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Genetic rat manipulation as an efficient model to unravel the role of Nogo-A and Staufen 2 proteins in cognitive processes.

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1. SUMMARY

Gene targeting technologies enabling a germline complete gene ablation mice had an enormous impact on the analysis of gene functions in vivo during the past two decades. However, the complete loss of a gene function often leads either to embryonic or early postnatal lethality or to molecular compensation that compensate for the function of the missing gene. Tissue or cell specific knockouts mostly avoid those drawbacks, but are currently confined mainly to mice as a model system. Indeed, development of gene inactivation technologies in rats is still far behind those available for mice, even if the elucidation of gene functions in transgenic rats would have several important advantages over using mice. The larger body size of a rat simplifies interventions such as microsurgery or multiple-electrode electrophysiological recording in vivo. Furthermore, higher order cognitive functions are more developed in this social rodent species than in the more solitarily living mice. Indeed, many behavioral tests are more advanced or validated for the rat, especially regarding the behavioral assessment of complex neuropsychiatric disease phenotypes, such as negative symptoms in schizophrenia or complex cognitive phenotypes.

In the present study we used two different miRNA-based knockdown rat models to study the impact of those genes on emotional behavior and learning and memory processes.

The first transgenic rat model is deficient for the Nogo-A protein within the entire animal. Nogo-A is expressed in CNS oligodendrocytes as well as in subpopulations of neurons and is known to suppress neurite growth and regeneration. In vivo studies in rats have shown successful regeneration of corticospinal tract axons over long distances and a significant enhancement of functional recovery using either neutralizing antibodies against Nogo-A or peptides blocking the Nogo receptor NgR. However, only few studies analyzed the role of Nogo-A on behavioral processes. Here, we show that Nogo-A deficient rats display behavioral phenotypes related to schizophrenia, such as difficulty in reversal learning, lower exploration and most importantly a reduced social

contact behavior. Our behavior observations extend those described for Nogo-A knockout mice.

In the second transgenic rat model were realized the first inducible tissue-specific gene inactivation rats described so far by knocking down Staufen2 (Stau2) protein production within excitatory neurons of the forebrain using an artificial miRNA targeting the respective protein. Staufen2 is a double-stranded RNA-binding protein essential for the localization of mRNAs in diverse cell types. In neurons, Stau2 regulates the dendritic localization and local translation of a subset of mRNAs that play a pivotal role in synaptic plasticity. In vitro experiments have shown that Stau2 is involved in the formation of dendritic spines, thereby modifying synaptic plasticity. However, no studies of Staufen function have been performed in vivo. Using our Stau2 deficient rats, we could show that the animals have an unaltered spatial reference memory and fear conditioning. However, Stau2 deficient rats have a highly significant impairment of spatial working memory. In addition, the transgenic animals have significant difficulties to detect spatial novelty. These behavioral finding fit very well to the in vivo electrophysiological data recorded by our collaborations, who could demonstrate that Stau2 deficient rats have an enhancement for LTP and an impairment of LTD. Together these findings suggest that Stau2 transported mRNAs are responsible for modulating synaