr. Michael A. Kiebler

THE ROLE OF STAUFEN 2 AND PUMILIO 2 IN HIPPOCAMPUS BASED LEARNING

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Antonia Franziska Demleitner
aus Regensburg

2021

Mit Genehmigung der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

Berichterstatter:	Prof. Dr. rer. nat. Michael A. Kiebler
Mitberichterstatter:	PD Dr. Swetlana Sirko PD Dr. Florence Bareyre
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. Bastian A. Popper
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung:	15.07.2021

Parts of this thesis have already been published in:

Follwaczny P. & Schieweck R., Riedemann T., Demleitner A., Straub T., Klemm AH, Bilban M., Sutor B., Popper B. & Kiebler MA (2017) *Deficiency of the RNA-binding protein Pumilio2 determines predisposition for epilepsy in mice*. Disease Models and Mechanisms 10:1333-1342.

Popper B. & Demleitner A., Bolivar VJ, Kusek G., Snyder-Keller A., Schieweck R., Temple S., Kiebler MA (2018) *Staufen2 deficiency leads to impaired response to novelty in mice.* Neurobiology of Learning and Memory 150:107-115.

Schieweck R., Riedemann T., Forné I., Harner M., Bauer K. E., Rieger D., Ang F.-Y., Hutten S., Demleitner A. F., Popper B., Derdak S., Sutor B., Bilban M., Imhof A. and Kiebler M. A. (2021) *Pumilio2 and Staufen2 selectively balance the synaptic proteome*. Cell Reports 35:109279.

Für meine Eltern

Table of Content

Abbreviations

3’-UTR	3'-Untranslated Region
AMPA	α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid
ASD	Autism Spectrum Disorders
BrdU	Bromodeoxyuridine
Calm3	Calmodulin 3
CaMKIIa	Ca2+/Calmodulin dependent Kinase II subunit alpha
CNS	Central Nervous System
CREB-1	cAMP Response Element Binding Protein 1
DCX	Doublecortin
DI	Discrimination Index
eIF-4E	translations initiations factor 4E
fEPSP	evoked field Excitatory Postsynaptic Potential
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GABA	γ-Aminobutyric Acid
IPC	Intermediate Progenitor Cells
LTD	Long Term Depression
LTP	Long Term Potentiation
Map1b	Microtubule Associated Protein 1B
MAPK	Mitogen-Activated Protein Kinase
mEPSP	miniature Excitatory Postsynaptic Potential
mGluR-LTD	protein synthesis dependent LTD
mRNA	messenger RNA
NMDA	N-Methyl-D-aspartate
NOL	Novel Object Location recognition
NOR	Novel Object Recognition
NRE	Nanos Responsive Element
OF	Open Field
p0, p21	postnatal day 0, 21
PKA	Protein Kinase A
Prox1	Prospero Homeobox Protein 1
PSD	Post-Synaptic Density
PUF	Pumilio and FBF
Pum1	Pumilio 1
Pum2	Pumilio 2
qRT-PCR	quantitative Real-Time PCR
RBP	RNA Binding Protein
RGC	Radial Glial Cell
Rgs4	Regulator of G protein Signaling 4
RNP	Ribonucleoprotein particle
SGZ	Subgranular Zone
Stau1	Staufen 1
Stau2	Staufen 2
TLE	Temporal Lobe Epilepsy
WT	Wildtype

List of Figures

List of Tables

Abstract English

The formation and consolidation of new memories relies upon a process called synaptic plasticity. Hereby, single polarized neurons, which form dendritic trees as input region and an axon as output region, respond and adapt to various stimuli. Depending on the duration and interval of a train of stimuli it receives, a synapse will be either strengthened or weakened, thereby altering circuit activity and memories contained in that circuit. During this process, localized mRNAs will be locally translated. Here, RNA-binding proteins (RBPs), which are able to regulate both dendritic localization and subsequent translation of their target mRNAs, have evolved as important players. Many RBPs have been associated with neurological diseases such as mental retardation, autism and epilepsy, highlighting their crucial role in circuit homeostasis. Staufen 2 (Stau2) and Pumilio 2 (Pum2) are two RBPs, which have been implicated in embryonal neurogenesis, dendritic spine morphogenesis as well as hippocampus associated long-term memory in various species.

The aim of my project was to gain insight into the role of Stau2 and Pum2 in hippocampal memory. For this, I used single and double Stau2 and Pum2 knock-down mouse models. I exploited extensive behavioral memory tests, immunohistochemistry of adult neurogenesis as well as molecular biological assays to screen for possible effects of the knock-down of either Stau2, Pum2 or both. Together, my data strongly suggest that Stau2 and Pum2 balance hippocampal-dependent memory.

Stau2 knock-down impairs hippocampus-dependent spatial long-term memory as well as novelty detection, which relies on proper function of not only the hippocampus but also para-hippocampal structures such as the perirhinal and medial pre-frontal cortex. Importantly, adult neurogenesis is unchanged in naïve $Stau2^{KD}$ mice and upregulated upon stimulation via behavioral testing. In Pum2 deficient mice, hippocampal memory is impaired. Adversely, adult neurogenesis is impaired in naïve mice, and increases after stimulation by behavioral testing. Strikingly, $Stau2^{KD}/Pum2^{KD}$ animals show unaltered hippocampal memory. Adult neurogenesis levels in naïve mice are comparable to those found in $Pum2^{KD}$ animals but remain unchanged upon stimulation. Taken together, my results highlight the role of both Stau2 and Pum2 in hippocampus-dependent memory. Stau2 and Pum2, however, serve functions that are rescued upon knock-down of both proteins, suggesting a balancing role in a pathway that is also mediated by other proteins. Unravelling the underlying mechanisms will therefore be key to better understand the role of Stau2 and Pum2 in hippocampal memory and their role in neuropsychiatric diseases like autism spectrum disorders or other types of mental retardation.

Zusammenfassung Deutsch

Für die Bildung und Konsolidation von neuen Gedächtnisinhalten ist der Prozess, der als synaptische Plastizität bekannt ist, von elementarer Bedeutung. Im Rahmen dieses Prozesses antworten einzelne polarisierte Neuronen, die einen Dendritenbaum als Eingangsregion und ein Axon als Ausgangsregion für und von Informationen bilden, auf Stimuli und passen sich diesen an. Abhängig von Dauer und Intervall der Einwirkung einer Reihe von Stimuli, kommt es entweder zur Kräftigung oder Schwächung von Synapsen. Diese Anpassung verändert die Aktivität in neuronalen Netzwerken und dadurch die Gedächtnisinhalte, die in diesen gespeichert sind. RNA Bindeproteine (RBPs), die die Fähigkeit haben, die Lokalisation und Translation ihrer Ziel-mRNAs zu beeinflussen, haben sich als wichtige Faktoren in diesem Feld etabliert. Sie sind auf einzigartige Weise dazu fähig, mRNAs und Proteine zu bestimmten Synapsen zu transportieren. Viele RBPs wurde bereits mit neurologischen Krankheiten wie geistiger Retardierung, Autismus und Epilepsie in Verbindung gebracht. Dies unterstreicht ihre wichtige Rolle in der Balance von neuronalen Netzwerken. Staufen 2 (Stau2) und Pumilio 2 (Pum2) sind zwei RBPs, die mit embryonaler Neurogenese, der Ausbildung von synaptischen Verbindungen (*dendritic spine morphogenesis*) sowie in mehreren Spezies mit hippocampus-abhängigem Lernen assoziiert wurden.

Das Ziel meines Projektes war es, Einblick in die Rolle, die Stau2 und Pum2 im hippocampalen Gedächtnis spielen, zu bekommen. Um dies zu erreichen, verwendete ich Einzel- und Doppel Stau2 und Pum2 Knock-Down Mauslinien. Ich führte umfangreiche Verhaltenstest durch, die das Gedächtnis der Tiere prüfen sollten. Zudem analysierte ich die adulte Neurogenese der Tiere mit Hilfe von immunhistochemischen Färbungen und quantifizierte gehirnspezifische Level von ausgewählten Ziel-mRNAs, um mögliche Effekte des Knock-Downs von sowohl Stau2 als auch Pum2 zu sehen. Zusammenfassend legen meine Daten nahe, dass Stau2 und Pum2 ausgleichende Rollen im hippocampalen Gedächtnis spielen. Die Herabregulierung von Stau2 führt zu Defiziten im hippocampus-abhängigen Langzeitgedächtnis sowie in der Erkennung von neuen Objekten. Besonders Letzteres ist auch auf die regelrechte Funktion einiger parahippocampaler Strukturen wie des perirhinalen und des medialen präfrontalen Kortex angewiesen. Tatsächlich zeigte sich die adulte Neurogenese in naiven Stau2KD Tieren unverändert, wohingegen sie nach der Stimulation durch Verhaltenstest hochreguliert war. Pum2 Knock-down Mäuse zeigen Defizite im hippocampus-abhängigen Lernen. Die adulte Neurogenese zeigte sich in naiven Tieren erniedrigt, ließ sich aber durch Verhaltenstest stimulieren. Interessanterweise ist in Stau2KD/Pum2KD Tieren das

räumliche Langzeitgedächtnis nicht beeinträchtigt und ist die Erkennung von neuen Objekten nur gering verschlechtert. Die Level von adulter Neurogenese lassen sich mit denen von Pum2KD Mäusen vergleichen, verändern sich allerdings nicht durch Stimulation durch Verhaltenstests. Zusammenfassend lässt sich sagen, dass meine Ergebnisse die Rolle von sowohl Stau2 als auch Pum2 im hippocampalen Gedächtnis hervorheben. Allerdings nehmen Stau2 und Pum2 Funktionen ein, die durch Knock-Down von beiden Proteinen das hippocampale Gedächtnis nur gering beeinflussen. Dies legt die Vermutung nahe, dass beide Proteine eine balancierende Rolle in diesem Signalweg innehaben und letztendlich andere Proteine eine dominantere Rolle in diesem spielen. Die zugrundeliegenden Mechanismen dieses Signalweges aufzudecken, wird deshalb entscheidend dafür sein, die Rolle, die Stau2 und Pum2 im hippocampalen Gedächtnis spielen, besser zu verstehen. Dies wird hoffentlich auch dazu führen, dass ihre Rolle bei der Entstehung von neuropsychiatrischen Krankheiten wie Autismus oder anderer Formen der geistigen Retardierung besser nachvollzogen werden kann.

1 Introduction

How do we make new memories? Years of research have shown, the answer to this question is not easy. Eric Kandel, a Nobel laureate recipient for his work on signal transduction during memory formation in the nervous system, used a reductionist approach to the question by using a very simple model organism, *Aplysia* (Kandel, 2001). In *Aplysia*, a marine snail, many of the cells in its nervous system are big, even visible with the human eye. Learning processes may include fewer than 100 of the 20.000 nerve cells that make up its entire nervous system. The human brain has approximately 86 billion nerve cells, mice approximately 71 billion. Learning processes are far more intricate, requiring numerous neurons and synapses. And while it is still difficult at this point in time to understand learning processes in humans or rodents in detail, research on memory formation and function in rodents has steadily increased as they are more similar to us humans than *Aplysia* is (Kandel, 2001).

1.1 The role of the hippocampus in learning and memory

Memory requires changes at the synapse, the connection between singular nerve cells – a process called synaptic plasticity (Malenka and Bear, 2004). Synapses that are used more will be strengthened in response to increases in their activity, whereas those, who are not used will be weakened. On a wider perspective these processes lead to changes on neuronal circuit level thereby altering the wiring of the brain. Depending on where exactly those synapses, neurons and circuits are located new memories may be created or old memories will be altered.

Generally, the brain has two major types of memory: declarative (episodic) memory for facts, events, places and persons and nondeclarative (implicit memory) for perceptual and motor skills (Kandel et al., 2014). Special interest has been put into the process of acquiring episodic memory. A break-through in localizing this aspect of memory was made thanks to a patient widely known as “H. M.”, who was suffering from intractable temporal lobe epilepsy since adolescence. In 1953, the neurosurgeon William Beecher Scoville removed most of H. M.’s left and right temporal lobes, including the majority of the hippocampal tissue (Scoville and Milner, 2000). Although his seizures were not halted completely, a at the time surprising finding resulted in him becoming famous. Unconfounded by any other mental disabilities, he lost the ability to acquire new episodic memories, in his mind staying the 27-year-old man he was the day before surgery. Importantly, other aspects of memory, especially implicit memory remained unaffected (Augustinack et al., 2014).

Since these first discoveries of the importance of the hippocampus in declarative memory, research has focused on rodents as model organisms for the different mechanistic processes behind this form of memory. Mainly two important findings were made: *O'Keefe et al.* discovered through electrophysiological recordings of alive and behaving rats that certain cells – later fittingly called "Place Cells" – are active when the animal is in a specific location and are no longer active if it leaves this area (O'Keefe and Dostrovsky, 1971). This marked the foundation of the discovery of the hippocampus' role in spatial memory. The second important discovery that occurred simultaneously was that of processes now known as Long- Term Potentiation (LTP) and Long- Term Depression (LTD) (Kandel et al., 2014). In brief, repetitive stimulation of certain pathways leads to strengthening of the corresponding synapses in response to their synaptic activity. We now distinguish an early phase of LTP, that responds to only limited trains of stimuli and therefore only retains short-term memory. The effects that are seen are caused by an increased incorporation of AMPA receptors, a well-known glutamate receptor subtype in the nervous system, into the postsynaptic density (PSD). Importantly, protein synthesis remains unaffected under these conditions (Kandel et al., 2014). Only when trains of stimuli are applied repetitively, the late phase of LTP is reached, wherein protein synthesis is initiated to yield long- term (days and longer) adaption of potentiated synapses thus resulting in long- term memory. Involved in this pathway are, among many others, the following kinases: Protein Kinase A (PKA), Mitogen-Activated Protein Kinase (MAPK) pathways signaling ultimately to the transcription factor termed cAMP response Element Binding Protein (CREB-1). This, in turn, modulates transcription and translation of specific target proteins (Kandel, 2001). Importantly, this is dependent upon the activation of NMDA receptors, another group of glutamate receptors, at the PSD and consequently of the Ca^{2+}/Calmodulin dependent Kinase II (CaMKII). Early studies have shown CaMKII deficient mice to have defective LTP leading to impaired performance in hippocampus based spatial memory tasks (Bach et al., 1995; Lisman et al., 2002). In turn, alteration or lack of stimuli can lead to weakening of synapses, a process now known as LTD (Gladding et al., 2009). An intricate balance between LTP and LTD, between strengthening and weakening of synapses is needed for proper acquisition and retention of new memories (Cooper and Bear, 2012). One form of LTD that is mediated by metabotropic glutamate receptors (mGluR) has been shown to be affected upon knock-down of the RNA-binding protein (RBP) Stau2 (Lebeau et al., 2011; Berger et al., 2017). Together, RBPs have emerged in recent times as important players in the field of synaptic plasticity.

1.2 The role of RBPs in neural function and disease

Research has revealed RBPs play important roles in the control of localization and translation of mRNAs in neurons (Kiebler and Bassell, 2006; Bramham and Wells, 2007). They do so by binding mRNAs, recognizing certain target sequences and structures preferentially in the 3'-untranslated region (3'-UTR), therefore influencing either the stability, localization and/or translational state of mRNAs. The precise role of RBPs is therefore highly dependent on the subset of mRNAs each one can bind. RBPs are a diverse group of proteins, spanning from Fragile X Mental Retardation Protein (FMRP), cause of a well-researched phenotype in humans including – among other symptoms – mental retardation (TD-BFX, 1994) to proteins like Staufen 2 (Stau2) and Pumilio 2 (Pum2), whose role in the human brain is less well known.

The goal is to provide a short, concise introduction to this class of proteins and their role in the nervous system, especially the hippocampus. Our lab has focused its research mainly on two proteins, Stau2 and Pum2, which are also subject of this thesis. Therefore, an emphasis will be made on elucidating the current scientific understanding on those proteins.

Neurons are highly specialized and polarized cells. Both the axonal and the dendritic compartments serve synergistically yet mechanistically completely different purposes requiring localization of numerous proteins. This could either be achieved by translation in the soma and then transporting the newly synthesized proteins to the desired location, or by local translation of transcripts at their final destination. The latter has been suggested to contribute to synaptic strengthening upon activation (called LTP), thereby providing an excellent explanation of how synaptic plasticity is achieved. There has been broad interest in finding the mechanisms that make this possible. In recent times, RBPs have emerged as a family of proteins capable of binding RNA and influencing its translational state. They do so by binding mRNAs in specific target sequences thereby forming so called neuronal ribonucleoprotein particles (RNPs). They represent larger protein and mRNA complexes, containing many distinct mRNAs as well as RBPs (Kiebler and Bassell, 2006; Kusek et al., 2012).

1.2.1 The role of the RBP Staufen 2 in the nervous system

Staufen (Stau) was one of the first RBPs to be linked to mRNA localization. It belongs to the family of double-stranded RBPs, binding long extended stem loops in the 3'-UTR region of mRNAs (Ramos et al., 2000). It was first described in 1995 that Stau is required for the localization of *prospero* and *bicoid* mRNAs in the *Drosophila* oocyte (St Johnston, 1995). It

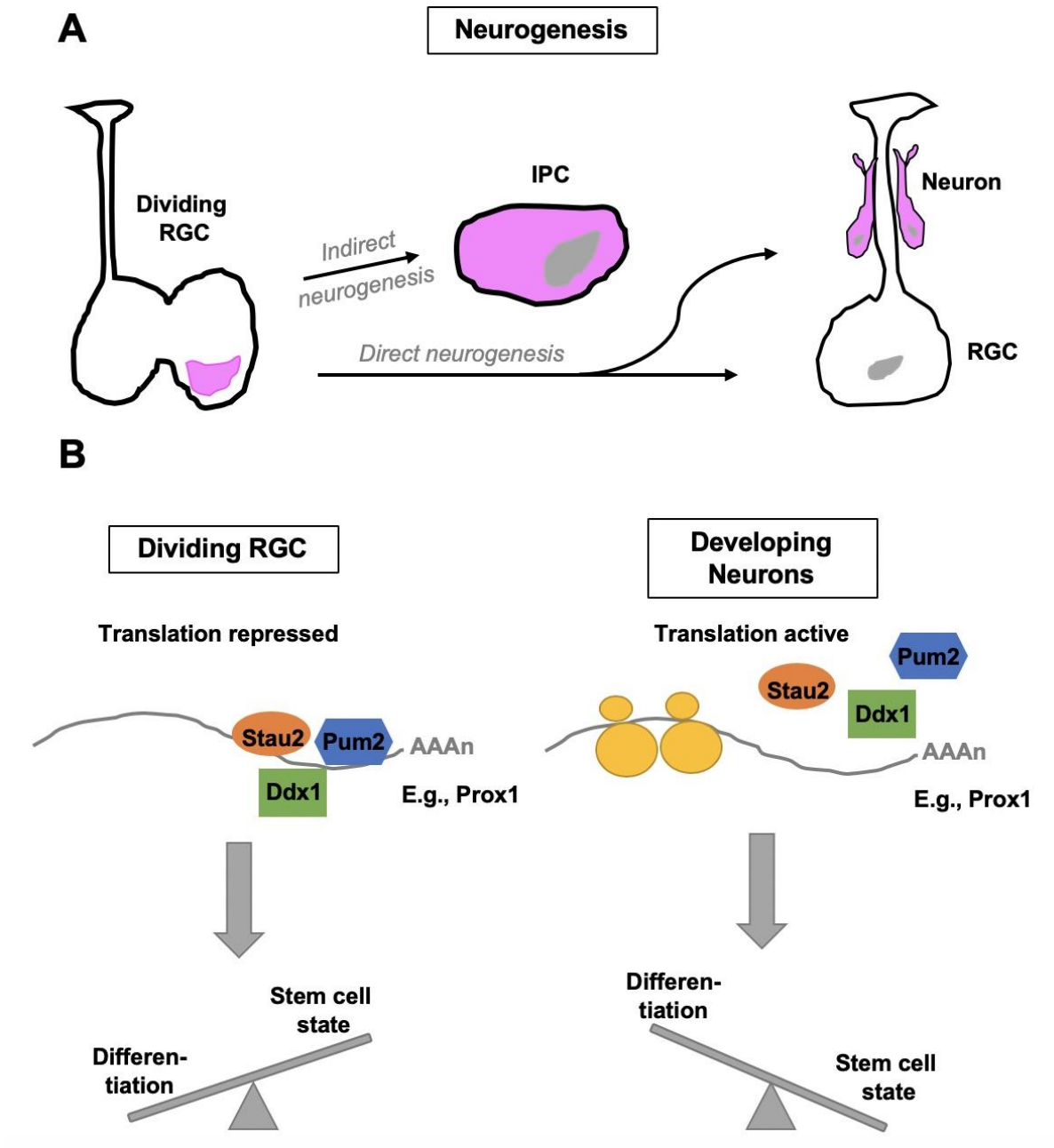


Figure 1: The role of Stau2 and Pum2 in embryonal neurogenesis in mice
(A) During embryonal mouse neurogenesis, radial glial cells (RGCs) either divide asymmetrically to produce another RGC and a post-mitotic neuron ("direct neurogenesis") or produce another RGC and an intermediate progenitor cell (IPC) ("indirect neurogenesis"). The IPC then divides once more symmetrically to produce two neurons. A complex containing (among others) Stau2, Pum2, Ddx1 and their target RNAs (e.g. the cell fate determinant Prox1) (pink shading) localises into the differentiating cell (IPC/neuron). **(B)** The localisation and translational repression of cell fate determinants like Prox1 through the Stau2, Pum2 and Ddx1 containing complex promotes the stem cell state and supresses differentiation in a dividing RGC (left). Upon segregation, the mRNAs encoding for cell fate determinants, are translated and promote differentiation and suppress the stem cell state (right side). Adapted from Heraud-Farlow and Kiebler (2014)

was later described, that this binding is dependent on a 3'UTR region of Stau and microtubule dependent (Ferrandon et al., 1994). *Li et al.* (1997) first showed that Stau is crucial for the asymmetric localization of *prospero* mRNA in *Drosophila* neuroblasts, the progenitor cells of the fly CNS.

Mammals express two orthologs of Stau: Staufen 1 (Stau1) and Staufen 2 (Stau2) (Heraud-Farlow and Kiebler, 2014). Stau2, unlike Stau1, is predominantly expressed in the nervous system as well as in gonads. *Kusek et al.* (2012) demonstrated, that Stau2 is also relevant for asymmetric lineage progression in mammalian stem cell division by binding specific mRNAs. Hereby, it forms a complex with Pum2 and DDX1, containing the mRNA *prox1* (mammalian *prospero*). Lack of any of the proteins causes premature differentiation of neurons (Vessey et al., 2012) (**Fig. 1**). Thus, Stau proteins have emerged as important localizers of cell fate determinants, therefore influencing embryogenesis and embryonal neurogenesis.

Stau2, as mentioned before, is localized within dendrites of mammalian neurons (Kiebler et al., 1999). Upon knock-down, dendritic spines, PSDs and miniature excitatory postsynaptic potentials (mEPSPs) are reduced in rat primary hippocampal neurons (Goetze et al., 2006). *Lebeau et al.* (2011) showed, that Stau2 is necessary for protein synthesis dependent LTD. This form of LTD is dependent on metabotropic glutamate receptors, which are G-Protein-coupled receptors and activated by glutamate. In mGluR-LTD mGluRs mediated translational changes at the synapse upon activation (Malenka and Bear, 2004). This may be coupled with the requirement of Stau2 for forming *Map1b* mRNA containing granules that are necessary for mGluR-LTD. *Heraud-Farlow et al.* (2013) revealed Stau2 to stabilize its target mRNAs. Importantly, they showed, most target mRNAs are localized dendritically, e.g. *Calm3* and *Rgs4* (Heraud-Farlow et al., 2013). Further studies revealed Stau2 to be associated with various translational repressors, namely FMRP, Pur-alpha, RBM14 and Pum2 indicating Stau2 RNPs are in a translationally repressed state (Fritzsche et al., 2013).

1.2.2 The role of the RBP Pumilio in the nervous system

Pumilio has previously been identified as a translational repressor in *Drosophila* germline oocytes (Asaoka-Taguchi et al., 1999; Parisi and Lin, 2000). It acts together with Nanos by repressing translation of *cyclin B* mRNA, thereby inhibiting pole cell division (Asaoka-Taguchi et al., 1999). Pumilio belongs to the PUF family (short for Pumilio and FBF, orthologs of the same protein in different species), a group of RBPs implicated in translational repression, activation and RNA transport. All members of the PUF family are characterized by specific recognition sequences in the 3'-UTR of their target mRNAs (Quenault et al., 2011). This

sequence contains a characteristic C-terminal domain with eight tandem repeats and binds RNA through the nanos responsive element (NRE), a defined nucleotide sequence motif, located in the 3′-untranslated region of target mRNAs (Zamore et al., 1997). In *Drosophila*, Pum mediates neuronal excitability, which increases upon down-regulation (Schweers et al., 2002). Additionally, Pum2 and Nanos regulate dendritic branching of higher order dendrites *Drosophila* peripheral neurons, and the dendritic tree loses complexity upon knock-down of either of these proteins (Ye et al., 2004). Interestingly, in *Drosophila* peripheral motor neurons Pum has been shown to bind and translationally repress the mRNA of the voltage-gated sodium channel *para* (Muraro et al., 2008). Further, at the neuromuscular junction, Pum binds and represses the mRNAs encoding for the translation initiation factor 4E (eIF-4E) and the glutamate receptor subunit GluRIIA, influencing excitability (Menon et al., 2009). Besides this ample information about Pum function in the nervous system of lower eukaryotes, research on the function in higher species is still lacking. In mammals, two orthologs exist, Pumilio 1 (Pum1) and Pum2 (Spassov and Jurecic, 2002). Little is known about Pum1 function. A microarray of target mRNAs and comparison of found Pum targets in *Drosophila* revealed only a roughly 14% overlap suggesting distinct function of the orthologs through binding different subsets of mRNAs (Morris et al., 2008). Using coimmunoprecipitation, over 60 Pum2 mRNA targets were revealed, several being associated with growth regulation (Fox et al., 2005). Pum2 is found in the dendrites and cell body of mammalian hippocampal neurons (Vessey et al., 2006). During cellular stress, much like Stau2, Pum2 temporarily assembles into stress granules (Vessey et al., 2006). In their follow up study, Vessey et al. showed that loss of Pum2 in immature mammalian hippocampal neurons leads to increased dendritic branching, whereas in adult neurons, it leads to decreased dendritic branching. Interestingly, mESPCs along the dendritic shaft of neurons were found to be increased upon knock-down, which was associated with the upregulation of mRNAs coding for eIF-4E and the voltage gated sodium channel $Na_v1.1$ (Vessey et al., 2010). Further studies showed, that loss of Pum2 also increases the sodium currents and action potential firing in rat visual cortical pyramidal neurons (Driscoll et al., 2013). Pum2 was shown to directly bind and suppress translation of the mRNA coding for the voltage gated sodium channel $Na_v1.6$, *Scn8a,* (Driscoll et al., 2013) thereby regulating neuronal excitability. In line with these findings, *in vivo* studies have shown Pum2 deficient mice to have spontaneous, age-dependent seizures in adulthood (Siemen et al., 2011). These mice also showed impaired novelty response (Siemen et al., 2011). More recently, brain tissue therapeutically resected from patients with temporal lobe epilepsy showed to have lower levels

of Pum2 protein as well, highlighting its probable role in the genesis of this, often intractable form, of epilepsy (Wu et al., 2015).

1.2.3 RBPs and their role in memory

In fact, many RBPs have been associated with memory deficits (Conlon and Manley, 2017). Fragile X Syndrome (FXS), a X-chromosomal dominantly inherited genetic silencing mutation via a triplet-repeat expansion within the 5'-UTR of the Fragile X mental retardation 1 gene (FMR1). It is characterized by mental retardation, behavioral deficits and somatic findings like an elongated face, large protruding ears and macroorchism (TD-BFX, 1994; Bassell and Warren, 2008; Tolino et al., 2012). It is the most common single cause of autism, which covers a diverse group of psychological findings such as impaired social interaction and language deficits. Five percent of individuals with autism spectrum disorders (ASD) carry a single gene mutation for FMR1 (Tolino et al., 2012). FMRP is an RBP that serves as translational repressor of distinct transcripts at the synapse dependent on mGLuR activation. The phenotype seen in affected patients as well as in knock-out mouse models is likely caused by a dysregulation of translation upon knock-down. Several targets have been identified, two of which – namely CaMKIIα and Arc – may be important for the observed learning deficits (Bassell and Warren, 2008).

RBP	**affected part of memory**	**behavioural tests**	**species**	**reference**
FMR1	Spatial long-term memory	Morris water maze	mouse	(TD-BFX, 1994)
Translin	Spatial novelty recognition	NOL	mouse	(Park et al., 2017)
NONO	Spatial long-term memory	Morris water Maze	mouse	(Mircsof et al., 2015)
Pum/Pum2	Long-term memory	Pavlovian conditioning	drosophila	(Dubnau et al., 2003)
	Hippocampal memory	nesting	mouse	(Siemen et al., 2011)
Stau	Long-term memory	Pavlovian conditioning	drosophila	(Dubnau et al., 2003)

Table 1: List of RBPs with their suggest roles in memory

A second example of an RBP involved in neural diseases is Rbfox1. Rbfox1, a splicing factor, has been identified as being crucial for mediating synaptic transmission and excitation through its target mRNAs, one of which is *Scn8a* (Gehman et al., 2011). Gehman et al. (2011) showed that knock-out of Rbfox1 in the dentate gyrus of mice increases susceptibility to spontaneous and kainic acid induced seizures. Interestingly, mutations in the Rbfox1 gene,

which is also known as Ataxin-2 Binding Protein 1 (A2BP1), have been associated with ASD, mental retardation and epilepsy (Bhalla et al., 2004; Sebat et al., 2007).

An increasing number of RBPs have been associated with mental retardation and memory deficits. Table 1 provides an overview over selected RBPs that have been implied roles in learning and memory in animal models, which part of memory is affected upon knock-down of said proteins and what model organism and testing procedure was used.

1.3 The role of Stau2 and Pum2 in hippocampal memory formation

Stau2 and Pum2 have been linked to memory for the first time in a study using *Drosophila*, which identified mutants carrying loss of function mutations for either of these proteins to show defective one day memory in an Pavlovian olfactory conditioning procedure (Dubnau et al., 2003). The authors then went on to propose an involvement of a joint Stau2/Pum2 pathway in memory. Detailed mechanistic data, however, for the role of the Stau2/Pum2 pathway in memory is sparse.

Siemen et al. (2011) published a first study, using a LacZ-mediated Pum2 knock-down mouse model, the same model I used in this study, conducting a first characterization of the phenotype of this line. This included – among others – a Novel Object Recognition (NOR) test, a Morris water maze, and nesting behavior testing. Mice showed decreased nesting scores indicating possible damage to hippocampal function and improved novelty detection in the NOR task, which is known to involve the hippocampus but also adjacent structures. Interestingly, in *Siemen et al.* $Pum2^{KD}$ mice showed superior performance in the Morris water maze, a hippocampus dependent spatial memory task (Siemen et al., 2011).

These first studies on the effect of Stau2 and Pum2 on hippocampal memory are rather preliminary and not yet conclusive. There is additional ample evidence hinting to possible roles of the two proteins in hippocampal memory. As aforementioned, both proteins are found in the dendritic compartment of hippocampal neurons and are involved in the localization and translation of their (joint) target mRNAs thereby affecting processes involved in synaptic plasticity such as dendritic spine morphogenesis (Ye et al., 2004; Vessey et al., 2006, 2010, 2012; Heraud-Farlow et al., 2013). Importantly, both proteins have been implicated in localizing cell fate determinants and consequently promoting lineage progression in embryonal neurogenesis upon knockdown (Li et al., 1997; Kusek et al., 2012; Vessey et al., 2012).

Taken together, increasing evidence hints to Stau2 and Pum2 contributing to the formation of hippocampal memory. They are both necessary for regulation of embryonal neurogenesis as

well as dendritic spine development. Further, knock-down animal models show impaired or altered olfactory (in *Drosophila*) and hippocampal (in mice) memory.

1.4 The role of neurogenesis in hippocampal memory

The hippocampus is one of the few regions in the brain, where neurogenesis occurs throughout life (Zhao et al., 2008; Kempermann et al., 2015). First discovered in rats by Altman and Das in 1965, it was later also shown in other mammalian species (Kornack and Rakic, 1999). In 1998, a first group was able to prove that new cells were generated in the hippocampus of humans (Eriksson et al., 1998).

New neurons are only generated in two areas of the brain. First, in the subventricular zone, where cells contribute to processes involved in olfactory learning (Zhao et al., 2008). Importantly, the second area, where new neurons are generated throughout life is the subgranular zone of the dentate gyrus, a specific and well-defined part of the hippocampus. Figure 2A gives an overview over the stages of development a newborn neuron goes through and important marker proteins different stages in development are associated with. They are generated from a pool of neural stem cells (type 1 cell), which show characteristics of radial glial cells (RGCs), a common type of developmental cells in embryonal neurogenesis expressing the marker proteins Nestin and GFAP (Kempermann et al., 2015). They pass through three consecutive stages of progenitor cells, which are capable of self-renewal but which are lineage determined (Type 2a, 2b and Type 3). Important marker proteins are NeuroD, PSA-NCAM and for Type 2b and 3 Doublecortin (DCX) (von Bohlen und Halbach, 2011; Kempermann et al., 2015). An important stimulus during that time is depolarizing GABA currents (Ge et al., 2007). The underlying mechanism depends on a chloride ion gradient generated by two transporters, NKCC1 and KCC2, respectively (Reynolds et al., 2008; Kim et al., 2014). This is later superseded by activation through glutamatergic input (Ge et al., 2007). By that time, cells begin to integrate into the existing synaptic circuitry (Song et al., 2005). These Type 4 cells are early postmitotic neurons, roughly 2-3 weeks old that express marker proteins such as DCX, NeuN and Calretinin (Ming and Song, 2011). They are only followed by mature neurons, expressing Calbindin as distinctive marker, thereby morphologically resembling older neurons and being functionally integrated into the circuitry. There are several checkpoints of survival: when entering the postmitotic phase and when cells are starting to form their dendritic arbor, which gains complexity as they mature (Ambrogini et al., 2004; Overstreet-Wadiche and Westbrook, 2006). During these times, survival of the neurons can be stimulated by certain hippocampus involving learning tasks such as trace eyeblink conditioning

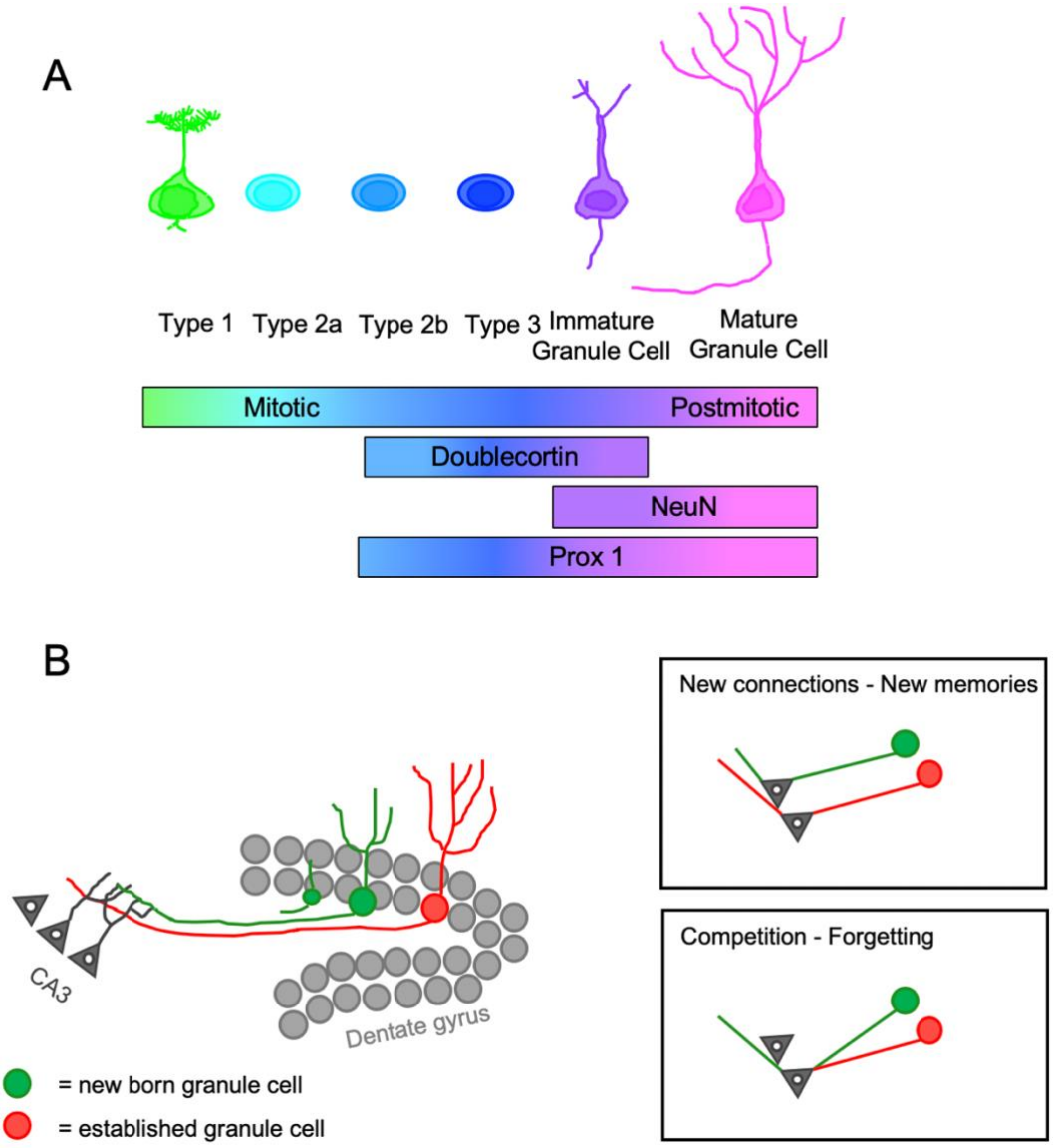


Figure 2: Mechanisms of adult neurogenesis in the hippocampus

(A) There are distinct stages in adult hippocampal neurogenesis, identifiable by certain marker proteins. Important examples and their expression through development of the neurons are depicted underneath a schematic view of the distinct morphological stages of a newborn neuron. Adapted from Ming and Song (2011) and von Bohlen und Halbach (2011). **(B)** There is evidence for different consequences to the integration of new neurons in the dentate gyrus. The newborn neurons form new connection and thereby contribute to the formation of new memories. At the same time, the newborn neurons compete with existing neurons for synaptic connection to the CA3 regions. This competition is believed to (to some extent) cause forgetting of already formed memories. Adapted from Frankland et al. (2013).

or the submerged Morris water maze, or environmental enrichment (Kempermann et al., 1997; Gould et al., 1999; Epp et al., 2007; Kee et al., 2007). Voluntary wheel running, in turn, has been shown to stimulate the proliferation and survival of newborn neurons (Gage et al., 1999).

As already mentioned, the hippocampus serves a crucial role in the generation of episodic memory as well as spatial based learning (Kandel et al., 2014). It has been shown, that new neurons at distinct stages in development are selectively removed and added to the hippocampus for proper performance of rodents in hippocampus based memory tasks (Deng et al., 2010). Importantly, it was shown, that only good performers in hippocampus based memory tasks show increased rates of neurogenesis (Sisti et al., 2007).
Relatively mature (4-6 weeks old) adult generated neurons are preferentially integrated into the hippocampal circuitry upon spatial learning, while younger cells are removed through apoptosis (Dupret et al., 2007; Kee et al., 2007). This suggests, a balance between integration and removal of newly born neurons, which are at different stages of development, represents a key mechanism by which hippocampal neurogenesis contributes to hippocampal learning.

Stimulating neurogenesis, however, can also negatively affect neurogenesis. Akers et al. (2014) showed, that mice subjected to voluntary wheel running had increased neurogenesis but performed worse in hippocampus-based memory tasks compared to their non-running littermates. It is believed that forming new memories involves processes in the alteration of the synaptic connectome, which interfere with already established circuitry. This may lead to forgetting of old memories in favor of newly formed ones (Frankland et al., 2013) (**Fig. 2B**).

1.5 Assessing hippocampal-based memory

An important question, when talking about hippocampal memory is how it can be tested. This is not an easy task and most tests, that are available now, have evolved over time. As previously described, the hippocampus plays an important role in visuo-spatial and verbal or narrative memory (Burgess et al., 2002). While narrative memory is a process difficult to be addressed in animal models and testing of these aspects of hippocampal memory is less common (Bunsey and Elchenbaum, 1996), various tests for visuo-spatial memory performance in animals have been developed (Sharma et al., 2010).

Although most of the tests were firstly developed for the use with rats, most have been adapted for the use in mice, as genetic models are nowadays very advanced in mice and they have become a more frequently used animal model.

The most widely used is the Morris water maze. It was first introduced for the use in rats (Morris, 1984) but has since been adapted for the use in mice. The testing setup consists of a pool filled with opaque fluid in which a platform is submerged to which the animal can escape. It does so by starting from various starting points and uses the animals natural drive to escape the open water. The animal learns the location of the platform using distant spatial cues as

indicated by a shortened latency to the escape platform. Importantly, nearly all animals will complete the test, lessening the problem of selection bias (Vorhees and Williams, 2014). Successful encoding of the location of the platform is tested with a delayed probe trial. Here, after runs with a platform in the pool, that was successfully located, the platform is removed. The time the animal spends in the quadrant, where the platform was once located, is measured, indicating place-memory of the platform if more time was spent in the corresponding quadrant. The delay used between the trial runs used for encoding of the location and the probe trial itself can be adapted to various intervals thus testing short or long term hippocampal-based spatial memory. One downside of the Morris water maze is the stress the swimming puts on the animals. Corticosterone levels, an important hormone in stress response, have been inversely correlated with testing performance (Harrison et al., 2009). Additionally, cognitive performance of the testing animals in the Morris water maze, has been shown to be affected upon prior exposure to stressful stimuli (De Quervain et al., 1998).

Therefore, another (less stressful) test was developed (Barnes, 1979). This paradigm consists of an elevated platform with holes around the outer perimeter. Under one hole is an escape box, in which the animal can hide. This test is based on the natural instinct of rodents to avoid open and brightly lit areas and as the Morris water maze, in most setups does not rely on food deprivation. As with all behavioral testing regimens, testing is affected by strain- and task- or setup-dependent differences (Sunyer et al., 2007).

Addressing the ability of rodents to remember spatial relationships and objects through the previously mentioned tasks using aversive stimuli does not reflect everyday memories typically affected in human diseases involving memory processes like Alzheimer's. Thus, other testing regimens have been developed to account for those processes. They are based on a rodent natural preference for novelty and in most setups differences between (a) the preference for a newly encountered object, so called novel object recognition or novelty detection and (b) the memory of an object's location, so called novel location recognition (NOL) (Vogel-Ciernia and Wood, 2014). Testing setup varies greatly between studies and compromises comparability (Antunes and Biala, 2012) which calls for a reliable control group to address and discuss possible effects. In general, the testing setup includes a habituation trial in which the animal is allowed to roam freely in a walled box containing to identical objects. For the novel object recognition trial one object is replaced by a novel object differing from the previous one in size and shape. For the novel object recognition trial, the position of one object is altered, while both objects are the same. For each trial time spent with the newly introduced or located object is compared to the time spent with the familiar object. If the novelty of the object or the location

is detected, more time will be spent with the novel object. It is important to recognize, that although novel location preference seems to be a hippocampus-dependent task (Sharma et al., 2010; Barker and Warburton, 2011), novel object preference seems to a bigger extent rely on extrahippocampal structures. Of significance for these tasks are the perirhinal cortex and medial prefrontal cortices (Brown and Aggleton, 2001; Barker and Warburton, 2011). Thus, if executed correctly, the novel object location and recognition paradigm may enable to make useful differentiations between hippocampal lesion and lesion of para-hippocampal areas.

Another easily operable test is the assessment of nesting behavior in mice. Nests are used widely by a variety of differently species to facilitate reproduction but also to provide shelter from cold temperatures and predators. Being able to build an appropriate nest thus is a quintessential task an animal has to master. Hippocampally lesioned mice have been shown to perform worse in a nesting paradigm (Deacon et al., 2002). Hereby mice are single caged for 24 hours and provided with single nestlet. After 24 h the use of the nestlet and complexity of the nest is judged by one single experimenter (Deacon, 2006). This test is less specific for hippocampal memory as effects have also been shown for lesions in the medial preoptic area (Numan, 1974; Slotnick and Nigrosh, 1975).

This brief overview does not take into account every test that has been established for testing hippocampal-based memory. Taken together, ample tests are validated for evaluating hippocampal-dependent memory. Most focus on specific aspects of hippocampal-dependent memory: spatial memory as a subtype of episodic memory.

1.6 Scope of the project

The mechanisms by which hippocampal neurogenesis influences the generation of hippocampus-based memories and vice versa is far from being understood. Both Stau2 and Pum2 have been implicated in embryonal neurogenesis (Kusek et al., 2012; Vessey et al., 2012). Furthermore, orthologs of both proteins have been linked to olfactory discrimination memory in *Drosophila* (Dubnau et al., 2003). Additionally, knock-down of Pum2 in mice has been shown to affect performance in memory tasks (Siemen et al., 2011).

Based on the existing evidence and our knowledge about the function of these proteins in synaptic plasticity and dendrite morphogenesis, a project for this thesis was designed to elucidate the role of both Pum2 and Stau2 in hippocampal memory. We decided to use the known $Pum2^{KD}$ mouse line, as well as an existing $Stau2^{KD}$ mouse line in the lab. Additionally, another member of the lab, Dr. Bastian Popper, had already crossed both mouse lines to

generate a double knock-down mouse line in order to delineate the respective functions of both RBPs *in vivo*.

The scope of this thesis was to perform a first *in vivo* study of all three knock-down mouse lines to look into the joint role of Pum2 and Stau2 in hippocampal memory in mice and possibly linking the effects to changes in adult neurogenesis, which had not been studied before.

In detail, the aims of my project were to:

(i) Provide a first general characterization of the Stau2/Pum2 knock-down mouse line used in our laboratory,

(ii) Elucidate whether the Stau2/Pum2 pathway is important for hippocampal memory function in rodents and

(iii) Investigate possible effects of the knock-down of Stau2 and Pum2 on adult neurogenesis.

For these aims, I first analyzed target protein and mRNA levels as well as several known target mRNAs of both proteins. To address aim 2, I implemented a 4 week-long behavioral testing battery focusing on hippocampus-based memory tasks, namely NOR and NOL testing as well as Barnes maze testing and a simple nesting behavior paradigm as screening tool. Choosing the right behavioral tests for the question at hand is crucial. There are not only interracial differences between mice and rats in behavioral tasks (Frick et al., 2000; Cohen and Stackman, 2015), also testing duration and delay between sample phases are important parameters that might affect the outcome (Antunes and Biala, 2012). Spatial long-term memory formation (Deng et al., 2010) and to a lesser extent novelty response (Barker and Warburton, 2011) are hippocampus-based memory tasks. Of great importance is the time interval (delay) between the different test runs. Longer delays (6h or more) have been shown to challenge hippocampal memory (Broadbent et al., 2004; Cohen and Stackman, 2015). Lastly, to get a first insight into adult neurogenesis in these animals and thereby addressing aim 3, I performed immunohistochemical analysis of DCX+ cells of adult mice (5 months).

The current work has been designed as a set of pilot experiments. By no means, it represents a complete analysis of hippocampal memory of theses mouse lines. If anything, it shall serve as valuable starting point for further investigations regarding this interesting topic. Furthermore, the use of single as well as a double knock-down mouse lines provided a unique opportunity in defining unique contributions of each RBP on its own as well as possible synergistic effects as part of an RBP network.

2 Materials and Methods

2.1 Animals

All breading and pairing were performed at Zentrale Tierhaltung LMU (Schillerstr. 42, Munich, Germany). Animals were held in individually ventilated cages (Type II long, Tecniplast, Hohenpeißenberg, Germany) at an 12h light dark cycle with unlimited access to food (Altromin, Lage, Germany) and water (autoclaved). Mice for the behavioral testing regimen were moved to the behavioral testing unit at Pettenkoferstr. 12 in Munich, Germany, at 15 weeks of age and kept in open cages with the same diet. They were probed after completion of testing at 20 weeks.

All experiments were approved by the institutional committees on animal care and were performed according to the German Animal Protection Laws, conforming to international guidelines on the ethical use of animals (as stated explicitely in: Follwaczny et al., 2017; Popper, Demleitner et al., 2018).

2.1.1 Generation of mouse lines

2.1.1.1 Stau2^{KD} mice

The transgenic Stau2$^{Gt(RRG396)Byg}$ mouse line was generated in the Sally Temple lab (Neural Stem Cell Institute, Rensselear, NY) and has been published (Popper, Demleitner et al., 2018). Mice were generated from a mouse ES cell line containing a gene trap vector inserting a beta galactosidase reporter gene in intron 7 of transcript EN- SMUST00000027052.12 predicted to result in a C-terminally truncated Stau2 protein lacking RNA binding domain (RBD) IV as well as the Tubulin binding domain and expressing a rearranged RBD V. Stau2^{KD} mice were back crossed with C57/Bl6 WT mice for at least six generations and kept as inbred line after. Genotyping was performed using specific primers for the genetrap vector insertion (Popper, Demleitner et al., 2018).

2.1.1.2 Pum2^{KD} mice

The transgenic Pum2$^{Gt(XE772)Byg}$ line was generated in the lab of Renee Preijo Pera (UCSF, San Francisco, CA). An inserted gene trap vector with a beta galactosidase reporter gene was inserted between exon 10 and 11 resulting in a truncated Pum2 Protein (Siemen et al., 2011). Pum2^{KD} mice were back crossed with C57/Bl6 WT mice for at least six generations and kept as inbred line after. Genotyping was performed using specific primers for the genetrap vector insertion (Follwaczny et al., 2017).

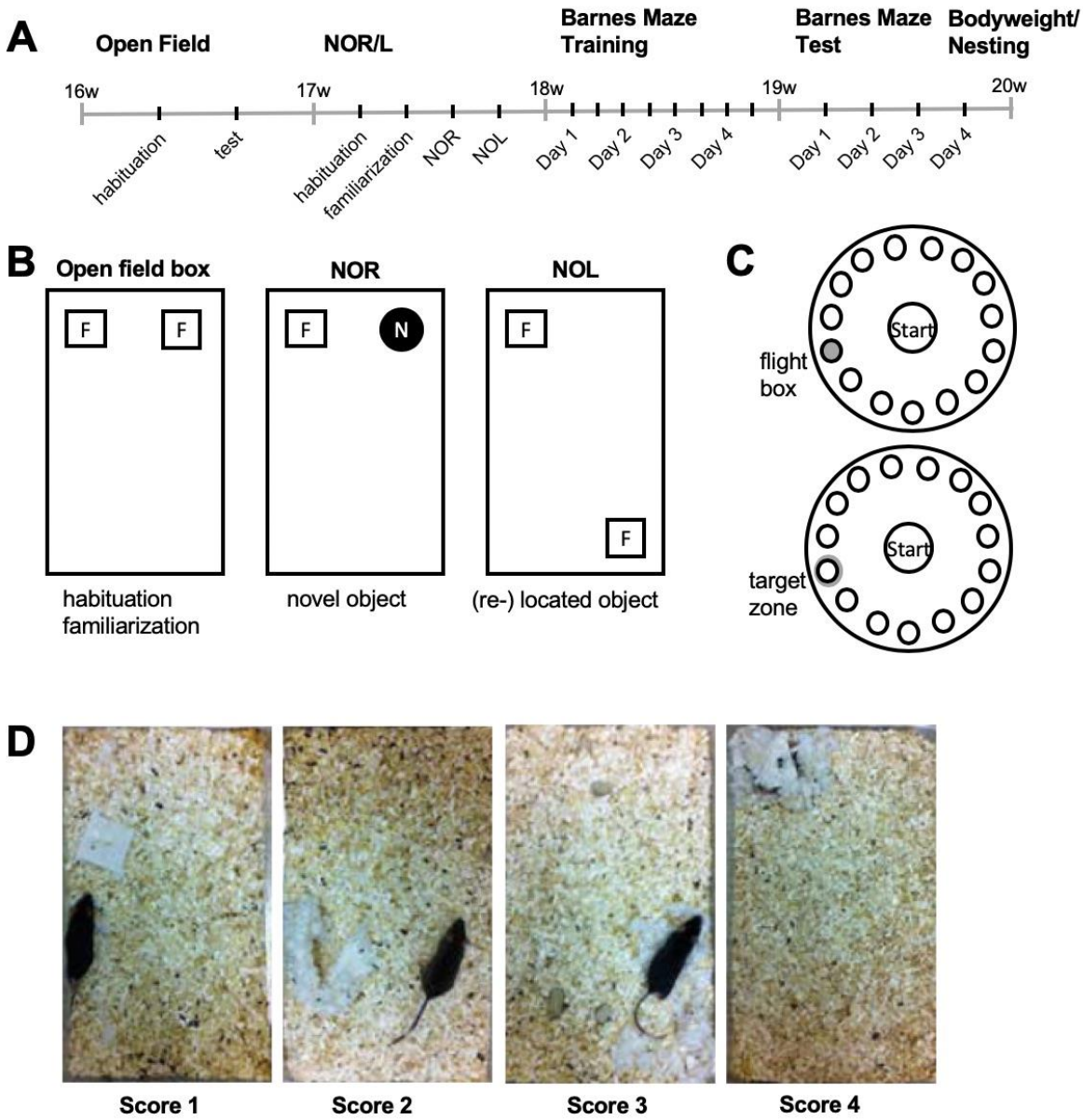


Figure 3: Overview over the behavioural testing battery
(A) Timeline of the behavioral testing setup. Mice were tested for a total of 4 weeks. Open field testing was followed by NOR/L testing and lastly training and testing phase, respectively, of the Barnes Maze. Usually, a 24h delay was in between test runs, but for training in the Barnes Maze mice were tested twice daily with an interval of 15min. **(B)** Testing setup for the NOR/L paradigm. **(C)** Testing setup for the Barnes Maze. Mice were located at the "start" area and had to find a target zone and enter a flight box located below one of the 20 holes. **(D)** Exemplary images of the assigned nesting scores (1-4). Abbrev.: W= weeks, NOR/L = Novel object recognition/location, F = familiar object, N = novel object. Adapted from *Popper*, *Demleitner et al.* (2018).

2.1.1.3 Stau2^{KD}/Pum2^{KD} mice

For Stau2^{KD}/Pum2^{KD} mice, homozygous Stau2^{KD} and Pum2^{KD} mice were crossed and subsequently back crossed to be on the same genetic background. Breeding was carried out by Bastian Popper at the local animal house at the time (Zentrale Tierhaltung, Schillerstraße). Homozygous mice, as confirmed by genetic testing using the specific primers for the genetrap vector insertion as mentioned above, were inbred to maintain the genetic background.

2.2 Behavioral Testing

The testing battery used has been published in (Popper, Demleitner et al., 2018). Mice for the behavioral testing regimen were moved to the behavioral testing unit at 15 weeks of age and probed after completion of testing at 20 weeks. Mice underwent a four-week behavioral testing period starting at postnatal week 16. Figure 1A shows an overview over the testing regimen. All behavioral tests were performed under identical environmental conditions and lighting (900 lux). Mice were kept in individual housing cages between tests. AnyMaze Software (Stoelting Co., Wood Dale, IL, USA) was used to track mice and to analyze the data.

2.2.1 Nesting

Analysis of nesting behavior was performed as described previously (Deacon, 2006). In brief, mice were kept in individual cages for 24 h and provided with a single nestlet (3 x 3 cm, Plexx B.V., Elst, The Netherlands). Nesting behavior was graded manually as followed: ‘1’ for no use of nestlet, ‘2’ for use of 0-40%, ‘3’ for 50-90% and ‘4’ for 100% use of the nestlet and building a nest higher than the mouse body (**Fig. 3D**). Grading was carried out by the same observer for all trials.

2.2.2 Open Field

The behavioral testing regimen was started in week one with Open Field (OF) Testing. For habituation, mice were placed in the center of the OF box (self-made, 80 x 40 cm clear plastic) and subsequently allowed to freely investigate the testing environment for 4 minutes (**Fig. 3B left**). After a 24 h delay, mice were again tested for 4 minutes. General ambulatory parameters such as total time mobile and distance travelled were analyzed using the automated video tracking software (AnyMaze, Stoelting, USA).

2.2.3 Novel Object Recognition and Novel Object Location

This paragraph is adapted from *Popper, Demleitner et al.* (2018). In the second week of testing, Novel Object Recognition (NOR) and Novel Object Location (NOL) tasks were performed in the already familiar open field environment. The procedure was done as described previously (Vogel-Ciernia and Wood, 2014) with slight adaptations. In brief, testing consisted of habituation and familiarization trials on the first two consecutive days, a NOL test on day three and NOR test on day four. In general, mice were placed in the center of the OF box and were allowed to investigate the objects for 4 minutes for each trial. For the habituation trials, two identical objects (identical in size and color) were placed in the same location of the OF box. For NOR, one object was replaced by a novel object differing in size and color but set in the same location. On day four, NOL was performed by placing one familiar object, instead of the novel object, in the opposite corner of the OF box (**Figure 3B middle and right**). The discrimination index ($DI=(Time_{novel}-Time_{familiar})/(Time_{novel}+Time_{familiar})$) was calculated based on total time spent with familiar and novel or relocated object, as described previously (Antunes and Biala, 2012).

2.2.4 Barnes Maze

Behavioral experiments were performed as described previously (Barnes, 1979) and adapted for mice as described previously (Bach et al., 1995). The Barnes maze platform adapted to mice was used for all experiments. First, mice were trained in the third week of the testing regimen. Mice were trained twice a day with a time interval of 15 min for four consecutive days. Here, mice were placed in the center of the platform and were given 5 min to complete the task for habituation (**Fig. 3C**). If the mouse failed to enter the flight box within the testing period, it was placed in the flight box to simulate escape and safety. To address long-term memory formation, mice were tested again in the fourth week once per day for four consecutive days and were given 4 min to complete the task. For all Barnes maze tests, recording was stopped either when the mouse entered the flight box or when the predetermined maximal test time was over. Relevant parameters such as mean speed for the whole test duration and time spent in the target zone as well as the latency to the first entry into the target zone as well as the flight box were measured using the automated video tracking system AnyMaze (Stoelting Co., USA).

2.3 Histology

2.3.1 Tissue preparation

For light microscopy weaned mice (postnatal day 21) were used. For immunohistochemistry adult mice (5 months old) were used. Mice were deeply anesthetized with CO_2.

Whole animal perfusion fixation was performed as described previously (Calzolari et al., 2015). First, mice were deeply anesthetized with CO_2. Then, animals were transcardially perfused with 4°C cold phosphate buffered saline (PBS) followed by 4°C cold 4% Roti®-Histofix (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Brains were then dissected and transferred in 4% Roti®-Histofix and post-fixed for additional 1 to 3 days.

2.3.2 Light microscopy

The exact procedure has been described previously (Popper, Demleitner et al., 2018). In brief, dissected brains were dehydrated in an increasing ethanol gradient (50–100% steps) and embedded in paraffin. Then, sections (approx. 5 µm thickness) were cut on a Reichert Jung Microtome (Leica Microsystems, Wetzlar, Germany). Subsequently, sections were mounted on glass slides (Thermo Fisher Scientific, Waltham, MA, USA), deparaffinized in a decreasing ethanol gradient (100–50% steps) and rehydrated, stained with hematoxylin, eosin (Sigma-Aldrich, St. Louis, MO, USA) and mounted with Histomount (Carl Roth, Germany). For image acquisition, a Leica DM2500 microscope equipped with a DMC2900 CMOS camera (Leica, Germany) was used.

2.3.3 Immunohistochemistry

For immunohistochemistry, brains were transferred to 30% glucose in ddH_2O and incubated at 4°C for dehydration. Brains were then mounted with Cryomount (Tissue-Tek® O.C.T. Compound, Sakura® Finetek, Japan) and frozen at -50°C. Hippocampi were cut in 20µm slices using a cryotome (Product CM1850, Leica, Germany) at -22°C and transferred into 24 well plates containing 4°C PBS. For long term storage, slices were transferred to storing solution (30% glycerol, 30% ethylene glycol, 30% ddH_2O, 10% 10x phosphate buffer (=0,25M NaH_2PO_4, pH 7,2-7,4), incubated at room temperature (RT) constantly shaking overnight (o/n) and then either processed directly or frozen until further processing at -20°C.

2.3.3.1 Staining

Antibody	Species	Company	Catalogue Number	Dilution
Stau2	Rabbit	(Heraud-Farlow et al., 2013)		1:500
Pum2	Rabbit	Abcam	ab10361	1:200
DCX	Rabbit	Abcam	ab18723	1:1000
NeuN	Chicken	Millipore	ABN91	1:500
$GABA_{A2}$	Rabbit	Alomone	AGA-002	1:200

Table 2: List of primary antibodies

Antibody	Species	Company	Dye	Dilution
Anti-Rabbit	Donkey	Life technologies	AF 488	1:500
Anti-Rabbit	Donkey	Molecular Probes (Life technologies)	AF 555	1:500
Anti-Chicken	Goat	Molecular Probes (Life technologies)	AF 647	1:500

Table 3: List of secondary antibodies

For staining, slices were washed in PBS at RT constantly shaking for 45 min (when staining was performed after cutting, this step was skipped). Slices were blocked in blocking solution (PBS + 0.5% Triton (v/v) + 1% bovine serum albumin (BSA, w/v)) for 45 min at RT constantly shaking. Primary antibodies were diluted in blocking solution (**Table 2**) and incubated at 4 °C o/n. Upon 3 times washing in PBS, slices were incubated with secondary antibody diluted in blocking solution (**Table 3**) at RT for 2h constantly shaking. Nuclei were counterstained with DAPI solution (Sigma-Aldrich, USA) for 5 min followed by three times washing in PBS for 10 min each. Slices were mounted on Superfrost slides (Thermo Fisher Scientific, USA) using Fluoromount^{TM} Aqueous Mounting Medium (Sigma, USA). Slides were dried o/n at 4°C before imaging. For further use slides were stored at 4°C while protected from light.

2.3.3.2 Image Acquisition and Analysis

The exact method for image acquisition and analysis has been described in (Follwaczny et al., 2017). In brief, confocal microscopy was performed with an inverted Leica SP8 microscope, equipped with lasers for 405, 488, 552 and 638 nm excitation. For image acquisition, either a 20x/0.75 or a 40x/1.30 oil objective were used. Pixel size was 80 nm. Recording was done sequentially to avoid possible bleed-through. The following fluorescence settings were used for detection: DAPI: 430-470 nm; AF488: 500-550, AF555: 560-600; AF647: 650-700. AF488, AF555 and AF647 were recorded with hybrid photo detectors (HyDs), DAPI with a conventional photomultiplier tube.

For neurogenesis analysis 7 sample tiles of every hippocampus were imaged using a z-stack of 10 µm with 15 individual images. For an accurate comparison of the preselected dentate gyrus, the same height of the dorsal hippocampus as determined by its shape was

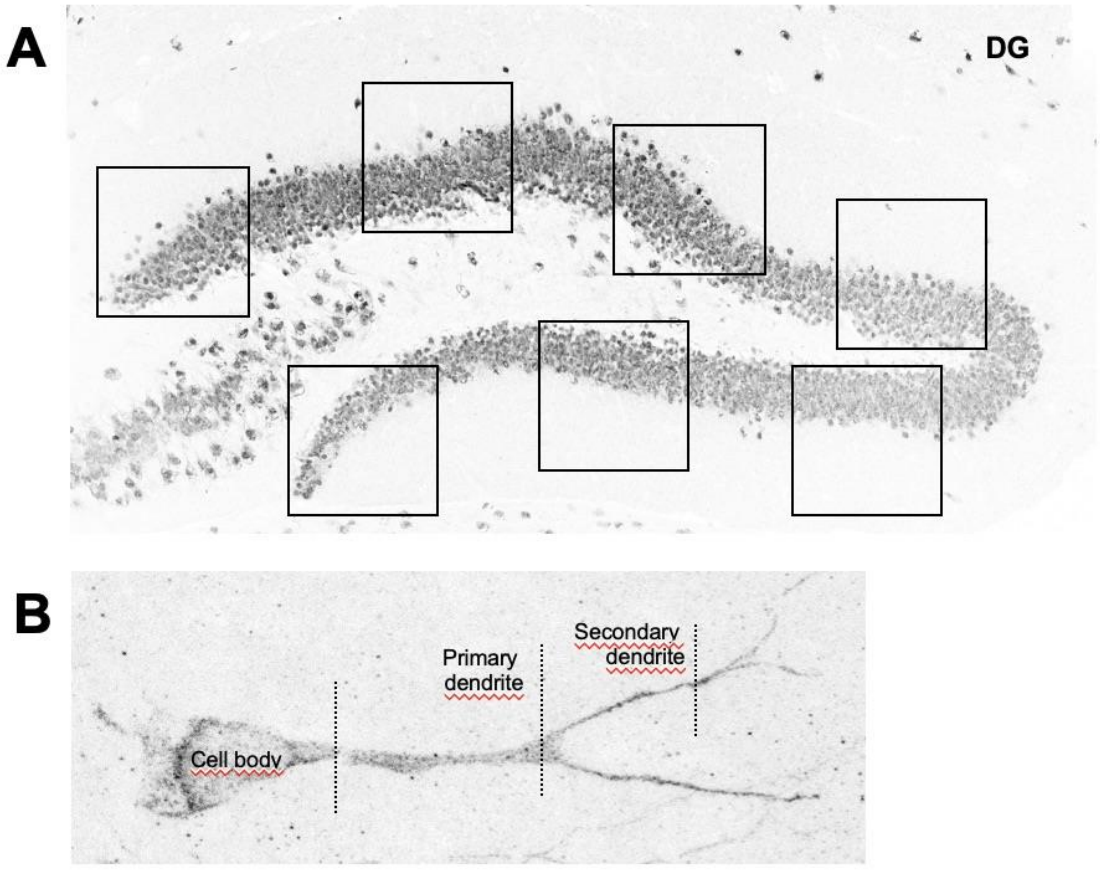


Figure 4: Representative images for the Doublecortin-based neurogenesis analysis
(A) Representative image of the NeuN-staining of a dentate gyrus (DG) marked with approximate location of the seven tile scans (see insets) made for each replicate. **(B)** Representative example of a Doublecortin-positive (DCX+) cell body and its dendritic arbor.

imaged (**Fig. 4A**). 7 Tiles (Tile size = 290.71x290.71µm) were placed in the same pattern on slides at the corresponding vertical plane to get an overview of the whole dentate gyrus (**Fig. 4A**). As dendrites do not sprout in a planar level the z-axis was recorded as well in z-stacks of 15 images per 9.98µm depth. This allowed for proper distinction between crossing or overlying dendrites and correlating the dendrites to the right cell bodies. Merged tile scans of Doublecortin (DCX), the neuronal marker NeuN and DAPI staining pattern of the whole dentate gyrus were acquired for overview.

For quantification only DCX signal in the tiles scans was recorded. As first rough estimate we used a cell profiler macro for DCX signal developed by a fellow M.D. student in our lab, Philipp Follwazcny, on maximum projections of the acquired z-stacks. For more in depth analysis images were quantified by hand using FIJI (Image J). Quantification was performed either using maximum projections or, for further distinction, z-stacks while moving through tiles. Parameters analyzed were total number of DCX+ cells, length of subgranular zone (SGZ) and length (µm) and number of primary and secondary dendrites. DCX+ cell number was further normalized to length of the SGZ. Criteria used for analysing dendritic length were DCX+ cell body, direct contact with the cell body from whom they arise and no interruption of staining along the dendrite. The First branching point of the dendrites was used as differentiation between primary and secondary dendrites (**Fig. 4B**).

For GABRA2, Stau2 and Pum2 stainings tile scans of the whole HC were merged using the stitching tool of the Leica built in software LAS X (Leica, Germany). Target signal was quantified using a FIJI macro developed in collaboration with the Core Facility for Bioimaging at the BMC (for complete code see *Appendices*). NeuN signal was used to determine cell layer from periphery.

2.4 Molecular Biology

For mRNA quantification, brains of postnatal day 0 and 21 as well as 5 months old mice were dissected and flash frozen in liquid nitrogen. Until further analysis brains were stored in RNase free Eppendorf 1.5ml tubes at -80°C.

2.4.1 RNA Isolation

Isolation of RNA was performed using TRIzol according to manufactures manual. In brief, brains were homogenized in 1 mL of TRIzol using lysing matrix D (MP Biomedicals LLC, Irvine, CA, USA) at 60 m/s for 40 s. Homogenate was mixed with 9 mL of TRIzol and incubated for 5 min at RT. RNA was extracted using 2% v/v chloroform-isoamylalcohol (24:1,

Sigma-Aldrich, USA). Upon centrifugation at 4100 g for 15 min at 4°C, upper phase was treated with 10% v/v isopropanol (Carl Roth, Germany) and incubated at RT for 10 min. Samples were spun at 2000 G for 15min at 4°C. RNA pellet was washed with ice-cold 75% Ethanol and dried at RT. Pellet was dissolved in 100µL RNase free H20 (Sigma-Aldrich, USA). Solution was heated for 5 min at 55°C. Concentration of RNA was measured using a NanoDrop 2000 photometer at 260 nm (Thermo Fisher Scientific, USA). For DNA depletion, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to manufactures manual for cleanup of previously isolated RNA including a step for on column DNase digestion. In brief, 80 µg of RNA were mixed with 500 µL RLT and 500 µL 70% ethanol and subsequently transferred to the binding column. Upon spinning and washing with 350 µL RW1 buffer, DNase reaction mixture (containing 27 KU DNase 1) was added and incubated for 15 min at RT. 350 µL RW1 buffer was added for washing and spun. Next, 500 µL RPE buffer was added to the column and centrifuged for 2 min at 120 g. This step was repeated once. RNA was eluted in 40 µL RNase-free water. The elution step was repeated once. Concentration was estimated using the NanoDrop for absorbance at 260 nm and RNA was stored at -80°C.

RNA quality was analyzed using a 1 % agarose RNA gel (dissolved in 1 x MOPS, 18% formaldehyde (c=36.5-38%, Sigma-Aldrich, USA) and DEPC water). In brief, 1 µg RNA was incubated in 15 µl of MOPS based loading buffer (50% v/v color formamide, 0.5 ng ethidium bromide, 17% v/v formaldehyde (Sigma, USA)) at RT for 5-10 min. Samples were then heated to 65°C and chilled on ice immediately. Samples were loaded and Gel was run at 80 V for 3-4 h.

2.4.2 cDNA Synthesis

For qRT-PCR cDNA synthesis was performed using superscript reverse transcriptase SSIII (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. In brief, 10% w/v (2 µg) RNA was mixed with 20 U RiboLock RNase inhibitor (Thermo Fisher Scientific, USA), 0.6 µM random primer (New England Biolabs, Ipswich, MA, USA) and 0.01 mM dNTP mix (New England Biolabs, USA). Samples were incubated at 65°C for 5 min and then cooled down to 4°C. Samples were mixed with 20% v/v (4 µL) 5x first strand buffer (Invitrogen, USA), 5% v/v (1 µL) 0.1 M DTT (Invitrogen, USA), 5% v/v (1 µL) RNA free water and 200 IU SSIII reverse transcriptase. Samples were incubated at 55°C for 60 min. Reaction was inactivated at 70°C for 15 min and cDNA was stored at -20°C until further use. To check for DNA contaminations, samples without added SSIII were prepared in parallel.

2.4.3 qRT-PCR

Reagent	Final Concentration
cDNA (Diluted)	5µl
Hot-Start *Taq*DNA Polymerase (New England Biolabs, USA)	0.04 U/µL
Hot-Start *Taq*DNA Polymerase Buffer (New England Biolabs, USA)	1x
Forward and reverse primer (each)	0.667 µM
BSA (New England Biolabs, USA)	0.2 µg/µl
Betaine (Sigma, USA)	0.5 M
dNTPs (New England Biolabs, USA)	80 µM
SYBR®-Green	0.3x

Table 4: Final concentration of regent per reaction used for qRT-PCR

Step	Temperature	Time
Initial Denaturation	95°C	5 min
Amplification (45 Cycles)	95°C	15 s
	60°C	15 s
	68°C	20 s
Melting Curve	95°C	10 s
	60°C	1 min
	60°C – 97°C	Continuous (Ramp Rate 0.2°C/s)
Cooling	37°C	30 s

Table 5: qRT-PCR program

Gene Name	Sequence
Stau2	fow.: 5'-AGTTGCGACTGGAACAGGAC-3' rev.: 5'- TGGACCACTCCATCCTTTGT-3'
Ppia	fow.: 5'-GTCAACCCCACCGTGTTCTT-3' rev.: 5'- CTGCTGTCTTTGGAACTTTG-3'
Rn18s	fow.: 5'-GAAACTGCGAATGGCTCATTAAA-3'

	rev.:5'- CCACAGTTATCCAAGTAGGAGAGGA-3'
Arc	fow.: 5'-CCTGGCCCCCAGTGATT-3' rev.: 5'- GTGTCATTCTCCTGGCTCTG-3'
Camk2a	fow.: 5'-AAACTGAAGGGAGCCATCCT-3' rev.: 5'-TCCATTGCTTATGGCTTCGATC -3'
Calm3	fow.: 5'-ATGCTGATGGCAATGGG-3' rev.: 5'-TTGTCAAAGACACGGAAGG-3'
Rgs4	fow.: 5'-GAGGAGAACATTGACTTCTGG-3' rev.: 5'-TGTAGATCTTCTTGGCTTTGG-3'
Kcna1	fow.: 5'-ATTCAGACTCTCCGCCGACT-3' rev.: 5'-CTGCAGCCCTTCTAGGACAC-3'
Kcna2	fow.: 5'-GGGACTGAGCTGCCTATTTG-3' rev.: 5'-AGGGAACTCGGTGCTGATAA-3'
Kcna4	fow.: 5'-GAAAAGGGGAAACAAATCACC-3' rev.: 5'-CACAGACAATGCCAGGTTGT-3'
Gabra2	fow.: 5'-GAAAGGCTCCGTCATGATAC-3' rev.: 5'-GCTTGTTCTCTGGCTTCTT-3'
Gabbr2	fow.: 5'-CTACGACGGTCTTACTCTCA-3' rev.: 5'-GGCCTCTCTCCTTTGTCTA-3'

Table 6: Overview of primers used for qRT-PCR

For qRT-PCR, a self-made SYBR green based qRT-PCR mix was used (for final concentration per reaction see **Table 4**) (Follwaczny et al., 2017; Popper, Demleitner et al., 2018). qRT-PCR was performed in two technical replicates, a non-reverse transcriptase and water control using a Lightcycler 96 (Hoffmann-La Roche AG, Basel, Switzerland). PCR program is depicted (**Table 5**). Primer were designed using the IDT Primer Quest Tool (IDT, USA) (for sequences used see **Table 6**). Primer pairs were rigorously tested for an efficiency of 2 ± 0.05 as calculated by the qRT-PCR analysis program (Roche, Switzerland). PCR product size and purity of assay was analyzed by running samples on an agarose gel. Signals of candidate genes were normalized to *PPIA* and 18S as reference genes using the comparative ΔΔCT method (Schmittgen and Livak, 2008). Both gave similar results, *PPIA* referenced values are displayed only.

2.5 Statistics and software

Statistics were calculated using the software GraphPad Prism (Version 7; GraphPad, San Diego, CA, USA). Data are presented as mean +/- SEM. Initially, data were tested for Gaussian distribution. If testing was positive, unpaired two-tailed Student's t-Test for comparison of two groups and One-Way ANOVA and Tukey Post Hoc for comparison of more than two groups were used to determine p-values. If data were not normally distributed, Mann-Whitney U-test for the comparison of two groups and for the comparison of more than two groups Kruskal-Wallis test was used to determine p-values. Kaplan Meier graphs for Barnes Maze testing were analyzed using Log-Rank test. For all behavioral tests Grubb's test (GraphPad, USA) was used to determine outliers. Unless stated otherwise, $p<0.05$ was considered statistically significant.

3 Results

3.1 Initial characterization of Stau2 and Pum2 knock-down mice

This project pioneered in evaluating the effects of knock-down of two key RNA binding proteins in mouse models. Based on what is known about the function of Stau2 and Pum2 by the work in our lab and in the literature, we decided to focus on the effects of the knockdown on learning, memory and - in a second step - neurogenesis. First, we performed an initial characterization analyzing the protein and target mRNA levels and distribution. Here we screened for levels of several known, learning, memory and epilepsy associated target mRNAs. We conducted gross microscopic analysis of the brains to screen for obvious structural, morphologic changes. Because of the implication of a common role of Pum2 and Stau2 in learning and memory (Dubnau et al., 2003), we set out to conduct hippocampal based learning tasks using adult mice. As behavioral studies are easily influenceable by a number of variables (O'Leary and Brown, 2012), we initially performed an open field task before commencing with our testing battery to screen for gross behavioral changes and get the mice adjusted to the testing environment.

For all experiments, the in *Materials and Methods* described $Stau2^{KD}$, $Pum2^{KD}$, $Stau2^{KD}/Pum2^{KD}$ and WT mouse lines, which were bred on the same genetic background, were used in parallel. General macroscopic histological analysis showed no obvious differences between the mice. We saw stimulus associated, *e.g.* environmental influences caused by cage opening tonic-clonic seizures in predominantly male $Pum2^{KD}$ mice with onset at 5 months of age as previously described (Siemen et al., 2011). This was also observed in the $Stau2^{KD}/Pum2^{KD}$ mice. None of the animals used in the behavioral testing regimen including control animals showed comparable seizures, although we did not have 24h monitoring.

3.1.1 Target gene expression

Here, we performed initial experiments using whole brain slices of 5 months old, male $Stau2^{KD}$ mice. As mentioned before, this mouse line has not been previously published. Therefore, we analyzed whole brain mRNA levels of the target gene and compared them to our control group to validate success of the gene trap (Popper, Demleitner et al., 2018). *Stau2* mRNA levels were significantly decreased by approx. 50% proving successful knock-down of our target gene (**Fig. 5A**). To prove this knockdown was also efficient on the protein level, we performed immunohistochemical stainings of the hippocampus for Stau2 and compared the $Stau2^{KD}$ line to our control group (**Fig. 5B**).

The Pum2^{KD} mouse has been published before (Siemen et al., 2011). As this was the first time the mouse line was used in our lab, I investigated *Pum2* mRNA levels for the whole brain compared to our control group (Follwaczny et al., 2017). Testing using qRT-PCR showed *Pum2* mRNA levels to be significantly reduced up to 90% to reference. In accordance to the down-regulation of mRNA levels, the corresponding Pum2 protein levels, tested with immunohistochemical staining of the hippocampus, were also decreased.

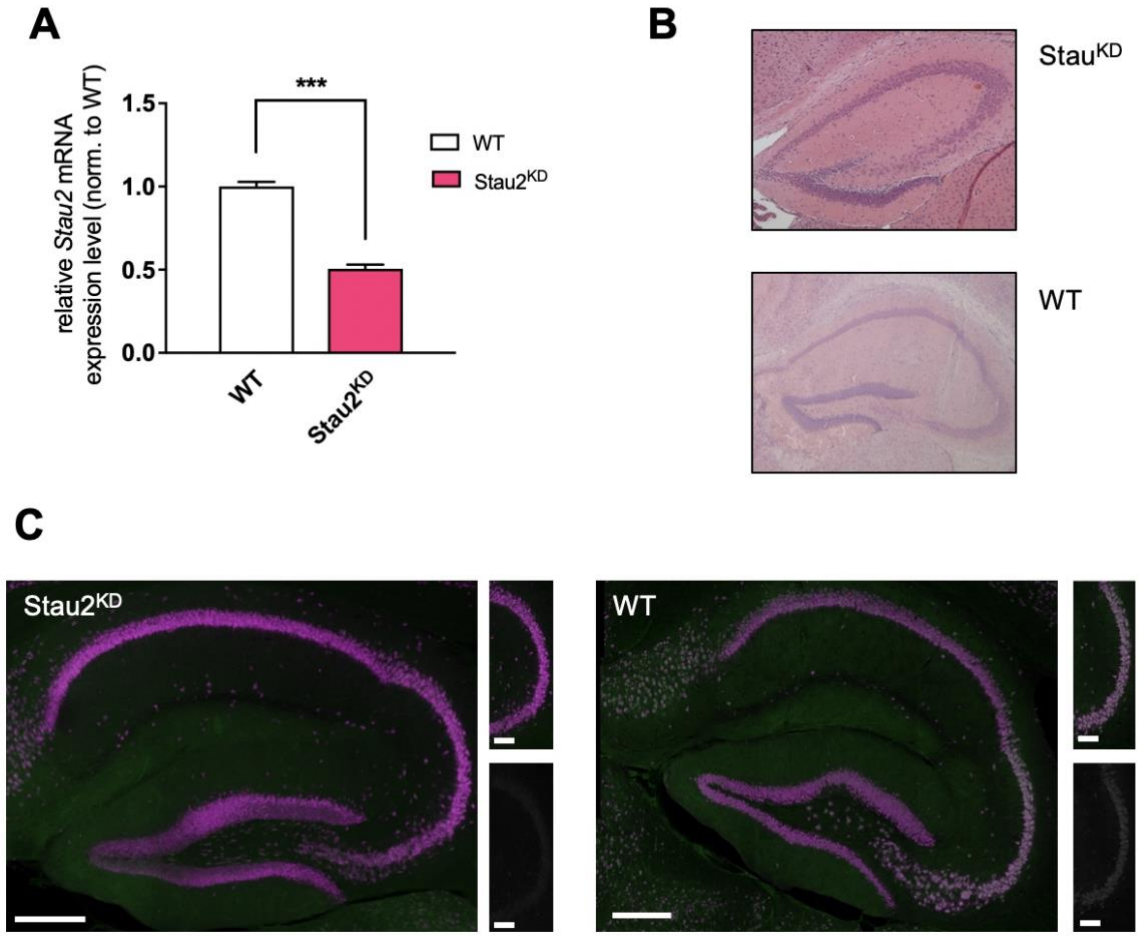


Figure 5: Stau2 levels are reduced in Stau2^{KD} mice.
(A) Stau2 mRNA levels in Stau2^{KD} and WT mouse brains (p= <0.0001). Statistics: Student's t-Test (n=8 for each group). Mean + SEM. ***p<0.001. **(B)** Representative images of H.E. stainings of the Stau2^{KD} (left) and WT (right) hippocampus of 21-day old mice. **(C)** Representative images of immunohistochemical staining of Stau2 protein in Stau2^{KD} (top) and WT (bottom) hippocampus. Big panels: overview. Stau2 = green, NeuN = magenta, scale bar = 200µm. Panels on the right: magnification of the CA3 subregion. Top panels = Merge of Stau2 (green) and NeuN (magenta) stainings. Bottom panels = Stau2 only (grey). Scale bar = 10µm.

When looking into the target protein and mRNA levels in whole brain isolates of the Stau2^{KD}/Pum2^{KD} mice both showed to be significantly reduced to about 30% and 10%

compared to levels in WT control mice proving success of the carry-over of the gene trap mechanism in the newly established Stau2^{KD}/Pum2^{KD} animals. Interestingly, Stau2 mRNA and protein levels seem to be lower than in same aged single Stau2^{KD} mice hinting to possible underlying synergistic effects of Stau2 and Pum2 (**data not shown**).

In conclusion, we could validate the success of the gene trap mechanism in all three knock-down mouse lines. While the knock-down is not a complete knock-out and Stau2 and Pum2 mRNA and protein levels vary upon target gene and mouse line, the incomplete knock-down might be better suitable model to study the mechanisms and roles of the proteins than a complete knock-out and draw assumptions to their role in humans as most symptomatic mutations a rarely a full knock-out.

3.1.2 General characterization

3.1.2.1 Weight

As part of the initial characterization of the mouse lines animal weight was measured. This parameter depends on multiple factors such as metabolism, activity and nourishment. All our animals had access to food and water ad libitum. Since mice were not monitored 24/7 daily activities were only observed during times of handling of the animals. To look out for gross differences between mouse lines, weight was recorded weekly during the behavioral testing regimen. Weight at time of sacrifice (recorded time point was 5 months) was used to compare the male specimen of Stau2^{KD}, Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mouse lines with a WT control group (**Fig. 6A**).

5 months old, male Stau2^{KD} mice did not differ significantly in weight compared to age and gender matched control animals, whereas 5 months old, male Pum2^{KD} mice in comparison weighed significantly less than age and gender matched control animals. Similar to Pum2^{KD}, 5 months old, male Stau2^{KD}/Pum2^{KD} show a significantly decreased bodyweight as compared to matched control animals. Stau2^{KD}/Pum2^{KD} animals also weighed observably less than Stau2^{KD} mice.

In brief, we found mice carrying a Pum2 gene-trap mutation, namely Pum2^{KD} and Stau2^{KD}/Pum2^{KD}, to weigh significantly less compared to WT control mice despite presumably normal food and water consumption. Further studies will be needed to tell, how daily activity levels, which could account for weight differences, compare between the 3 mouse lines and to a control group. We were excited to look into general locomotive behavior in the open field

test scheduled as a base assessment in our behavioral testing battery to screen for possible differences.

3.1.2.2 Nesting behavior

As described in the *Introduction*, nesting behavior can be used to broadly assess hippocampal function in mice. Mice with lesions in the hippocampus have been shown to score worse in nesting behavior (Deacon et al., 2002). This is independent from sex, as nesting is not solely a maternal task in mice. Nests appear to play a role for both sex types as they are needed for temperature control and shelter (Deacon, 2006).

Since both Stau2 and Pum2 proteins are expressed in the hippocampus and are suggested to play a role in memory processes at synapses, we used the nesting test as a convenient early screening tool for possible impairment (**Fig. 6B**). Stau2^{KD} mice exhibited better nesting performance than WT controls. Pum2^{KD}, in contrast, did not score differently when compared to WT, but did so when compared to Stau2^{KD}. Finally, Stau2^{KD}/Pum2^{KD} mice showed higher average nesting scores than Pum2^{KD}, but no significant difference to WT or Stau2^{KD}.

In conclusion, double knock-down mice show a rescue effect in performance in the nesting task as they perform significantly better than Pum2 single knock-down mice and in line with Stau2^{KD} and WT control mice. The nesting behavior assay we used is generally thought to be rather unspecific. A later chapter will cover the results in the better researched and more specific learning tasks we performed.

3.1.2.3 Open field assay

The open field (OF) test is a well-established behavioral test (Walsh and Cummins, 1976; Seibenhener and Wooten, 2015). It is commonly used to assess locomotor function and anxiety like behavior (Belzung and Griebel, 2001; Choleris, E, A. W. Thomas, M. Kavaliers, 2001). As it reflects basic behavioral patterns, it is a good reference for more complex tasks. We used the open field test to establish a baseline of the spontaneous ambulatory activity, but also to familiarize mice with the testing environment for the novel object recognition and novel object location (NOR/L) tasks. Mice are naturally driven to explore unknown environments, but fear wide, open spaces. This decreases with time as they get used to their surrounding (Choleris, E, A. W. Thomas, M. Kavaliers, 2001).

Testing setup of the OF task has already been explained. In brief, mice were placed in the center of a rectangular shaped and topless Plexiglas box under light and were allowed to roam the box freely during a 4 min period. The same process was repeated on the following day.

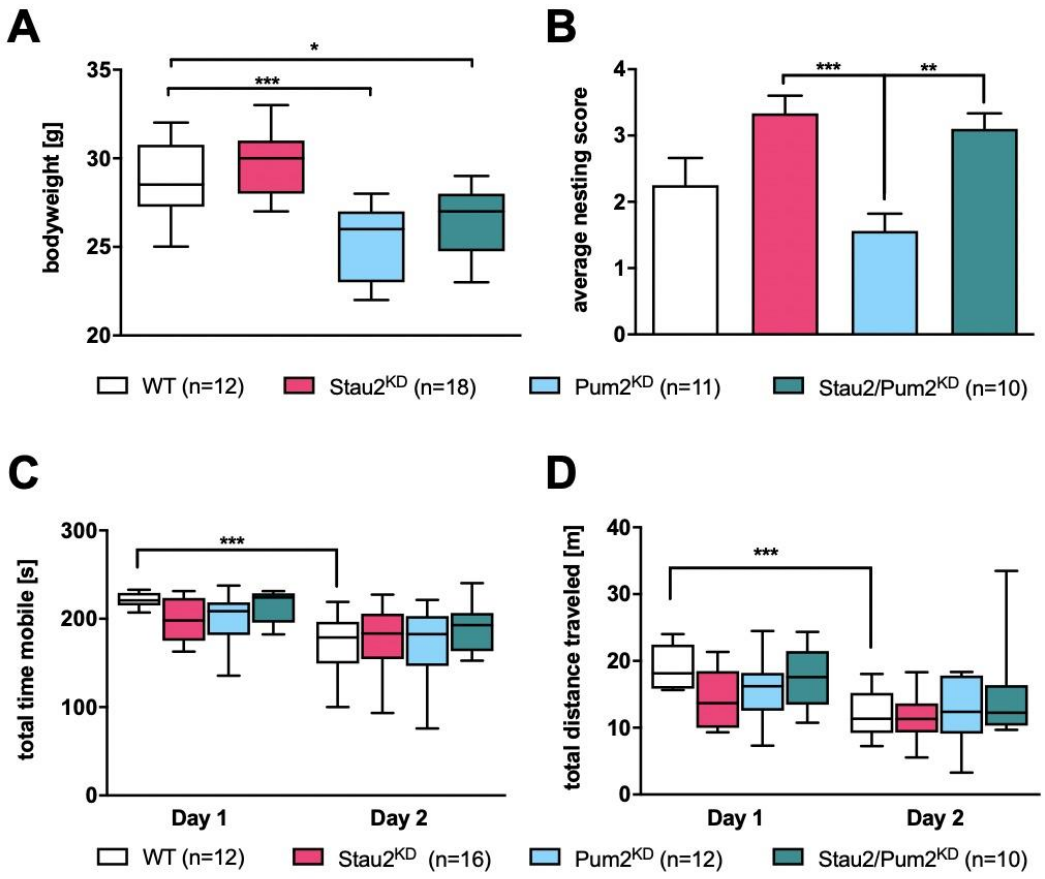


Figure 6: Stau2^{KD}/Pum2^{KD} mice show distinct anatomical and basic behavioral characteristics similar to Pum2^{KD} and Stau2^{KD}

(A) Bodyweight is significantly reduced in Stau2/Pum2^{KD} mice, similar to Pum2^{KD} mice. Average bodyweight was measured at 5 months of age for WT control, Stau2^{KD}, Pum2^{KD} and Stau2/Pum2^{KD} mice. Statistics: One-way ANOVA and Tukey post hoc (n-numbers are depicted). Mean + SEM. *p<0.05, ***p<0.001. **(B)** Stau2/Pum2^{KD}, as Stau2^{KD} mice(p=0.0003), show enhanced nesting scores compared to Pum2^{KD} mice. Nesting scores (1-4) were obtained after a 24h time period of individually housed animals at 20 weeks of age. Statistics: Mann-Whitney U-test (n-Numbers are depicted). Mean + SEM. ***p<0.001. **(C and D)** Stau2^{KD}, Pum2^{KD}, Stau2/Pum2^{KD} compared to WT mice (p= 0.0043 in **C** and p=0.0003 in **D**) fail to habituate in the Open Field (OF) paradigm. Total time spent mobile (C)and total distance (D) an animal traveled were analyzed for all three lines during the 4 min testing period for the first and second day of testing. Statistics: Kruskal-Wallis test (n-Numbers are depicted). Mean + SEM. ***p<0.001.

Basic ambulatory parameters, such as total distance travelled and total time mobile were recorded using an automated video tracking software.

First, we looked at the distance a mouse moves within the testing box in one trial on testing day 1 and 2, respectively, and compared the knock-down lines to their WT control littermates. WT showed signs of habituation as the distance a mouse travelled significantly decreased on the second day. All transgenic mouse lines, namely Stau2^{KD}, Pum2^{KD} and Stau2^{KD}/Pum2^{KD}

mice, travel less distance on the first day but match WT control littermates on day 2, suggesting failure to habituate (**Fig. 6D**).

As a second parameter, we determined the total time an animal spent moving within the open field during test duration ("total time mobile"). Similar patterns were observed as above. WT control mice showed signs of habituation on the second trial as the parameter 'time spent mobile' decreased. Again, Stau2^{KD}, Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice did not show significant habituation, but lower values on the first day of testing. Consequently, the control parameter 'time spent mobile' did not show any significant decrease (**Fig. 6C**).

Taken together, our results in the open field test show similar, decreasing patterns in moving time and distance for all mouse lines on both testing days, with smaller reductions for the transgenic mouse lines suggesting increased exploratory activity throughout the habituation process.

3.1.3 General morphology

As mentioned before, our mouse lines had not yet been thoroughly investigated. Therefore, we performed basic light microscopy stainings such as hematoxylin eosin (H.E.) and Nissl staining using 5 µm paraffin-fixed slices of whole mouse brains of all lines at the time point p21 to search for gross microscopic differences. We did not detect any major differences between the lines. Structure and built-up of the hippocampus seemed intact as compared to the control group. For reference, we show staining for WT and Stau2^{KD} mice (**Fig. 5B**).

As next step, we continued the general characterization of our mouse lines on the molecular level.

3.1.4 Initial molecular screening

As previously mentioned, this was an initial project to characterize the Stau2^{KD}/Pum2^{KD} mouse line, as well as – as a control – the Pum2^{KD} and Stau2^{KD} single knock-down mouse lines.

Therefore, we firstly set out to evaluate the effects of the respective knock-down of the RBP on known target mRNAs.

3.1.4.1 Expression levels of learning and Stau2 target mRNAs remain unchanged in Stau2^{KD} mice

For Stau2, we chose established target mRNAs, namely *Calm3* and *Rgs4* and additionally looked into two well-known players, *e.g. CaMKIIα* and *Arc/Arg3*.1, that often serve as

molecular markers for synaptic plasticity (Lisman et al., 2002; Plath et al., 2006). In *Drosophila*, Stau2 has been linked to *CaMKII*α before (Timmerman et al., 2013). Furthermore, we used *Arc/Arg3.*1, a well-known early marker for long-term potentiation (Bramham et al., 2008), for our screening. Here, I compared mRNA expression levels of whole brain lysates of WT and Stau2^{KD} mice at different time points in development, namely postnatal days 0, 21, and 5 months, respectively, via qRT-PCR.

For *Arc/Arg3.1*, Stau2^{KD} show similar expression patterns as WT with a significant 30-fold increase from p0 to p21 and slightly reduced levels at 5 months compared to p21 **(Fig. 7A).** Similar patterns were observed for *CaMKII*α*.* Expression levels both in Stau2^{KD} and our control group rise sharply with p21. For 5 months old animals, levels were further increased, which is significant for WT, but not for Stau2^{KD} (**Fig. 7B**).

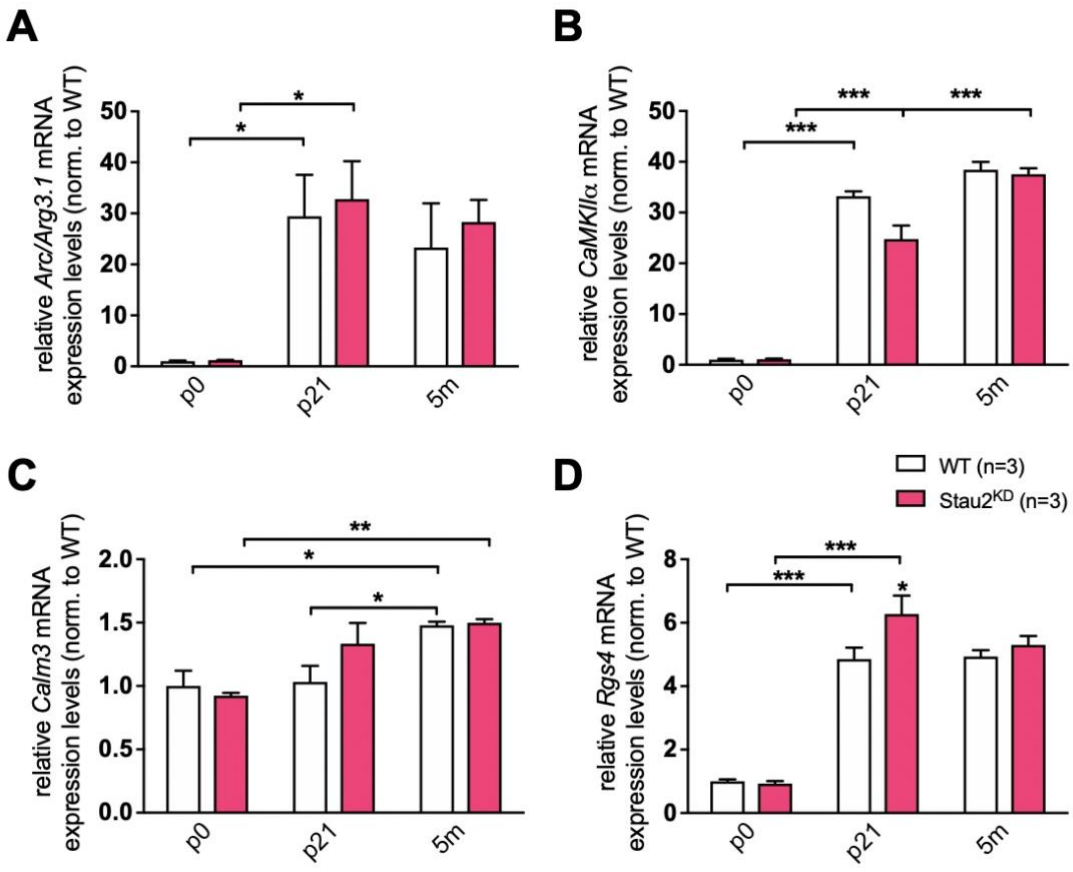


Figure 7: Stau2 associated target mRNAs show age dependent changes upon down-regulation of Stau2. qRT-PCR mRNA expression analysis for a known learning induced target, Arc/Arg3.1 (A), and several Stau2 associated targets, namely CaMKIIα (B), Calm3 (C) and Rgs4 (D) in total brain lysates obtained from newborn (p0), weaned (p21) and 5 months old WT and Stau2^{KD} mice, respectively. n-numbers are depicted. Statistics: ANOVA + Tukey post hoc. Mean + SEM. *p<0.05, **p<0.01, ***p<0.001.

Calm3 in our control group shows a steady increase from p0 to 5 months leading to a 1.5-fold change compared to base levels. Interestingly, this is matched for Stau2^{KD} **(Fig. 7C).** *Rgs4* levels show an increase of 5-fold from p0 to p21 in our control group and steady levels for 5 months. Stau2^{KD} show similarly increasing levels (**Fig. 7D**).

In conclusion, expression patterns of known Stau2 target mRNAs, namely *Calm3* and *Rgs4*, remain unchanged upon Stau2 knock-down. Well established markers for synaptic plasticity, such as *Arc/Arg3.1* and *CaMKII*α, also show significant changes upon Stau2 or Pum2 knock-down and similar expression patterns.

3.1.4.2 Epilepsy associated mRNA levels show changes in expression in Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice

Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice both exhibit age dependent, spontaneous tonic-clonic seizures. Therefore, we decided to firstly screen for changes of mRNA and protein levels of epilepsy associated targets on whole brain level and distribution within the hippocampus. Massive differences to WT control animals would suggest big changes in synaptic circuitry. Epileptogenesis is known to take place ahead of actual seizures (Goldberg and Coulter, 2013), which manifests at the age of around 5 months in our animals . Since this could potentially effect learning and memory formation, we wanted to exclude these effects before going ahead with extensive behavioral analysis.

The studies for the sodium channel family Na$_v$1 were carried out by Philipp Follwaczny in our lab and have already been published (Follwaczny et al., 2017). In my work I focused on the expression of voltage gated potassium channels and GABA$_A$-receptors.

3.1.4.2.1 Potassium channels (K$_v$)

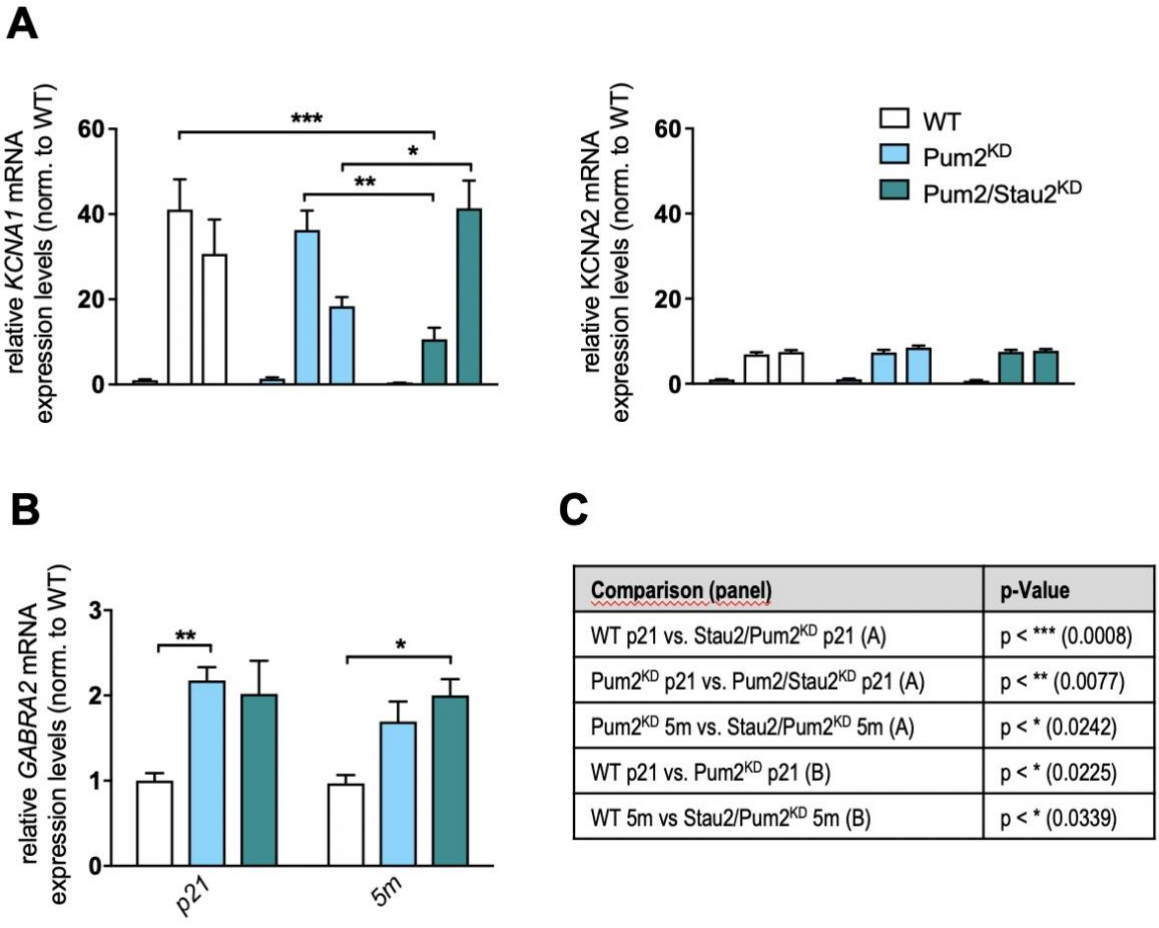
Voltage-gated potassium channels play an important role in the initiation and propagation of action potentials (Bean, 2007) as well as the regulation of intrinsic excitatory properties in neurons (Llinás, 1988). In mammalian models, K$_v$1.1 and K$_v$1.2 knock-down have revealed their importance in balancing neural excitability as knock-down leads to severe seizure phenotypes (Wenzel et al., 2007; Robbins and Tempel, 2012). Firstly, we explored, whether our Pum2^{KD} mice show changes in whole brain mRNA levels of these channels. I therefore determined mRNA concentrations at different points during development, namely at birth (p0), after weaning (p21), and at the onset of the seizures (5 months). *K$_v$1.1* mRNA levels in our WT control group show a 40-fold increase at p21 which is roughly maintained at 5m. Pum2^{KD} mice show similar changes at p21, but levels are not maintained and drop at 5 months (**Fig. 8A left**). In contrast, Stau2^{KD}/Pum2^{KD} mice only show a small increase from p0 to p21, which is

significantly less than WT controls, but recover to control levels with 5 months (**Fig. 8A left**). As for *K_V1.2* mRNA levels, our WT control group shows a 7-fold increase from p0 to p21, which is maintained into adulthood (5m). Pum2^{KD} and Stau2^{KD}/Pum2^{KD} show similar trends at p21 and 5m (**Fig. 8A right**).

Expression patterns of K_v1.1 are altered inversely in Pum2^{KD} and Stau2/Pum2^{KD} thus not giving conclusive evidence. K_v1.2 show similar trends in our Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mouse lines compared to the WT control group.

3.1.4.2.2 GABA receptors

$GABA_A$ receptors are ionotropic chloride ion transporters predominantly expressed in inhibiting interneurons in the hippocampus (Loddenkemper et al., 2014; Staley, 2015).



Comparison (panel)	p-Value
WT p21 vs. Stau2/Pum2^{KD} p21 (A)	p < *** (0.0008)
Pum2^{KD} p21 vs. Pum2/Stau2^{KD} p21 (A)	p < ** (0.0077)
Pum2^{KD} 5m vs. Stau2/Pum2^{KD} 5m (A)	p < * (0.0242)
WT p21 vs. Pum2^{KD} p21 (B)	p < * (0.0225)
WT 5m vs Stau2/Pum2^{KD} 5m (B)	p < * (0.0339)

Figure 8: Epileptogenic factors are mis-regulated in Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice.
(A and B) Expression analysis for KCNA1 (A left), KCNA2 (A right) and GABRA2 (B) coding for K_v1.1, K_v1.2 and GABRA2, respectively in total brain lysates obtained from newborn (p0), weaned (p21) and 5 months-old WT, Pum2^{KD} and Stau2/Pum2^{KD} mice (n=6 for GABRA2, n=3 for KCNA1 and 2). Statistics: One-way ANOVA. *p<0.05, **p<0.01, ***p<0.001. **(C)** Table listing detailed p-values shown in graphs (A and B).

Epileptic activity has been associated with loss of GABAergic interneurons and corresponding changes in $GABA_A$ receptor distribution (Bouilleret et al., 2000). As a first screening for potential target mRNAs, a fellow PhD student in our lab, Rico Schieweck, performed transcriptome analysis on whole brains of $Pum2^{KD}$ mice. These experiments revealed a significant upregulation of *Gabra2* levels, which codes for the alpha2 subunit of the $GABA_A$ receptor (Follwaczny et al., 2017). To further investigate this interesting finding, I conducted qRT-PCR on whole brain mRNA. Importantly, also $Stau2^{KD}$/$Pum2^{KD}$ mice were included in the analysis, since those animals also exhibited spontaneous seizures. Here, I checked *Gabra2* levels at three points in development (p0, p21 and 5 months) to see whether possibly changes are age- and seizure-onset dependent. Interestingly, *Gabra2* levels significantly increased in weaned and adult animals (p21 and 5 months) in both mouse lines (**Fig. 8B**). This effect is specific for the $GABA_A$ receptor subtype since *Gabbr2*, coding for a subunit of the $GABA_B$ receptor, remained unaffected (**data not shown**).

In collaboration with Philipp Follwazcny, a fellow M.D. student in our lab, I showed in immunohistochemical stainings of $GABA_A$ receptors on coronal whole-brain slices in our $Pum2^{KD}$ mice, that this up-regulation corresponds to an increase at protein level and especially manifests in a dendritic localization in the CA1 subregion of the hippocampus (Follwaczny et al., 2017). The occurrence of seizures is usually preceded by a process called epileptogenesis, wherein alteration in circuit function and homeostasis build up to eventually lead to aberrant excitability and seizures (Goldberg and Coulter, 2013).

Taken together, *Gabra2* mRNA is upregulated in both $Pum2^{KD}$ and $Stau2^{KD}$/$Pum2^{KD}$ mice in p21 and adult animals compared to control, suggesting *Gabra2* to be regulated by Pum2. These findings go in line with the Gabra2 protein level as $Pum2^{KD}$ mice show an up-regulation of $GABA_A$ in the dendritic compartment of the CA1 region the hippocampus. Together, these data could suggest changes of the balance of excitation and inhibition within the neural network of the hippocampus possibly giving rise to the seizures observed in adult animals.

In conclusion, we think that our results justify going on to perform behavioral studies with animals that are below five months of age, thus before the onset of spontaneous seizures. We therefore went ahead and conducted a 4-week behavioral testing regime with younger animals of all 3 transgenic mouse lines and one WT control line.

3.2 Effect of the RBP knock-down on spatial learning and memory

Hippocampal adult neurogenesis is known to contribute to learning and memory, especially to spatial long-term memory formation (Deng et al., 2010) and novelty response (Barker and Warburton, 2011). A previous study performed in *Drosophila* has linked Stau2 and Pum2 to long-term memory formation (Dubnau et al., 2003). We therefore set out to perform a battery of hippocampal specific memory tasks with the above-mentioned transgenic mouse lines.

In brief, we started with the previously described open field task to get the animals accustomed to their surroundings. In the following week, we performed multi-stage Novel Object Recognition (NOR) and Novel Object Location (NOL) tasks. These tasks are well established for investigating hippocampal memory (Cohen and Stackman, 2015). In the third week, we trained mice in the Barnes Maze task and completed the battery of assays with testing mice in the Barnes Maze in the fourth week. The Barnes Maze is a well-established alternative to the traditional Morris Water Maze with the advantage of not using strong aversive stimuli such fear or food deprivation which could alter test performance (O'Leary and Brown, 2012).

3.2.1 Novel Object Recognition and Novel Object Location

The object recognition task was first implemented in rats (Ennaceur and Meliani, 1988) and later adapted for the use in mice. It is based on the natural behavior of mammals to be attracted to novel objects. Exploration of these objects creates new memories, which can be recalled at a later point in time depending on the time of exposure (Antunes and Biala, 2012; Moscardo et al., 2012). As for the novel object paradigm, an animal will spend more time with an object it has not previously encountered (NOR) or with an object that is familiar but in a different location (NOL) (Vogel-Ciernia and Wood, 2014) (**Fig. 9**). Depending on the time spent with these objects, a discrimination index accounting for the time spent with the familiar object (T_F) as opposed to the novel object (T_N) can be calculated comparing the two time intervals ($DI=(T_N-T_F)/(T_N+T_F)$) (Antunes and Biala, 2012). If the animal spent more time with the novel object, the DI is positive. It turns negative, if more time was spent with the familiar object.

Mice with hippocampal lesions have been shown to perform worse in NOL tasks then control groups (Barker and Warburton, 2011). However, it has remained somewhat controversial as to what point parahippocampal structures, e.g. the perirhinal cortex are involved in object recognition and location as well as novelty response (Cohen and Stackman, 2015). Since both RNA-binding proteins, namely Stau2 and Pum2, are predominantly expressed in the hippocampus, we used the NOR/NOL paradigm to determinate whether any

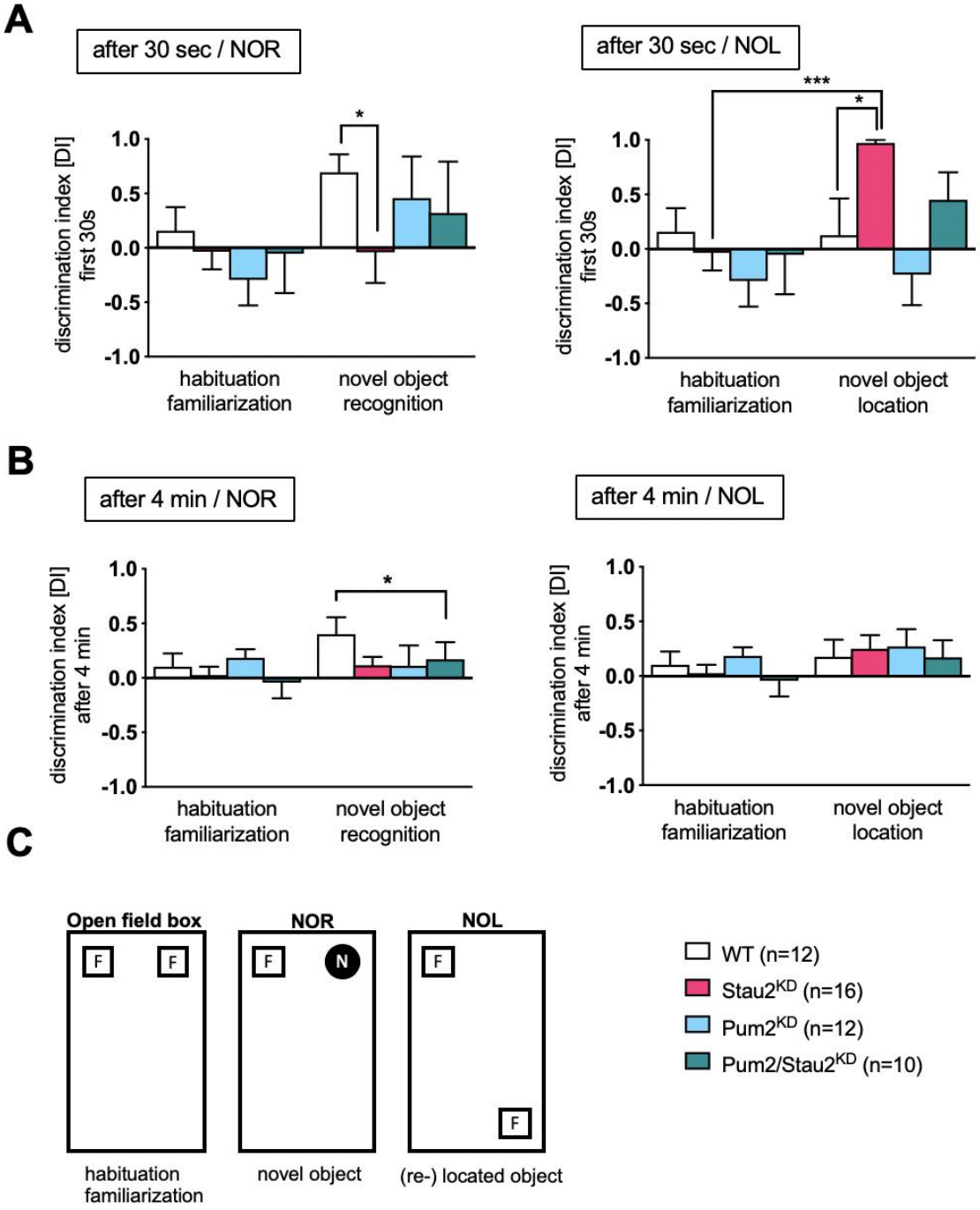


Figure 9: Novelty detection is impaired in Stau2^{KD} and Stau2^{KD}/Pum2^{KD} mice.
(A and B) Discrimination indexes (DI=(Time$_{novel}$-Time$_{familiar}$)/(Time$_{novel}$+Time$_{familiar}$)) for NOR (left) and NOL (right), respectively, were calculated for the first 30 s of each test condition (A) and for the entire 4 min testing period (B). Statistics: Mann-Whitney U-test. Mean+SEM. (A left) $p_{Stau2KD}$ = *0.0263, (A right) $p_{Stau2KD}$ < *** (0.0005=, $p_{Stau2KD}$ < * (0.0430), (B left) $p_{Stau2/Pum2KD}$ < * (0.0260). **(C)** Testing setup for the NOR/L paradigm. Abbrev.: NOR = Novel object recognition, NOL = Novel object location, F = familiar object, N = novel object.

change in performance in the NOL or the NOR can be detected. We performed both NOR and NOL with Stau2^{KD}, Pum2^{KD}, and Stau2^{KD}/Pum2^{KD} mouse lines and compared the results to those for our control group in the previously described test setup. As mice are known to interact more with novel objects in the first 30 s of their encounter (Cohen and Stackman, 2015), we compared the DI of the first 30 s with the DI after the full 4min testing period.

In the NOR task, our control group showed an expected increase in the DI in the first 30s (**Fig. 9A left**). Similar trends were observed when looking at the full 4min testing period (**Fig. 9B left**). Stau2^{KD} mice, in contrast, showed no increase in the DI when looking at the 30s interval and a significantly decreased DI compared to the control group (**Fig. 9A left**). Similar – though not statistically significant – observations were made when looking at the full 4 min testing period (**Fig. 9B left**). Pum2^{KD} mice showed an increase in DI from the familiarization to the NOR testing in the first 30s, but not for the 4 min testing period (**Fig. 9A,B left**). Stau2^{KD}/Pum2^{KD} mice showed similar trends as Pum2^{KD} mice, as they showed an increase in DI for the NOR task for the 30 sec interval as well as the 4 min testing period (**Fig. 9A,B left**).

In the NOL task, our control group showed no difference in the time of interaction with the familiar object as opposed to the newly located object indicated by a similar DI (**Fig. 9A,B right**). In contrast, Stau2^{KD} mice showed a significant increase in DI within the first 30 s of the NOL task that was also significantly higher than the corresponding DI for WT controls. Similar although weaker effects were seen for the 4 min testing period (**Fig. 9A,B right**). Pum2^{KD} mice, similar to our control group, showed no difference in the DI for the NOL (**Fig 9A,B right**). Interestingly, for the NOL task Stau2 ^{KD}/Pum2^{KD} mice showed similar effects as Stau2^{KD} mice. In detail, Stau2 ^{KD}/Pum2^{KD} mice showed increased interaction with the familiar object as opposed to the newly located object indicated by an increased DI in the NOL. This was apparent after 30 s and to a lesser extent maintained within in the full 4 min testing period (**Fig. 9A,B right**).

Taken together, for NOR, Stau2/Pum2^{KD} mice showed similar performance to our WT control. This is resembled in Pum2^{KD} mice, but Stau2^{KD} mice perform worse, suggesting compensatory effects upon double KD of Stau2 and Pum2. For the hippocampal dependent NOL task, Stau2^{KD}/Pum2^{KD} and Stau2^{KD} perform better than our WT control group and Pum2^{KD}, again indicating a synergistic, compensatory effect in the Stau2/Pum2 memory pathway.

3.2.2 Barnes Maze

At last, we performed the Barnes Maze, which is widely used for testing spatial memory formation. In contrary to similar spatial memory tests, the Barnes Maze does not use strong aversive stimuli or food deprivation, which could potentially alter test performance. It relies on a rodents drive to escape bright light and open surfaces (O'Leary and Brown, 2012). Using spatial cues, the animal learns to navigate the holes and find the escape box. This task depends on the formation of visuo-spatial memory, requiring hippocampal functioning (O'Keefe and Dostrovsky, 1971; Broadbent et al., 2004). Experiments were carried out adapted for mice as previously described (Bach et al., 1995).

General locomotive parameter such as mean speed or time spent in the target zone remain basically unchanged for all mouse lines further ruling out baseline shifts in performance comprising comparability or significance of results throughout (**Fig. 10**). Stau2^{KD} and Pum2^{KD} mice, respectively, showed no difference in success of entering the target zone surrounding the flight box compared to control (**Fig. 10A,B right**). Importantly, both mouse lines showed a significant reduction in the success rate of entering the flight box (**Fig. 10A,B left**). In contrast Stau2^{KD}/Pum2^{KD} showed similar success rates compared to control animals (**Fig. 10B left**). Interestingly Stau2^{KD}/Pum2^{KD} were significantly faster in entering the target zone compared to control animals (**Fig. 10B right**). All transgenic mouse lines implemented similar searching strategies as our control group directed to the flight box with episodes of trial and error (**data not shown**) (Popper, Demleitner et al., 2018).

Taken together, Stau2^{KD} and Pum2^{KD} animals took more time to find the target box and zone compared to WT control animals suggesting altered formation of spatial memory. Stau2^{KD}/Pum2^{KD}, perform similar if not better than our control group. This further suggests KD of a single gene of the Stau2/Pum2 pathway affects hippocampal memory, but performance is salvaged upon knockdown of both genes.

3.3 The role of RBPs in (induced) neurogenesis

Learning and memory in adults have been linked to adult hippocampal neurogenesis. Physiologically, neurogenesis is most active during embryonal development and then declines with age. The hippocampus is one of the few places in the adult brain, where neurogenesis continues to take place well into adulthood (Altman and Das, 1965; Kornack and Rakic, 1999). Learning has been shown to promote survival of newborn neurons 1 to 2 week after mitosis and to thereby facilitate adult neurogenesis (Gould et al., 1999; Kempermann et al., 2015).

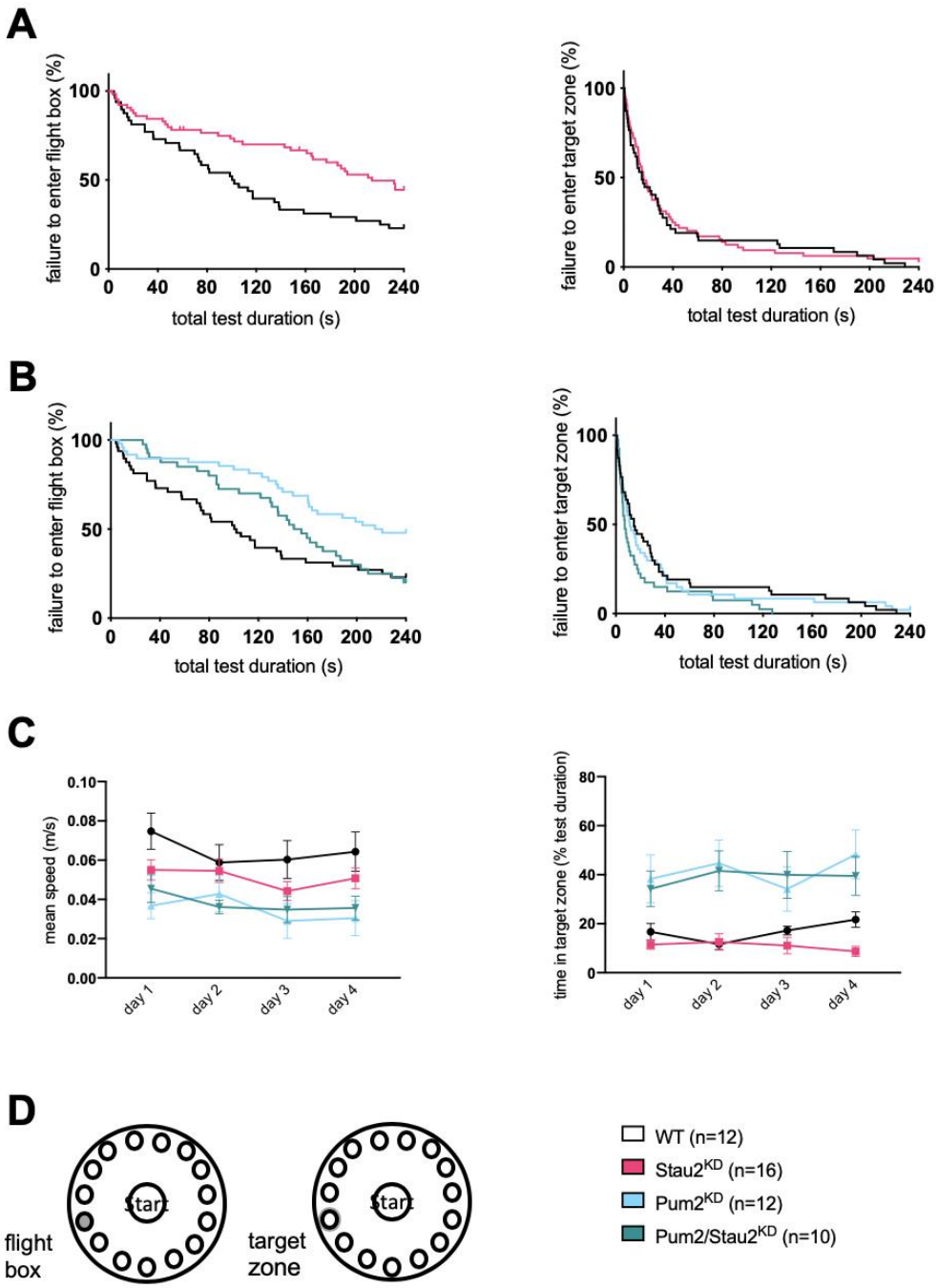


Figure 10: Long term memory is unchanged upon Pum2 down-regulation.
(A and B) Kaplan Meier Curves for WT, Stau2^{KD} (A), Pum2^{KD} and Stau2/Pum2^{KD} (B) mice for successful entry into the target zone (left) and afterwards into the flight box (right). Statistics: Log rank test (n-numbers are depicted). $p_{Stau2KD}$ < ** (0.0044) and p_{Pum2KD} < ** (0.0012) for entering the flight box (A and B left). $p_{Stau2/Pum2KD}$ < * (0.0368) for entering the target zone (B right). **(B and C)** Mean speed (B) and time in target zone (C) for WT and Pum2^{KD} mice. n-numbers are depicted. Statistics for repeated observations on day 1 through 4 were calculated using Kruskal Wallis followed by Dunn's multiple comparison. Mean + SEM. **(D)** Testing setup for the Barnes Maze. Mice were located in the "start" area and had to find the target zone and enter the flight box located below one of the 20 holes. Figure adapted from *Popper, Demleitner et al.* (2018).

Especially temporal lobe epilepsy (TLE) has been linked to disturbed neurogenesis (Parent et al., 1997; Cho et al., 2015). The acute phase of TLE is associated with increased neurogenesis, whereas in the chronic phase a decrease in newly generated neurons has been reported (Hattiangady and Shetty, 2008). During maturation, adult born neurons show proper connectivity, mostly to the CA3 area by developing their dendritic tree in this region (Markakis and Gage, 1999; Toni et al., 2008). Proper dendritic branching of newborn neurons is seen, when they are properly integrated into an existing circuitry (Zhao et al., 2006). This process, however, is disrupted, when epileptic activity occurs and cells show altered dendritic branching (Jessberger et al., 2007; Murphy et al., 2011).

In this thesis, the impact of RBPs in these processes has been experimentally evaluated. The role of Stau2 in embryonal neurogenesis has already been investigated (Kusek *et al.*, 2012, also Vessey 2012). In these two ground-breaking studies, Stau2 KD promotes the transition from RGCs to IPCs in embryonal neurogenesis, therefore leading to premature neuronal maturation. Combined neural-specific knock-out of Pum1 and Pum2 has been shown to decrease the number of neuronal stem cells in the hippocampus and consequently the number of newly generated newborn cells (Zhang et al., 2017). Both Stau2 and Pum2 are predominantly expressed in the hippocampus (as well as in testis) and have been shown to be involved in processes contributing to synaptic plasticity (Vessey et al., 2010; Heraud-Farlow and Kiebler, 2014). $Pum2^{KD}$ and $Stau2^{KD}/Pum2^{KD}$ mice both exhibit spontaneous age-dependent seizures. We therefore wanted to take a look into adult neurogenesis in these mouse lines.

We did so by immunohistochemical staining of coronal slices of adult (5 months old) mouse brains. As marker for neurogenesis, we used Doublecortin (DCX), a well-established marker for 2 to 6 week old newborn neurons (von Bohlen und Halbach, 2011). DCX is specific for newborn neurons from the early stages of differentiation and migration, as DCX positive cells lack antigens for glial, undifferentiated or apoptotic cells (von Bohlen und Halbach, 2011).

Figure 11 displays an overview of the immunostained hippocampus of either WT **(Fig. 11A),** $Stau2^{KD}$ **(Fig. 11B**), $Pum2^{KD}$ (**Fig. 11C**) or $Stau2^{KD}/Pum2^{KD}$ (**Fig. 11D**) mice, respectively. The locus of adult neurogenesis is clearly visible as a band of DCX+ cells in the subgranular zone (SGZ) in the overview picture on the left side of each row. The middle and right panel, respectively, show representative magnifications of the DCX+ cells and the dendritic arbor of naïve **(middle)** and trained mice (**right**). Tile scans of predefined areas of the hippocampus were obtained and analyzed as described under *Materials and Methods*.

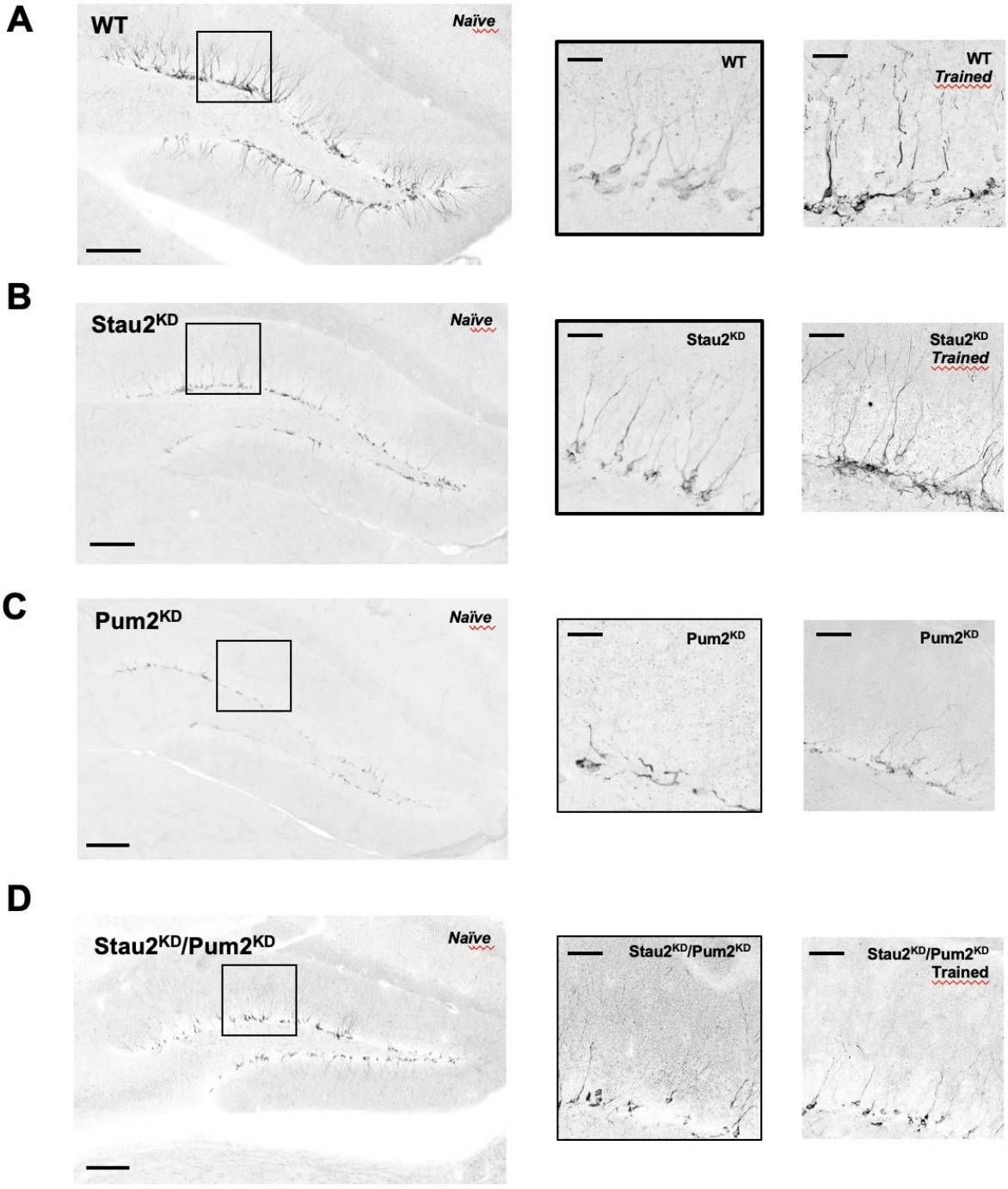


Figure 11: DCX staining of the hippocampus of naïve and trained WT and KD mice.
Pictured are (from left to right) a representative overview of DCX staining of the entire DG (scale bar = 250 µm) for naïve WT (**A**), Stau2^{KD} (**B**), Pum2^{KD} (**C**) and Stau2^{KD}/Pum2^{KD} (**D**) mice, respectively. Boxes indicate location of magnified inset shown in the middle. The right small boxes show a comparable inset from a trained mouse, scale bar = 25µm.

For Stau2^{KD} mice, we started the analysis of these images with a rough estimate of DCX positive cells in our tile scans using a macro (‘cell profiler’) generated by my colleague Philipp Follwaczny. Stau2^{KD} mice, which had been exposed to the behavioral testing regimen, showed a significant increase in DCX intensity in the dentate gyrus as compared to an age matched Stau2^{KD} control group. Surprisingly, these levels were also significantly above the levels of control group WT mice, that had been exposed to the same testing regimen (**data not shown**). As this data was only a first rough estimate substituting total cell numbers by signal intensity, we looked for an approach that was less influenced by background signal. We opted for manually quantifying cell numbers normalized to the length of the SGZ as well as length of primary and secondary dendrites to gain first insight into both development and maturation of the newborn neurons using the analysis program FIJI.

3.3.1 Cell density of DCX+ neurons in the SGZ of the hippocampus

Naïve SGZs of the hippocampus of adult Stau2^{KD} mice (5m) showed no significant difference in normalized cell numbers compared to WT controls. In Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice, DCX+ cells are significantly decreased when compared to WT littermates (**Fig. 12A**). Interestingly, Stau2^{KD} mice, which were exposed to the behavioral testing battery, showed a markedly increased number of DCX+ cells in the SGZ as compared to age matched Stau2^{KD} or WT control animals, respectively. A similar trend was observable in Pum2^{KD} mice. This increase was neither seen in our WT control group, nor, surprisingly, in Stau2^{KD}/Pum2^{KD} mice (**Fig. 12B**).

In conclusion, knock-down of either Stau2 or Pum2 leads to reduced generation of new neurons in the adult hippocampus. The effects of both proteins are not additive as Stau2^{KD}/Pum2^{KD} naïve mice show slightly more DCX+ cells than Pum2^{KD} mice. Upon exposure to a 4-week learning focused behavioral testing regimen as stimulus, only Stau2^{KD} and Pum2^{KD} mice yielded increased cell counts. This may point to a different response of neural progenitor cells in the SGZ to stimuli upon knock-down of either Stau2 or Pum2, that is rescued when both proteins are lacking.

3.3.2 Dendritic complexity of DCX+ neurons

Next, we looked into the establishment of the dendritic arbor of newly generated neurons in our mice as sign for proper integration and function of the cells. Interestingly, less newly generated neurons in Stau2^{KD}/Pum2^{KD} mice show primary dendrites as compared to WT

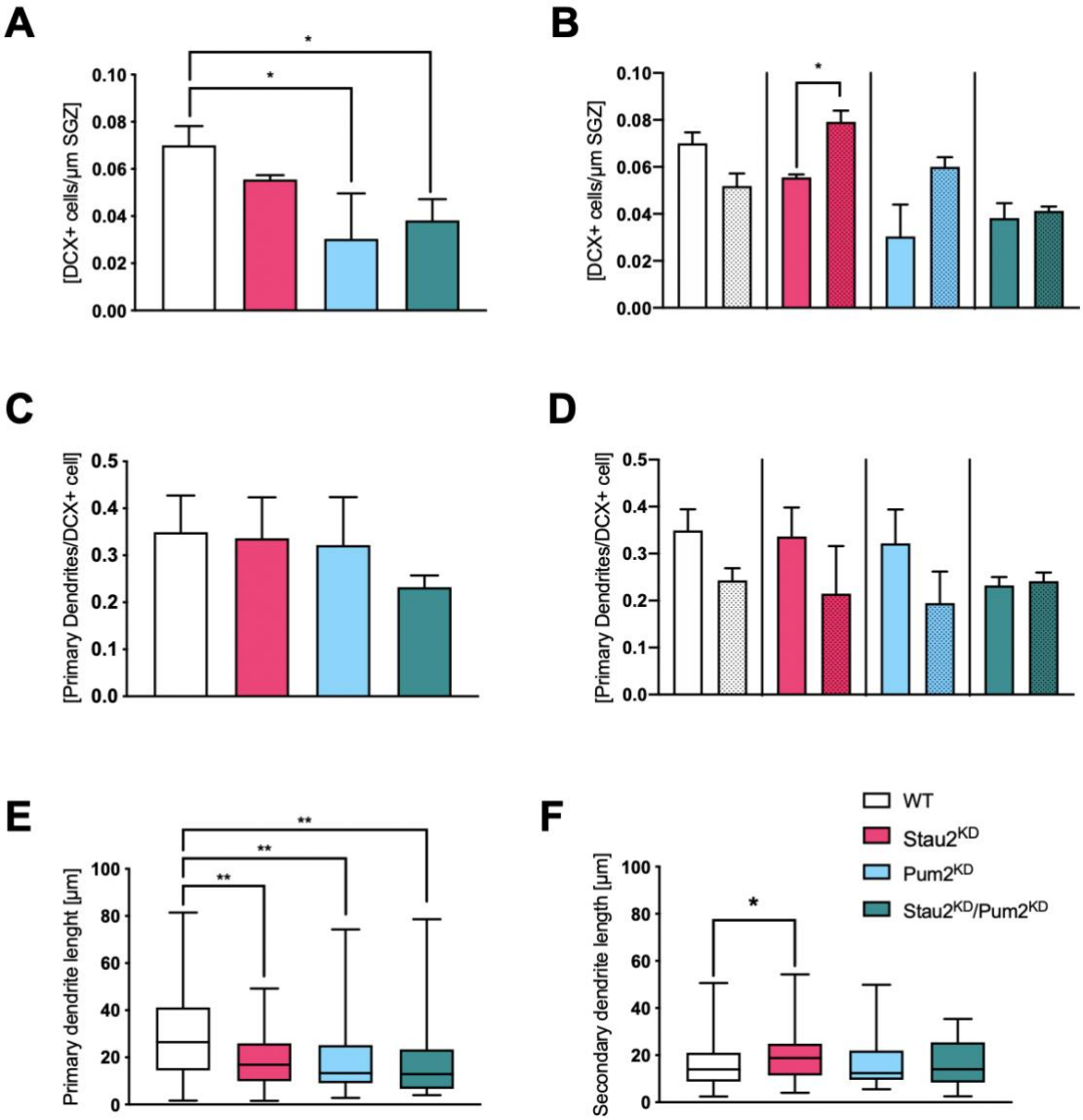


Figure 12: Stau2^{KD} mice show enhanced neurogenesis after learning while Pum2 down-regulation leads to impaired adult neurogenesis in mice.
Trained mice, which underwent the behavioral testing paradigm (marked with dotted bars), were compared with naïve controls. (**A and B**) shows the Quantification of DCX+ cells averaged to the length of the subgranular zone for naïve (**A**) mice and a comparison between naïve and trained mice (**B**). **(C and D**) show the ratio between counted primary dendrites and counted DCX+ cells per biological replicate for naïve mice **(C**) and - in comparison - with trained mice **(D**). **(E**) indicates the average length of the primary dendrites and **(F**) the average length of secondary dendrites analyzed in naïve animals. Unicolor columns: naïve, Dotted columns: learning. Statistics: Student's t-Test (A-D) and Kruskal Wallis + Dunn's multiple comparison (E and F). Mean + SEM. P-values: (A) p_{Pum2KD} < * (0.0438), $p_{Stau2KD/Pum2KD}$ < * (0.0259), (B) $p_{Stau2KD}$ < * (0.0407), (E) $p_{Stau2KD}$ < ** (0.0015), p_{Pum2KD} < ** (0.0098), $p_{Stau2KD/Pum2KD}$ < ** (0.0015), (F) $p_{Stau2KD}$ < * (0.0106)

control, Stau2^{KD} or Pum2^{KD} mice, respectively (**Fig. 12C**). When comparing the ratio of naïve animals with those of trained animals, that had been exposed to the behavioral testing battery, Stau2^{KD}/Pum2^{KD} mice show no obvious change in primary dendrites per DCX+ cell. WT,

Stau2^{KD} and Pum2^{KD} mice, respectively, show a reduction of primary dendrites relative to the number of cells generated (**Fig. 12D**). Next, we looked into the length of the dendrites.

Interestingly, all transgenic mouse lines have significantly shorter primary dendrites than our WT control animals (**Fig. 12E**). The length of secondary dendrites remains basically unchanged. Stau2^{KD} mice, however, have significantly longer dendrites compared to our control group (**Fig. 12F**).

Taken together, generation of new neurons in the hippocampus is reduced in all transgenic mouse lines. Stimulation by a 4-week long behavioral testing battery resulted only in altered neurogenesis in Stau2^{KD} and Pum2^{KD} mice, but not Stau2^{KD}/Pum2^{KD} mice, respectively. The observed dendritic arbor of those cells is altered in all our transgenic mouse lines, especially upon knock-down of both Stau2 and Pum2. Fewer newly generated neurons in Stau2^{KD}/Pum2^{KD} animals have primary dendrites, compared to the other transgenic lines and our WT control group. A finding that is conserved after stimulation by the behavioral testing battery. For the other mouse lines, the ratio is reduced upon learning, suggesting more newly born neurons, that have yet to develop a properly aligned primary dendrite, are present. Thus, both Stau2 and Pum2 affect adult neurogenesis in different, possibly additive ways.

As this was only a first characterization of the neurogenesis observed in our transgenic mouse lines many open questions remain. Of great interest will be, whether the newborn neurons in our mouse lines integrate properly into the existing synaptic circuitry thereby aiding the functions of the hippocampus such as pattern separation. It will be crucial to understand, whether the changes I observed contribute to the defective hippocampal memory seen in Stau2^{KD} and Pum2^{KD} animals. Though this will not be the only answer as neurogenesis is affected in Stau2^{KD}/Pum2^{KD} animals, but hippocampal memory is intact in those mice.

4 Discussion

As aforementioned in the *Introduction*, RBPs have been attributed important roles in neural function and disease (Tolino et al., 2012). Stau2 and Pum2, but also FMRP amongst others, have been linked to impaired performance in hippocampus based memory tasks including long-term spatial memory and object recognition memory (TD-BFX, 1994; Siemen et al., 2011; Berger et al., 2017).

Because of the increasing evidence that the functions of Pum2 and Stau2 in embryonal neurogenesis and in hippocampal memory might be intertwined, I decided to take deeper insight into a possible joint role of Pum2 and Stau2 in hippocampal memory and subsequently in adult neurogenesis. To my knowledge, this study is the first to employ a double knock-down mouse model for both Stau2 and Pum2 to gain insight into the role of the Stau2/Pum2 pathway in cognition in higher eukaryotes.

4.1 Stau2 and Pum2 play a modulating role in hippocampal memory

A study published while this thesis was in progress showed conditional Cre-mediated Stau2^{KD} rats have impaired spatial memory long-term memory (Berger et al., 2017). In brief, rats were trained in the Morris water maze using a fixed platform. Here, a platform is immersed in one quadrant of a round pool just below water level. Rats that are placed into the pool cannot see the platform, but try to escape from the water. Eventually, they hit the platform and stay on it. For all trials, the platform remains in the same location. Interestingly, Stau2^{KD} rats perform worse after a 30 min and after a 6 h delay between individual test runs compared to wt littermates. When rats, however, were tested with a shortened interval of 1 min between tests, memory performance remained intact.

In line with this finding is that our Stau2^{KD} mice showed intact short-term spatial memory as observed in the Barnes Maze training phase, where animals were tested with a delay of 15 min (**data not shown**). Upon longer delays (24 hours), where performance depends on hippocampal long-term memory, our Stau2^{KD} mice, too, performed worse compared to WT control animals. These findings suggest robust disruption of hippocampal spatial memory, as reduced Stau2 levels led to impaired performance in two separate spatial memory tasks in two different studies. In our congenital Stau2^{KD} mice, novel object recognition (NOR), but not spatial novelty recognition (NOL) was impaired, whereas the rats used by *Berger et al*. show impaired spatial novelty recognition. but not novel object recognition. Besides interracial differences in behavioral tasks (Frick et al., 2000; Cohen and Stackman, 2015), testing duration

and delay between sample phases are important parameters that may alter performance (Antunes and Biala, 2012). As test duration and retention delay were shorter in the protocol used by *Berger et al*. (2017), the obtained data is not really comparable. Additionally, the conditional knock-down used in their study was only activated by administration of tamoxifen by the time the rats were born. As previously mentioned, Stau2 has been attributed an important role in embryonal neurogenesis. The effects this might have on hippocampal based learning tasks has yet to be determined.

In line with *Siemen et al.* (2011), the Pum2^{KD} mice used in this study showed decreased nesting scores and good novelty recognition in the NOR. Interestingly, Pum2^{KD} mice showed superior performance in the Morris Water maze, an spatial memory based, hippocampus-dependent task (Siemen et al., 2011). In contrary, Pum2^{KD} mice in our study showed impaired performance in the Barnes maze, a comparable, stress-reduced spatial memory task. Pum2^{KD} mice are smaller and show spontaneous seizures with an onset at 5 months of age. The Morris water maze is a test known to cause stress – which may trigger seizures – and potentially weight loss (Harrison et al., 2009). Considering these limitations to the Morris water maze, it is questionable, whether the testing procedure used by *Siemen et al.* study is truly reliable for this mouse model. A less stressful alternative, like the Barnes maze used in our study, may be a better choice for evaluating hippocampal memory. *Zhang et al.* (2017) also exploited a low-stress cognitive test focusing on spatial memory – the Lashley III maze – in their study on Pum1/Pum2 knock-down mice. Here, mice showed impaired spatial memory. This is in line with our findings further supporting a possible role for Pum2 in hippocampal memory formation. The drastic effects seen by *Zhang et al.* (2017) may be in part due to the co-joined Pum2 and Pum1 knock-down, which both play important roles in neurons, though their distinct roles are less elusive. However, Pum1 levels remain unchanged in our mice (Schieweck et al., *unpublished*).

In Stau2^{KD}/Pum2^{KD} mice, both object recognition as well as object location memory is intact- Consequently, mice performed equally good – if not better – compared to WT control animals in short- and long-term spatial memory tasks. Taking together, double knock-down mice of Stau2 and Pum2 appear to rescue the effects on hippocampus-based memory seen in the respective single knock-down mouse lines.

On a side-note, it is interesting to speculate, what might be the reason Stau2^{KD} mice fail in both spatial memory and novelty detection but Pum2^{KD} mice only show weaker performance in the Barnes maze spatial memory task. Stau2 is needed for proper performance in object recognition and spatial memory tasks. Pum2 appears to be only needed for proper performance

in spatial memory tasks. Both proteins are predominantly expressed in the hippocampus (Siemen et al., 2011; Popper, Demleitner et al., 2018). The hippocampus plays a key role in the formation of especially long term spatial memory and – to a lesser extent – of novelty response (Broadbent et al., 2004; Cohen and Stackman, 2015). Novelty response or object recognition memory is in our current understanding greater affected by lesions in the peri- and entorhinal cortex, structures also belonging to the hippocampal memory axis (Winters et al., 2008; Barker and Warburton, 2011). Novelty response seems to be less dependent on hippocampal function tissue than spatial learning (Broadbent et al., 2004). It is therefore tempting to speculate that Pum2 function plays only a minor role in hippocampal memory compared to Stau2, as knock-down of Pum2 only affects spatial memory but not novelty response.

Besides these considerations about possible differences in novelty detection and spatial memory, an intriguing finding is that simultaneous knock-down of Stau2 and Pum2 did not impair hippocampal memory. Stau2 and Pum2 on their own are both needed for proper performance in spatial memory tasks as shown in several different studies using different testing regimens (Siemen et al., 2011; Berger et al., 2017; Zhang et al., 2017). This raises clearly important questions. Either the pathway containing Stau2 and Pum2 might not be crucial for hippocampal memory, or, alternatively, Stau2 and Pum2 play inter-dependent roles that – upon knock-down of both proteins – can rescue each other. Evidence for this theory might be that $Stau2^{KD}/Pum2^{KD}$ mice show slightly impaired performance in the NOR/L, but not in the Barnes maze. An interesting publication by Abel and colleagues showed a similar mechanism with two different proteins, Translin/Trax and the activin A receptor type 1C (ACVR1C). Translin/Trax is an RNA-binding protein complex consisting of two proteins, which inhibits translational repression of target mRNAs through microRNAs (Asada et al., 2014). It has been shown to localize to dendrites upon stimulation (Wu et al., 2011). *Park et al.* (2017) showed that Translin-KO mice have impaired long-term object spatial memory and showed upregulation of several miRNAs, which – among others – target ACVR1C (Park et al., 2017). Down-regulation of ACVR1C in WT mice impaired spatial long-term memory, but down-regulation of ACVR1C in Translin-KO mice did not impair spatial long term memory (Park et al., 2017), much like long-term spatial memory stays vastly unaffected in $Stau2^{KD}/Pum2^{KD}$ mice. Although expression of ACVR1C is activity and Translin-dependent and Stau2 and Pum2 – as far as is known – are constitutionally expressed, this study provides an example of how two proteins might independently affect hippocampal memory but that - upon knock-down of both proteins - memory can remain intact. The detailed mechanism behind

this remains unclear and will need further research to find answers. The study by Tully and colleagues (Dubnau et al., 2003) was the first project to imply the role of Stau2 and Pum2 in memory. As we learn more about the role of Stau2 and Pum2 in hippocampal-dependent memory, it has become clear that the pathways and effects involved are far more complex and possibly involve additional proteins.

A possible alternative candidate that might be relevant in this pathway is the RBP FMRP, as $Pum2^{KD}$, $Stau2^{KD}$ and $FMRP^{KD}$ rodents, respectively, all show disrupted long-term spatial memory (Siemen et al., 2011; Berger et al., 2017; Tian et al., 2017). There is also evidence of a potential connection of Pum2, Stau2 and FMRP on a molecular level: Pum2 and FMRP are both found in stress granules (Soong et al., 2006; Vessey et al., 2006) and enriched in Stau2-RNPs (Fritzsche et al., 2013). Further studies will be needed to determine, whether and if so to what extent FMRP is involved in the Stau2/Pum2 affected pathway in memory and what role it might play in hippocampal memory.

4.2 Stau2 and Pum2 have distinct roles in adult hippocampal neurogenesis

The formation of new memory depends on a variety of different processes. On a cellular level, learning takes place in form of alterations of different synapses, thereby altering synaptic input to a neuron (Kandel, 2001). Herein, RBPs play crucial roles, as they are widely thought to be responsible for the regulation of various aspects of posttranscriptional gene regulation including local protein synthesis at the synapse. Stau2 is involved in the transport of mRNA along microtubules to dendrites (Heraud-Farlow and Kiebler, 2014), whereas Pum2 serves primarily as translational regulator (Zhang *et al.*, 2017; Zahr *et al.*, 2018; Schieweck *et al.*, unpublished).

Another important process in hippocampal memory formation is adult neurogenesis, the generation of new neurons in the subgranular zone of the dentate gyrus and their subsequent integration into the existing synaptic circuitry. New neurons are generated throughout life in the subgranular zone of the dentate gyrus in the hippocampus (Zhao et al., 2008; Kempermann et al., 2015) and are functionally integrated as indicated by physiological recordings displaying similar properties to mature neurons (van Praag et al., 2002; Overstreet-Wadiche and Westbrook, 2006). A fine-tuned balance between the addition and removal of new cells is needed for proper memory acquisition and retention (Dupret et al., 2007).

Newly generated neurons go through different stages of development (**Fig. 2A**). This can be visualized using distinct markers, which are only expressed during different stages of development. Doublecortin (DCX) is expressed in type 2b progenitor cells, which are still

mitotic, until cells become early immature post-mitotic granule cell and begin to integrate into the existing synaptic circuitry (Kempermann et al., 2004; von Bohlen und Halbach, 2011). DCX is therefore a widely used marker for quantifying adult neurogenesis. During their time of DCX+ positivity, primary dendrite length grows to approximately 300 µm (Overstreet-Wadiche and Westbrook, 2006).

In our adult animals (5 months old), base levels of neurogenesis were slightly below WT levels for Stau2^{KD} animals and significantly more for Pum2^{KD} and Stau2^{KD}/Pum2^{KD} animals. The rate of neurogenesis declines all through adulthood (Mathews et al., 2017), although learning tasks have been shown to increase adult neurogenesis (Gould et al., 1999; Epp et al., 2007). *Gould et al.* (1999) used trace eyeblink paired and unpaired conditioning as well as Morris water maze place and cue learning tasks on rats and analyzed BrdU labeled cells after exposure of the animals to the tasks. Hippocampus dependent tasks led to an increase in BrdU positive cells. It is tempting to speculate that the observed increase in neurogenesis in our mice is caused by an increased susceptibility to stimulation of neurogenesis upon our behavioral testing regimen. Further analysis of the property of these newly generated cells is needed to determine what factors actually drive adult neurogenesis. As mentioned before, data on the role of Stau2 and Pum2 in adult neurogenesis is sparse. *Kusek et al.* (2012) showed that Stau2 plays an important role in embryonic neurogenesis: Stau2 becomes asymmetrically distributed during mitosis – together with its bound mRNAs – in the neuroblast progenitor cell. Upon knock-down of Stau2, differentiation of newborn neural progenitor cells into neurons is promoted (Kusek et al., 2012). One target mRNA of Stau2 and Pum2 is *Prox1* (Vessey et al., 2012), which regulates Notch1-mediated inhibition of neurogenesis (Kaltezioti et al., 2010). By asymmetric localization of *Prox1*, Stau2 and Pum2 therefore mediate the neural differentiation of type 2, DCX+ neural progenitor cells. *Prox1* is expressed in the dentate gyrus during development and into adulthood and its presence in the adult brain leads to granule cell maturation in the dentate gyrus (Lavado et al., 2010). *Lavado et al.* (2010) showed that ubiquitous, conditional expression of *Prox1* in mice led to an increased cell count of mostly NeuN+ neurons and to a lesser extent DCX+ neurons in the SGZ of the hippocampus. It is tempting to hypothesize that the decreased DXC+ cell count is caused by failure of asymmetric localization of *Prox1* and other target mRNAs by a Pum2, Stau2 and DDX1 containing complex (Vessey et al., 2012), which then could lead to premature differentiation. Asymmetric segregation of fate determinants such as Numb and Notch has been shown before (Zhong et al., 1997), which is a well-known principle of cell fate determination.

The reduction of neurogenesis in Pum2^{KD} mice is in line with *Zhang et al.* (2017), who showed mice lacking expression of both Pum1 and Pum2 in the brain had severely decreased DCX+ cells in the postnatal hippocampus and increased perinatal apoptosis of neurons in the hippocampus. Additionally, the volume of the dentate gyrus was found to be significantly reduced in this study. It has previously been shown that mice carrying a homologous knock-down for Pum2 are bigger in size than mice carrying a homologous knock-down for Pum1 and that also brain size is affected, though brain body size ratio remains unchanged (Siemen et al., 2011; Zhang et al., 2017; Lin et al., 2019). Our Pum2^{KD} mice are also smaller in body and brain size compared to WT control animals (Schieweck *et al*., *unpublished*). Pum1 levels, however, remain unchanged in our mouse line (Schieweck *et al., unpublished*). As previously mentioned, Pum2 has been implicated in translational repression in mammalian neural stem cells (Vessey et al., 2012; Zahr et al., 2018; Lin et al., 2019). In detail, it does so by binding relevant target mRNAs. Examples for important target mRNAs in mammalian neurons are cell fate determinants such as *Prox1* (Vessey et al., 2010), but also the cell cycle inhibitor *Cdknb1*. *Cdknb1* inhibits translation and thus causes cell cycle progression and proliferation (Lin et al., 2019). The effects on proliferation are global leading to the above-mentioned differences in body and brain weight (Lin et al., 2019). Taken together, this suggests that the effects on neurogenesis seen in our Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice might not solely depend on the failed asymmetric distribution of cell fate determinants but also on a global cell cycle inhibitory effect, thereby explaining the more pronounced reduction in DCX+ cells as well as in bodyweight.

Importantly, GABAergic depolarization is an important developmental signal cascaded during adult neurogenesis (Ge et al., 2007). The underlying mechanism depends on a chloride ion gradient generated by two transporters, NKCC1 and KCC2, respectively (Kim et al., 2012). During development, NKCC1 expression is down-regulated and KCC2 expression is subsequently up-regulated. Congenital upregulation of KCC2 has been shown to lead to more immature neurons, reduced neurogenesis and smaller brain size in *zebrafish* (Reynolds et al., 2008). Interestingly, in cultured rat cortical neurons, upon knock-out of Pum2, KCC2 is also upregulated. Though data on expression levels in our mice is lacking, it is tempting to speculate that this finding contributes to the level and morphology of the neurogenesis we observed in Pum2^{KD} and Stau2^{KD}/Pum2^{KD} mice.

When new neurons are generated, they require synaptic input and stimulation in order to survive (Zhao et al., 2008). The survival of neurons can be facilitated by environmental enrichment or spatial learning (Kee et al., 2007). In our study, neurogenesis is enhanced upon

stimulation by a 4-week long behavioral testing battery in Stau2^{KD} and Pum2^{KD} animals, but to a lesser extent in Stau2^{KD}/Pum2^{KD} animals. *Kee et al.* (2007) used BrdU labeling of newborn neurons in mice undergoing the Morris water maze testing. The study showed that once cells are 4 weeks old, they are more likely to be integrated into the existing synaptic circuitry as indicated by co-labeling of Fos+ and BrdU+ cells. We used the Barnes maze, a comparable spatial memory task in our mice. The increased cell count we observed in mice undergoing the behavioral testing regimen might therefore be explained by the preferential integration and longer survival of newborn neurons upon stimulation.

Importantly, knock-down of Stau2 and Pum2 led to not only reduced adult neurogenesis, but also altered cell morphology. All transgenic mouse lines show shorter primary dendrites. Additionally, Stau2^{KD}/Pum2^{KD} animals have fewer primary dendrites per DCX+ cell. It has been shown that through the development of newborn neurons and as they become integrated in the existing synaptic circuitry, the dendritic tree grows and becomes more complex (Ambrogini et al., 2004; Overstreet-Wadiche and Westbrook, 2006). This is indicated by longer dendrites and increased dendritic branch points (Overstreet-Wadiche and Westbrook, 2006). As described previously, neurons that fail to integrate properly become apoptotic and fail to mature (Ming and Song, 2011; Kempermann et al., 2015). It is tempting to speculate that the reduced length of DCX+ neurons observed in our mouse lines can be attributed to lacking functional integration of newborn neurons and subsequent apoptosis. Furthermore, detailed analysis will be needed as preliminary TUNEL-staining remained inconclusive (**data not shown**).

4.3 Effects of altered neurogenesis on hippocampus dependent learning

Another important finding is that although neurogenesis was increased in Stau2^{KD} mice after learning, they performed worse than control animals in spatial memory tasks. In contrary, Stau2^{KD}/Pum2^{KD} mice showed good hippocampal learning abilities, but at the same time reduced adult neurogenesis, whereas reduced neurogenesis in Pum2^{KD} animals was associated with worse performance in hippocampus-based memory tasks. Data on a correlation between neurogenesis and hippocampal memory performance is sparse and controversial. Into adulthood, preserved neurogenesis has first been described by *Altman et al.* (1965). Since then, the role of newly generated neurons in the hippocampus has been a research topic of broad interest. Because of the complexity of the topic, advances have only been made in recent times (Ming and Song, 2011; Gonçalves et al., 2016). We know that adequate neurogenesis is important for spatial memory. Several studies have shown that relatively mature (4 to 6 weeks

old) adult generated granule cells are preferentially integrated into the hippocampal circuitry upon spatial learning (Dupret et al., 2007; Kee et al., 2007). For memory acquisition to be successful, a balance between integration and apoptosis of newly born neurons is necessary (Dupret et al., 2007).

As reviewed by *Toda and Gage* (2018), several approaches in ablating adult neurogenesis, either by radiation or administration of antimitotic agents showed that treated animals perform worse than their healthy control group. *Shors et al.* (2001) treated rats with the antimitotic agent methylazoxymethanol acetate (MAM) and subjected them to hippocampus dependent trace conditioning. Hereby, a temporal gap between the conditioned stimulus (white noise) and unconditioned stimulus (periorbital eyelid stimulation) requires a trace memory of the conditioned stimulus to be paired with the unconditioned stimulus (Shors et al., 2001). This requires proper hippocampal function (Weiss et al., 1999). Mice treated with MAM, thereby lacking adult neurogenesis, showed fewer conditioned responses (eyeblinks) to the conditioned stimulus alone (white noise) after repeated trace stimuli compared to control animals. The effect was rescued upon discontinuation of the MAM treatment and recovery of the cell production (Shors et al., 2001). This resembles our Pum2^{KD} mice, as they show reduced neurogenesis and perform worse in hippocampus dependent memory tasks. On the other hand, although conditions of elevated glucocorticoids or aging reduce cell proliferation in the dentate gyrus, memory performance is not necessarily worse (Leuner et al., 2006). Although our Stau2^{KD}/Pum2^{KD} mice show robust hippocampal memory, neurogenesis did not exceed the levels seen in our Pum2^{KD} mice. Stau2^{KD}/Pum2^{KD} mice have spontaneous seizures at 2-3 months of age, which is earlier than Pum2^{KD} mice starting to exhibit seizures at 5 months (**data not shown**). It is tempting to speculate that network activity is higher in these animals, taking into account the earlier seizures but also the improved memory functions. Hippocampus specific memory tasks such as trace eyeblink conditioning and the submerged Morris water maze enhance adult hippocampal neurogenesis in trained rats (Gould et al., 1999). A similar pattern can be observed in our Stau2^{KD} and Pum2^{KD} mice, which show enhanced neurogenesis after stimulation with Barnes maze testing together with other subsequent behavioral tests. This finding, however, was not found in WT control animals, further providing evidence that not all behavioral tests lead to stimulated neurogenesis. Importantly, *Sisti et al.* (2007) showed that only good performers in learning tasks had increased levels of BrdU labeled newborn neurons. Our study was not designed to account for differences in performing memory tasks when analyzing neurogenesis rates. As more than 10 animals per group were tested in the behavioral

testing regimen, but only 3 each were used for immunohistochemical analysis, a potential bias cannot be excluded at the present time.

Importantly, possible links between forgetting and neurogenesis have been drawn (Frankland et al., 2013; Akers et al., 2014). A proposed model using computational techniques takes into account increased forgetting in young animals (and humans) and relates it to high levels of neurogenesis present at this point in development (Frankland et al., 2013). In computational models, adding more cells to an existing circuitry appears to be capable of disrupting existing circuits thereby deleting already encoded memory. Further evidence for this theory provided the study by *Akers et al.* (2014), which compared memory performance in a context fear learning task with the count of newly generated neurons in mice exposed to voluntary wheel running. Wheel running is known to enhance adult neurogenesis (Gage et al., 1999), compared to same aged animals, which were not using a running wheel. Mice, which did additional voluntary wheel running, showed increased neurogenesis but also weaker performance in the memory task, thereby providing evidence that not all new generated neurons are beneficial in memory performance (Akers et al., 2014). This could explain, why memory in Stau2^{KD} is worse to control group mice, although they did show enhanced neurogenesis upon learning.

The development of dendritic spines is yet another crucial step in the integration of newborn neurons into an existing circuitry (Zhao and Overstreet-Wadiche, 2008). EPSCs serve as useful parameter to evaluate the synaptic function as they will increase upon successful integration (Kandel et al., 2014). As already described, Pum2 and Stau2 have been both implicated in dendritic spine morphogenesis (see *Introduction*). *Goetze et al.* (2006) showed that upon Stau2 knock-down in cultured rat hippocampal neurons, the density of dendritic spines is reduced as well as the number of mEPSPs. Interestingly, *Berger et al.* (2017) recorded enhanced fEPSP slopes in functional hippocampal slices of Stau2 knock-down rats, hence increased LTP, but disrupted hippocampal memory. Similar observations have been made with PSD95 mutant mice (Migaud et al., 1998). PSD95 is a postsynaptic protein, involved in anchoring the NMDA-receptor to the postsynaptic density. While localization of the receptor remained unchanged in the mice, PSD95 deficient mice showed enhanced LTP, but performed worse in Morris water maze hippocampal memory testing (Migaud et al., 1998). This finding was complemented by a study suggesting that Stau2 was necessary for mGluR dependent LTD (Lebeau et al., 2011). The correlation appeared differently for Pum2. Pum2^{KD} mice showed impaired hippocampal memory. *Vessey et al.* (2010) showed that Pum2 knock-down in rat hippocampal neurons leads to increased mEPSCs as well as increased dendritic arborization

and reduced dendritic spines. Interestingly, in functional hippocampal slices of Pum2^{KD} mice, LTP is reduced (Schieweck et al., 2021). In *Drosophila* peripheral motor neurons, knock-down of Pum2 leads to loss of class III and IV dendritic branches (Ye et al., 2004). On further notice, Pum2 has also been implicated to play a role in axonal development as knock-down reduces axonal crossing and also leads to mis-location of certain mRNAs in the axon (Martínez et al., 2019). Taken together, there is ample evidence of the involvement of Stau2 and Pum2 in the development and maturation of neurons, especially in dendritic branching and dendritic spine morphogenesis with consequential effects on the formation of LTP and LTD, respectively. In the few animal studies available, this led to impaired hippocampal memory, which we found in our mice as well. An intricate balance between LTP and LTD is needed for adequate acquisition of memories (Kandel et al., 2014). Thus, it can be hypothesized that the disrupted balance in LTP and LTD is the cause for the memory problems in Stau2^{KD} and Pum2^{KD} mice. As Pum2 and Stau2 seem to shift the balance to different sides of the BCM curve (Goetze et al., 2006; Vessey et al., 2010; Lebeau et al., 2011; Berger et al., 2017), it is tempting to speculate that the effects compensate for each other upon knock-down of both proteins, therefore enabling normal memory performance in Stau2^{KD}/Pum2^{KD} mice.

4.4 Conclusions

In sum, this thesis provides direct evidence that Stau2 and Pum2 both play important roles in the formation of hippocampus-based memory. Importantly, upon double knock-down of Stau2 and Pum2, hippocampal memory remains unaffected. Taken together, this suggests that Stau2 and Pum2 are both contributing players in the Stau2/Pum2 memory pathway as suggested by *Dubnau et al. (2003)*, but knock-down of both proteins can at least partially rescue downstream effects (**Fig. 13**). Their roles are probably not driven by their function in adult neurogenesis as knock-down of Pum2, but not Stau2, led to reduced adult neurogenesis, though both single knock-down mouse lines have impaired hippocampal memory. Further, although neurogenesis is reduced in the double knock-down mouse model, hippocampal memory remained intact. Because of the distinct contributions of Stau2 and Pum2 to mRNA localization and translation, the key factor to their function in memory may lie upon the distinct properties and roles of their target mRNAs and how those are influenced by Stau2 and Pum2.

The detailed mechanism by which Stau2 and Pum2 influence synaptic integrity and thereby hippocampal memory is unknown and an intense topic of current research. Dendritic localization of Stau2 (Tang et al., 2001; Goetze et al., 2006; Lebeau et al., 2011; Heraud-Farlow et al., 2013; Sharangdhar et al., 2017; Bauer et al., 2019) and Pum2 (Ye et al., 2004; Vessey et

al., 2006, 2010; Schieweck et al., 2021) and some of their important target mRNAs has been demonstrated. *Ca^{2+}/Calmodulin-dependent kinase II alpha* (*CaMKIIα*) mRNA, which is an important factor in the establishment of LTP, is also enriched in Stau2-RNPs (Fritzsche et al., 2013). The dendritic localization of Stau2 target mRNAs appears to be activity dependent, which was proven for two separate target mRNAs involved in synaptic plasticity, *Rgs4* and *Calm3* (Sharangdhar et al., 2017; Bauer et al., 2019). Interestingly, dendritic Calm3 protein levels remain unaffected (Berger et al., 2017). This provides evidence, that although Stau2 affects mRNA localization, its effects on the protein level of its target mRNAs might be less extensive.

As previously mentioned, FMRP1 – an RBP implicated in a congenital, monogenetic mental retardation has been found to be enriched in Stau2 granules (TD-BFX, 1994; Fritzsche et al., 2013). Pum2 co-localizes with FMRP in stress granules (Vessey et al., 2006). Consequently, FMRP knock-out mice showed impaired spatial learning ability in the Morris

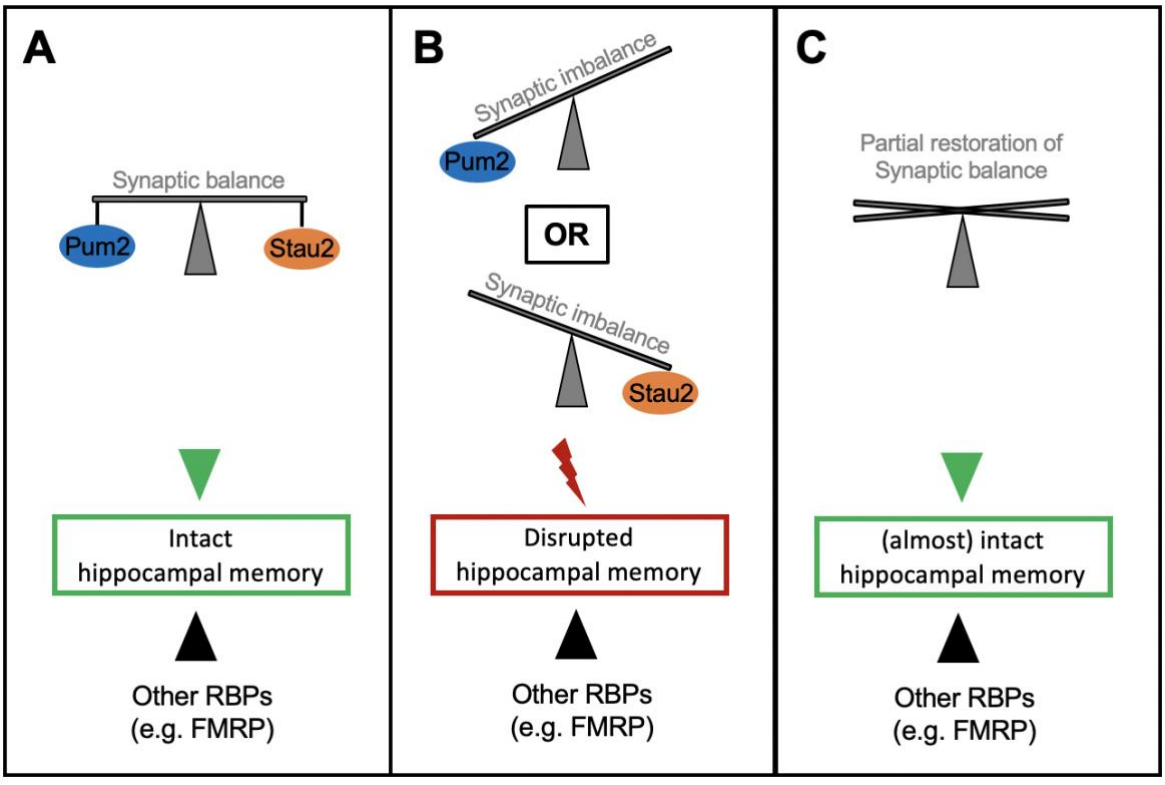


Figure 13: The role of Stau2 and Pum2 in hippocampal learning
Current working hypothesis for the effect of Stau2 and Pum2 on hippocampal memory: Stau2 and Pum2 affect synaptic balance and thereby influence hippocampus-dependent memory formation. Other RBPs might influence the Stau2 and Pum2 containing memory pathway. One possible member is FMRP. **(A)** A balance of Stau2 and Pum2 is relevant for proper function of a Stau2/Pum2 containing pathway. **(B)** Knock-down of either Stau2 or Pum2 disrupts formation of hippocampal memory in mice. **(C)** Upon knock-down of both Stau2 and Pum2 synaptic balance is partially restored and hippocampal memory is almost completely restored.

water maze as well as reduced LTP and increased LTD (Tian et al., 2017). It is therefore tempting to speculate Stau2 and Pum2 contribute to hippocampal learning in conjunction with FMRP. Since knock-down of both Stau2 and Pum2 leaves hippocampal memory unaffected, FMRP might be the main player, being modulated by Pum2 and Stau2. Further studies will therefore be necessary to experimentally validate this hypothesis in order to gain insight into the mechanisms by which Stau2 and Pum2 influence hippocampal learning and neurogenesis.

.

Appendices

Macro 1

```
//gets overall shape of the hippocampus
Ddir = getDirectory("Choose Destination Folder");
getDimensions(width, height, channels, slices, frames);
Original_width=width;
Original_height=height;
run("Gaussian Blur...", "sigma=180");
setAutoThreshold("Default");
setAutoThreshold("Default dark");
run("Convert to Mask");
run("Fill Holes");
run("Analyze Particles...", "size=100000-Infinity show=Outlines display summarize add");
newImage("Mask", "8-bit black", width, height, 1);
roiManager("select", 0);
run("Create Mask");
run("Invert LUT");
path_Mask = Ddir + "_green_Mask.tif";
saveAs("tif", path_Mask);
run("Select None");
run("Invert");
path_Mask_inverted = Ddir + "_green_Mask_inverted";
saveAs("tif", path_Mask_inverted);
```

Macro 2

```
Ddir = getDirectory("Choose Destination Folder");
//print(Ddir);
getDimensions(width, height, channels, slices, frames);
Original_width=width;
Original_height=height;
//small_width =floor(width/10);
//small_height =floor(height/10);
```

```
//run("Size...", "width=small_width height=small_height constrain average interpolation=None");
roiManager("select", 0);
run("Clear Outside");
run("Median...", "radius=2");
setAutoThreshold("Default dark");
run("Convert to Mask");
for(i=0; i<15;i++){run("Dilate");}
run("Gaussian Blur...", "sigma=80");
//run("Gaussian Blur...", "sigma=20");
setAutoThreshold("Default");
//setThreshold(70, 255);
run("Convert to Mask");
path_red=Ddir + "_red";
saveAs("tif", path_red);
waitForUser("Pause","Load _green_Mask_inverted.tif and then press OK");
imageCalculator("Add create", "_green_Mask_inverted.tif","_red.tif");
run("Invert");
path_calculated=Ddir + "_calculated";
saveAs("tif", path_calculated);
```

Table 7: Macros for quantification of Stau2, Pum2 and Gabra2 immunostaining

Bibliography

Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang HL, Wheeler AL, Guskjolen A, Niibori Y, Shoji H, Ohira K, Richards BA, Miyakawa T, Josselyn SA, Frankland PW (2014) Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science (80-) 344:598–602.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319–335.

Ambrogini P, Lattanzi D, Ciuffoli S, Agostini D, Bertini L, Stocchi V, Santi S, Cuppini R (2004) Morpho-functional characterization of neuronal cells at different stages of maturation in granule cell layer of adult rat dentate gyrus. Brain Res 1017:21–31.

Antunes M, Biala G (2012) The novel object recognition memory: Neurobiology, test procedure, and its modifications. Cogn Process 13:93–110.

Asada K, Canestrari E, Fu X, Li Z, Makowski E, Wu YC, Mito JK, Kirsch DG, Baraban J, Paroo Z (2014) Rescuing dicer Defects via Inhibition of an Anti-Dicing Nuclease. Cell Rep 9:1471–1481.

Asaoka-Taguchi M, Yamada M, Nakamura A, Hanyu K, Kobayashi S (1999) Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. Nat Cell Biol 1:431–437.

Augustinack JC, van der Kouwe AJW, Salat DH, Benner T, Stevens AA, Annese J, Fischl B, Frosch MP, Corkin S (2014) H.M.'s contributions to neuroscience: A review and autopsy studies. Hippocampus 24:1267–1286.

Bach ME, Hawkins RD, Osman M, Kandel ER, Mayford M (1995) Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal ltp in the range of the θ frequency. Cell 81:905–915.

Barker GRI, Warburton EC (2011) When Is the Hippocampus Involved in Recognition Memory? J Neurosci 31:10721–10731.

Barnes CA (1979) Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. J Comp Physiol Psychol 93:74–104.

Bassell GJ, Warren ST (2008) Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. Neuron 60:201–214.

Bauer KE, Segura I, Gaspar I, Scheuss V, Illig C, Ammer G, Hutten S, Basyuk E, Fernández-Moya SM, Ehses J, Bertrand E, Kiebler MA (2019) Live cell imaging reveals 3′-UTR dependent mRNA sorting to synapses. Nat Commun 10:3178.

Bean BP (2007) The action potential in mammalian central neurons. Nat Rev Neurosci 8:451–465.

Belzung C, Griebel G (2001) Measuring normal and pathological anxiety-like behaviour in mice: A review. In: Behavioural Brain Research, pp 141–149.

Berger SM, Fernández-Lamo I, Schönig K, Fernández Moya SM, Ehses J, Schieweck R, Clementi S, Enkel T, Grothe S, von Bohlen und Halbach O, Segura I, Delgado-García JM, Gruart A, Kiebler MA, Bartsch D (2017) Forebrain-specific, conditional silencing of Staufen2 alters synaptic plasticity, learning, and memory in rats. Genome Biol 18:222.

Bhalla K, Phillips HA, Crawford J, McKenzie OLD, Mulley JC, Eyre H, Gardner AE, Kremmidiotis G, Callen DF (2004) The de novo chromosome 16 translocations of two patients with abnormal phenotypes (mental retardation and epilepsy) disrupt the A2BP1 gene. J Hum Genet 49:308–311.

Bouilleret V, Loup F, Kiener T, Marescaux C, Fritschy JM (2000) Early loss of interneurons and delayed subunit-specific changes in GABA(A)-receptor expression in a mouse model of mesial temporal lobe epilepsy. Hippocampus 10:305–324.

Bramham CR, Wells DG (2007) Dendritic mRNA: transport, translation and function. Nat Rev Neurosci 8:776–789.

Bramham CR, Worley PF, Moore MJ, Guzowski JF (2008) The immediate early gene arc/arg3.1: regulation, mechanisms, and function. J Neurosci 28:11760–11767.

Broadbent NJ, Squire LR, Clark RE (2004) Spatial memory, recognition memory, and the hippocampus. Proc Natl Acad Sci U S A 101:14515–14520.

Brown MW, Aggleton JP (2001) Recognition memory: What are the roles of the perirhinal cortex and hippocampus? Nat Rev Neurosci 2:51–61.

Bunsey M, Elchenbaum H (1996) Conservation of hippocampal memory function in rats and humans. Nature 379:255–257.

Burgess N, Maguire EA, O'Keefe J (2002) The human hippocampus and spatial and episodic memory. Neuron 35:625–641.

Calzolari F, Michel J, Baumgart EV, Theis F, Götz M, Ninkovic J, Gotz M, Ninkovic J (2015) Fast clonal expansion and limited neural stem cell self-renewal in the adult subependymal zone. Nat Neurosci 18:490–492.

Cho K-O, Lybrand ZR, Ito N, Brulet R, Tafacory F, Zhang L, Good L, Ure K, Kernie SG, Birnbaum SG, Scharfman HE, Eisch AJ, Hsieh J (2015) Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. Nat Commun 6:6606.

Choleris, E, A. W. Thomas, M. Kavaliers FSP (2001) A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. Neurosci Biobehav Rev 25:235–260.

Cohen SJ, Stackman RW (2015) Assessing rodent hippocampal involvement in the novel object recognition task. A review. Behav Brain Res 285:105–117.

Conlon EG, Manley JL (2017) RNA-binding proteins in neurodegeneration: Mechanisms in aggregate. Genes Dev 31:1509–1528.

Cooper LN, Bear MF (2012) The BCM theory of synapse modification at 30: Interaction of theory with experiment. Nat Rev Neurosci 13:798–810.

De Quervain DJF, Roozendaal B, McGaugh JL (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394:787–790.

Deacon RMJ (2006) Assessing nest building in mice. Nat Protoc 1:1117–1119.

Deacon RMJ, Croucher A, Rawlins JNP (2002) Hippocampal cytotoxic lesion effects on species-typical behaviours in mice. Behav Brain Res 132:203–213.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339–350.

Driscoll HE, Muraro NI, He M, Baines RA (2013) Pumilio-2 Regulates Translation of Nav1.6 to Mediate Homeostasis of Membrane Excitability. J Neurosci 33:9644–9654.

Dubnau J, Chiang A-S, Grady L, Barditch J, Gossweiler S, McNeil J, Smith P, Buldoc F, Scott R, Certa U, Broger C, Tully T (2003) The staufen/pumilio pathway is involved in Drosophila long-term memory. Curr Biol 13:286–296.

Dupret D, Fabre A, Döbrössy MD, Panatier A, Rodríguez JJ, Lamarque S, Lemaire V, Oliet SHR, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons Moser E, ed. PLoS Biol 5:1683–1694.

Ennaceur A, Meliani K (1988) A new one-trial test for neurobiological studies of memory in rats. III. Spatial vs. non-spatial working memory. Behav Brain Res 51:83–92.

Epp JR, Spritzer MD, Galea LAM (2007) Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation. Neuroscience 149:273–285.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317.

Ferrandon D, Elphick L, Nüsslein-Volhard C, St Johnston D (1994) Staufen protein associates with the 3′UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell 79:1221–1232.

Follwaczny P, Schieweck R, Riedemann T, Demleitner A, Straub T, Klemm AH, Bilban M, Sutor B, Popper B, Kiebler MA (2017) Pumilio2-deficient mice show a predisposition for epilepsy. Dis Model Mech Mech 10:1333–1342.

Fox M, Urano J, Reijo Pera RA (2005) Identification and characterization of RNA sequences to which human PUMILIO-2 (PUM2) and deleted in Azoospermia-like (DAZL) bind. Genomics 85:92–105.

Frankland PW, Köhler S, Josselyn SA (2013) Hippocampal neurogenesis and forgetting. Trends Neurosci 36:497–503.

Frick KM, Stillner ET, Berger-Sweeney J (2000) Mice are not little rats: Species differences in a one-day water maze task. Neuroreport 11:3461–3465.

Fritzsche R et al. (2013) Interactome of two diverse RNA granules links mRNA localization to translational repression in neurons. Cell Rep 5:1749–1762.

Gage FH, van Praag H, Kempermann G (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.

Ge S, Pradhan DA, Ming G li, Song H (2007) GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci 30:1–8.

Gehman LT, Stoilov P, Maguire J, Damianov A, Lin C, Shiue L, Jr MA, Mody I, Black DL (2011) The splicing regulator Rbfox1 (A2BP1) controls neuronal excitation in the mammalian brain. Nat Genet 43:706–711.

Gladding CM, Fitzjohn SM, Molnár E (2009) Metabotropic glutamate receptor-mediated long-term depression: Molecular mechanisms. Pharmacol Rev 61:395–412.

Goetze B, Tuebing F, Xie Y, Dorostkar MM, Thomas S, Pehl U, Boehm S, Macchi P, Kiebler MA (2006) The brain-specific double-stranded RNA-binding protein Staufen2 is required for dendritic spine morphogenesis. J Cell Biol 172:221–231.

Goldberg EM, Coulter DA (2013) Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction. Nat Rev Neurosci 14:337–349.

Gonçalves JT, Schafer ST, Gage FH (2016) Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. Cell 167:897–914.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260–265.

Harrison FE, Hosseini AH, McDonald MP (2009) Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. Behav Brain Res 198:247–251.

Hattiangady B, Shetty AK (2008) Implications of decreased hippocampal neurogenesis in chronic temporal lobe epilepsy. Epilepsia 49:26–41.

Heraud-Farlow JE, Kiebler MA (2014) The multifunctional Staufen proteins: conserved roles from neurogenesis to synaptic plasticity. Trends Neurosci 37:470–479.

Heraud-Farlow JE, Sharangdhar T, Li X, Pfeifer P, Tauber S, Orozco D, Hörmann A, Thomas S, Bakosova A, Farlow AR, Edbauer D, Lipshitz HD, Morris QD, Bilban M, Doyle M, Kiebler MA (2013) Staufen2 regulates neuronal target RNAs. Cell Rep 5:1511–1518.

Jessberger S, Zhao C, Toni N, Clemenson GD, Li Y, Gage FH (2007) Seizure-Associated, Aberrant Neurogenesis in Adult Rats Characterized with Retrovirus-Mediated Cell Labeling. J Neurosci 27:9400–9407.

Kaltezioti V, Kouroupi G, Oikonomaki M, Mantouvalou E, Stergiopoulos A, Charonis A, Rohrer H, Matsas R, Politis PK (2010) Prox1 regulates the Notch1-mediated inhibition of neurogenesis Palmer TD, ed. PLoS Biol 8:e1000565.

Kandel ER (2001) The molecular biology of memory storage: A dialogue between genes and synapses. Science (80-) 294:1030–1038.

Kandel ER, Dudai Y, Mayford MR (2014) The molecular and systems biology of memory. Cell 157:163–186.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10:355–362.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

Kempermann G, Song H, Gage FH (2015) Neurogenesis in the Adult Hippocampus. Cold Spring Harb Perspect Biol 7:a018812.

Kiebler MA, Bassell GJ (2006) Neuronal RNA granules: movers and makers. Neuron 51:685–690.

Kiebler MA, Hemraj I, Verkade P, Köhrmann M, Fortes P, Marión RM, Ortín J, Dotti CG (1999) The mammalian Staufen protein localizes to the somatodendritic domain of cultured hippocampal neurons: Implications for its involvement in mRNA transport. J Neurosci 19:288–297.

Kim JM, Kim DH, Lee Y, Park SJ, Ryu JH (2014) Distinct roles of the hippocampus and perirhinal cortex in GABAA receptor blockade-induced enhancement of object recognition memory. Brain Res 1552:17–25.

Kim JY, Liu CY, Zhang F, Duan X, Wen Z, Song J, Feighery E, Lu B, Rujescu D, St Clair D,

Christian K, Callicott JH, Weinberger DR, Song H, Ming GL (2012) Interplay between DISC1 and GABA signaling regulates neurogenesis in mice and risk for schizophrenia. Cell 148:1051–1064.

Kornack DR, Rakic P (1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A 96:5768–5773.

Kusek G, Campbell M, Doyle F, Tenenbaum SA, Kiebler M, Temple S (2012) Asymmetric segregation of the double-stranded RNA binding protein Staufen2 during mammalian neural stem cell divisions promotes lineage progression. Cell Stem Cell 11:505–516.

Lavado A, Lagutin O V., Chow LML, Baker SJ, Oliver G (2010) Prox1 Is required for granule cell maturation and intermediate progenitor maintenance during brain neurogenesis Palmer TD, ed. PLoS Biol 8:43–44.

Lebeau G, Miller LC, Tartas M, McAdam R, Laplante I, Badeaux F, DesGroseillers L, Sossin WS, Lacaille JC (2011) Staufen 2 regulates mGluR long-term depression and Map1b mRNA distribution in hippocampal neurons. Learn Mem 18:314–326.

Leuner B, Gould E, Shors TJ (2006) Is there a link between adult neurogenesis and learning? Hippocampus 16:216–224.

Li P, Yang X, Wasser M, Cai Y, Chiat W (1997) Inscuteable and staufen mediate asymmetric localization and segregation of prospero RNA during drosophila neuroblast cell divisions. Cell 90:437–447.

Lin K, Qiang W, Zhu M, Ding Y, Shi Q, Chen X, Zsiros E, Wang K, Yang X, Kurita T, Xu EY (2019) Mammalian Pum1 and Pum2 Control Body Size via Translational Regulation of the Cell Cycle Inhibitor Cdkn1b. Cell Rep 26:2434–2450.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Llinás RR (1988) The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science (80-) 242:1654–1664.

Loddenkemper T, Talos DM, Cleary RT, Joseph A, Sánchez Fernández I, Alexopoulos A, Kotagal P, Najm I, Jensen FE (2014) Subunit composition of glutamate and gamma-aminobutyric acid receptors in status epilepticus. Epilepsy Res 108:605–615.

Malenka RC, Bear MF (2004) LTP and LTD: An embarrassment of riches. Neuron 44:5–21.

Markakis EA, Gage FH (1999) Adult-generated neurons in the dentate gyrus send axonal projections to field CA3 and are surrounded by synaptic vesicles. J Comp Neurol 406:449–460.

Martínez JC, Randolph LK, Iascone DM, Pernice HF, Polleux F, Hengst U (2019) Pum2

Shapes the Transcriptome in Developing Axons through Retention of Target mRNAs in the Cell Body. Neuron 104:1–16.

Mathews KJ, Allen KM, Boerrigter D, Ball H, Shannon Weickert C, Double KL (2017) Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers. Aging Cell 16:1195–1199.

Menon KP, Andrews S, Murthy M, Gavis ER, Zinn K (2009) The translational repressors Nanos and Pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition. J Neurosci 29:5558–5572.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RGM, Morrison JH, O'Dell TJ, Grant SGN (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396:433–439.

Ming G li, Song H (2011) Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. Neuron 70:687–702.

Mircsof D et al. (2015) Mutations in NONO lead to syndromic intellectual disability and inhibitory synaptic defects. Nat Neurosci 18:1731–1736.

Morris AR, Mukherjee N, Keene JD (2008) Ribonomic Analysis of Human Pum1 Reveals cis-trans Conservation across Species despite Evolution of Diverse mRNA Target Sets. Mol Cell Biol 28:4093–4103.

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47–60.

Moscardo E, Salvetti B, Becchi S, Bertini G, Fabene PF (2012) The Novel Object Recognition Test in Rodents : Which Are the Essential Methodological Aspects ? Proc Meas Behav 2012:476–478.

Muraro NI, Weston AJ, Gerber AP, Luschnig S, Moffat KG, Baines RA (2008) Pumilio binds para mRNA and requires Nanos and Brat to regulate sodium current in drosophila motoneurons. J Neurosci 28:2099–2109.

Murphy BL, Pun RYK, Yin H, Faulkner CR, Loepke AW, Danzer SC (2011) Heterogeneous integration of adult-generated granule cells into the epileptic brain. J Neurosci 31:105–117.

Numan M (1974) Medial preoptic area and maternal behavior in the female rat. J Comp Physiol Psychol 87:746–759.

O'Keefe J, Dostrovsky J (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res 34:171–175.

O'Leary TP, Brown RE (2012) The effects of apparatus design and test procedure on learning and memory performance of C57BL/6J mice on the Barnes maze. J Neurosci Methods 203:315–324.

Overstreet-Wadiche LS, Westbrook GL (2006) Functional maturation of adult-generated granule cells. Hippocampus 16:208–215.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727–3738.

Parisi M, Lin H (2000) Translational repression: A duet of Nanos and Pumilio. Curr Biol 10:81–83.

Park AJ, Havekes R, Fu X, Hansen R, Tudor JC, Peixoto L, Li Z, Wu YC, Poplawski SG, Baraban JM, Abel T (2017) Learning induces the translin/trax RNase complex to express activin receptors for persistent memory. Elife 6:e27872.

Plath N et al. (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52:437–444.

Popper B, Demleitner A, Bolivar VJ, Kusek G, Snyder-Keller A, Schieweck R, Temple S, Kiebler MA (2018) Staufen2 deficiency leads to impaired response to novelty in mice. Neurobiol Learn Mem 150:107–115.

Quenault T, Lithgow T, Traven A (2011) PUF proteins: Repression, activation and mRNA localization. Trends Cell Biol 21:104–112.

Ramos A, Grünert S, Adams J, Micklem DR, Proctor MR, Freund S, Bycroft M, Johnston DS, Varani G (2000) RNA recognition by a Staufen double-stranded RNA-binding domain. EMBO J 19:997–1009.

Reynolds A, Brustein E, Liao M, Mercado A, Babilonia E, Mount DB, Drapeau P (2008) Neurogenic role of the depolarizing chloride gradient revealed by global overexpression of KCC2 from the onset of development. J Neurosci 28:1588–1597.

Robbins CA, Tempel BL (2012) Kv1.1 and Kv1.2: similar channels, different seizure models. Epilepsia 53 Suppl 1:134–141.

Schieweck R, Riedemann T, Forné I, Harner M, Bauer KE, Rieger D, Ang F yee, Hutten S, Demleitner AF, Popper B, Derdak S, Sutor B, Bilban M, Imhof A, Kiebler MA (2021) Pumilio2 and Staufen2 selectively balance the synaptic proteome. Cell Rep 35:109279.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108.

Schweers BA, Walters KJ, Stern M (2002) The Drosophila melanogaster translational

repressor pumilio regulates neuronal excitability. Genetics 161:1177–1185.

Scoville WB, Milner B (2000) Loss of recent memory after bilateral hippocampal lesions. 1957. J Neuropsychiatry Clin Neurosci 12:103–113.

Sebat J et al. (2007) Strong Association of De Novo Copy Number Mutations with Autism. Science (80-) 316:445–449.

Seibenhener ML, Wooten MC (2015) Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice. J Vis Exp:e52434.

Sharangdhar T, Sugimoto Y, Heraud-Farlow J, Fernández-Moya SM, Ehses J, Ruiz de los Mozos I, Ule J, Kiebler MA (2017) A retained intron in the 3′- UTR of Calm3 mRNA mediates its Staufen2- and activity-dependent localization to neuronal dendrites. EMBO Rep 18:1762–1774.

Sharma S, Rakoczy S, Brown-Borg H (2010) Assessment of spatial memory in mice. Life Sci 87:521–536.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372–376.

Siemen H, Colas D, Heller HC, Brüstle O, Pera RAR (2011) Pumilio-2 function in the mouse nervous system. PLoS One 6:e25932.

Sisti HM, Glass AL, Shors TJ (2007) Neurogenesis and the spacing effect: learning over time enhances memory and the survival of new neurons. Learn Mem 14:368–375.

Slotnick BM, Nigrosh BJ (1975) Maternal behavior of mice with cingulate cortical, amygdala, or septal lesions. J Comp Physiol Psychol 88:118–127.

Song H, Kempermann G, Wadiche LO, Zhao C, Schinder AF, Bischofberger J (2005) New neurons in the adult mammalian brain: Synaptogenesis and functional integration. In: Journal of Neuroscience, pp 10366–10368.

Soong HK, Dong WK, Weiler IJ, Greenough WT (2006) Fragile X mental retardation protein shifts between polyribosomes and stress granules after neuronal injury by arsenite stress or in vivo hippocampal electrode insertion. J Neurosci 26:2413–2418.

Spassov DS, Jurecic R (2002) Cloning and comparative sequence analysis of PUM1 and PUM2 genes, human members of the Pumilio family of RNA-binding proteins. Gene 299:195–204.

St Johnston D (1995) The intracellular localization of messenger RNAs. Cell 81:161–170.

Staley K (2015) Molecular mechanisms of epilepsy. Nat Neurosci 18:367–372.

Sunyer B, Patil S, Höger H, Luber G (2007) Barnes maze, a useful task to assess spatial reference memory in the mice. Protoc Exch 198:58–68.

Tang SJ, Meulemans D, Vazquez L, Colaco N, Schuman E (2001) A role for a rat homolog of staufen in the transport of RNA to neuronal dendrites. Neuron 32:463–475.

TD-BFX (1994) Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. Cell 78:23–33.

Tian Y, Yang C, Shang S, Cai Y, Deng X, Zhang J, Shao F, Zhu D, Liu Y, Chen G, Liang J, Sun Q, Qiu Z, Zhang C (2017) Loss of FMRP impaired hippocampal long-term plasticity and spatial learning in rats. Front Mol Neurosci 10:269.

Timmerman C, Suppiah S, Gurudatta B V, Yang J, Banerjee C, Sandstrom DJ, Corces VG, Sanyal S (2013) The Drosophila transcription factor Adf-1 (nalyot) regulates dendrite growth by controlling FasII and staufen expression downstream of CaMKII and neural activity. J Neurosci 33:11916–11931.

Tolino M, Köhrmann M, Kiebler MA (2012) RNA-binding proteins involved in RNA localization and their implications in neuronal diseases. Eur J Neurosci 35:1818–1836.

Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF (2008) Neurons born in the adult dentate gyrus form functional synapses with target cells. Nat Neurosci 11:901–907.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030–1034.

Vessey JP, Amadei G, Burns SE, Kiebler MA, Kaplan DR, Miller FD (2012) An Asymmetrically Localized Staufen2-Dependent RNA Complex Regulates Maintenance of Mammalian Neural Stem Cells. Cell Stem Cell 11:517–528.

Vessey JP, Schoderboeck L, Gingl E, Luzi E, Riefler J, Di Leva F, Karra D, Thomas S, Kiebler MA, Macchi P (2010) Mammalian Pumilio 2 regulates dendrite morphogenesis and synaptic function. Proc Natl Acad Sci U S A 107:3222–3227.

Vessey JP, Vaccani A, Xie Y, Dahm R, Karra D, Kiebler MA, Macchi P (2006) Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. J Neurosci 26:6496–6508.

Vogel-Ciernia A, Wood MA (2014) Examining object location and object recognition memory in mice. Curr Protoc Neurosci 69:8.31.1-8.31.17.

von Bohlen und Halbach O (2011) Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. Cell Tissue Res 345:1–19.

Vorhees C V., Williams MT (2014) Assessing spatial learning and memory in rodents. ILAR J 55:310–332.

Walsh RN, Cummins RA (1976) The Open-Field Test: a critical review. Psychol Bull 83:482–

504.

Weiss C, Knuttinen MG, Power JM, Patel RI, O'Connor MS, Disterhoft JF (1999) Trace eyeblink conditioning in the freely moving rat: Optimizing the conditioning parameters. Behav Neurosci 113:1100–1105.

Wenzel HJ, Vacher H, Clark E, Trimmer JS, Lee AL, Sapolsky RM, Tempel BL, Schwartzkroin PA (2007) Structural consequences of Kcna1 gene deletion and transfer in the mouse hippocampus. Epilepsia 48:2023–2046.

Winters BD, Saksida LM, Bussey TJ (2008) Object recognition memory: Neurobiological mechanisms of encoding, consolidation and retrieval. Neurosci Biobehav Rev 32:1055–1070.

Wu X-L, Huang H, Huang Y-Y, Yuan J-X, Zhou X, Chen Y-M (2015) Reduced Pumilio-2 expression in patients with temporal lobe epilepsy and in the lithium-pilocarpine induced epilepsy rat model. Epilepsy Behav 50:31–39.

Wu YC, Williamson R, Li Z, Vicario A, Xu J, Kasai M, Chern Y, Tongiorgi E, Baraban JM (2011) Dendritic trafficking of brain-derived neurotrophic factor mRNA: Regulation by translin-dependent and -independent mechanisms. J Neurochem 116:1112–1121.

Ye B, Petritsch C, Clark IE, Gavis ER, Jan LY, Jan YN (2004) Nanos and Pumilio are essential for dendrite morphogenesis in Drosophila peripheral neurons. Curr Biol 14:314–321.

Zahr SK, Yang G, Kazan H, Borrett MJ, Yuzwa SA, Voronova A, Kaplan DR, Miller FD (2018) A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification. Neuron 97:520-537.e6.

Zamore PD, Williamson JR, Lehmann R (1997) The Pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. RNA 3:1421–1433.

Zhang M, Chen D, Xia J, Han W, Cui X, Neuenkirchen N, Hermes G, Sestan N, Lin H (2017) Post-transcriptional regulation of mouse neurogenesis by Pumilio proteins. Genes Dev 31:1354–1369.

Zhao C-S, Overstreet-Wadiche L (2008) Integration of adult generated neurons during epileptogenesis. Epilepsia 49:3–12.

Zhao C, Deng W, Gage FH (2008) Mechanisms and Functional Implications of Adult Neurogenesis. Cell 132:645–660.

Zhao C, Teng EM, Summers RG, Ming G-L, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3–11.

Zhong W, Jiang MM, Weinmaster G, Jan LY, Jan YN (1997) Differential expression of

mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. Development 124:1887–1897.

Acknowledgements

This thesis was a long time coming. Therefore, there are a lot of people I would like to express my gratitude to:

Prof. Dr. Michael Kiebler for generously giving me the opportunity to pursue my MD thesis in his lab, for fostering my passion for neuroscience and for his continuous guidance and encouragement of my professional interests over the years.

The Förderprogramm für Forschung und Lehre of the Medical Faculty of the LMU Munich and all the people involved for supporting my project and providing a platform for enriching discussions with fellow students.

Drs. Bastian Popper and Rico Schieweck for their continued guidance and support of my project, for inspiring discussions and their challenging scientific questions.

My fellow MD students Philipp Follwaczny, Helena F. Pernice and Surina Frey for their experimental support.

My collaborators for their help and support over the years. In particular, I thank Prof. Dr. Magdalena Götz, Dr. Leda Dimou and Dr. Sarah Schneider.

Ulrike Kring, Jessica Weigel (geb. Olberz), Christin Illig, Daniela Rieger and Sabine Thomas for their excellent experimental support.

Dr. Saskia Hutten for her patience while introducing me to basic practical work in the laboratory and her continued support.

Dr. Tobias Straub for excellent statistical support.

Janina Ehses and Ryan Boehler, PhD, for critical comments on my thesis.

All members of the Kiebler lab for their intellectual input and helpful discussions.

Last, but not least: my family for their continued motivation and support not only of my thesis and my medical endeavours in general, but in all matters of life.

Eidesstattliche Erklärung

Ich, Antonia Franziska Demleitner, erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

“The Role of Staufen 2 and Pumilio 2 in Hippocampus based Learning”

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zu Erlangung eines akademischen Grades eingereicht wurde.

München, 23.07.2021	Antonia Franziska Demleitner
Ort, Datum	Unterschrift

Curriculum Vitae

Name	Antonia Franziska Demleitner
Birthdate/-place	May 9th, 1994 in Regensburg

Education

10/2014 – 02/2017	Doctoral thesis project in the Department of Anatomy and Cell Biology at the LMU Munich (supervisor Prof. Dr. Michael Kiebler) supported by the FöFoLe of the LMU Munich (dissertation scholarship program).
Since 04/2020	Resident, Department of Neurology, Klinikum rechts der Isar, Hospital of the TU Munich
11/2019	Passing of the third state examination, Ärztin (MD)
10/2012 – 11/2019	Medical studies at the LMU und TU Munich with rotations at the Neurological Institute, Columbia University, New York; Guy's Hospital, King's College, London; Hospital for Special Surgery, Cornell University, New York and University Hospital Zurich
2012	High School Graduation

Scientific Contributions

Congress contributions

Demleitner A., Schieweck R., Popper B., Hutten S., Maderthaner S, Kiebler M. (2015). *Einfluss von RNA-Bindeproteinen auf die Entwicklung von epileptischen Anfällen in Pumilio2- und Staufen2-defizienten Mauslinien*, 88. Congress of the German Society for Neurology, Düsseldorf, 09.23.2015-09.26.2015

Demleitner A. & Follwaczny P., Schieweck R., Popper B. & Kiebler M. (2016). *Einfluss der RNA-Bindeproteine Pumilio2 und Staufen2 auf die Entwicklung von epileptischen Anfällen im Mausmodell*, 53. Annual meeting of the German Society for Epileptology e.V, Jena, 03.02.2016-03.05.2016

Poster prize

Demleitner A., Follwacny P., Schieweck R., Klemm A., Straub T., Popper B. & Kiebler M. (2016). *Role of the RNA-binding proteins Pumilio 2 and Staufen 2 in epileptogenesis – Impacts on neurogenesis and long-term memory*, 12th European Congress on Epileptology, Prague, 09.11.2016-09.15.2016

Publications

Ikenberg B.* & **Demleitner A. F.***, Thiele T., Wiestler B., Götze K., Mößmer G., Lingor P. (2021) *Cerebral venous sinus thrombosis after ChAdOx1 nCov-19 vaccination with a misleading first cerebral MRI scan.* Stroke and Vascular Neurology svn-2021-001095 (* shared first authorship)

Schieweck R., Riedemann T., Forné I., Harner M., Bauer K. E., Rieger D., Ang F.-Y., Hutten S., **Demleitner A. F.**, Popper B., Derdak S., Sutor B., Bilban M., Imhof A. and Kiebler M. A. (2021) *Pumilio2 and Staufen2 selectively balance the synaptic proteome*. Cell Reports 35(12):109279

Popper B.* & **Demleitner A.*,** Bolivar VJ, Kusek G., Snyder-Keller A., Schieweck R., Temple S., Kiebler M. A. (2018) *Staufen2 deficiency leads to impaired response to novelty in mice.* Neurobiology of Learning and Memory 150:107-115 (* shared first authorship)

Follwaczny P. & Schieweck R., Riedemann T., **Demleitner A.,** Straub T., Klemm A. H., Bilban M., Sutor B., Popper B. & Kiebler M. A. (2017) *Deficiency of the RNA-binding protein Pumilio2 determines predisposition for epilepsy in mice*. Disease Models and Mechanisms 10(11):1333-1342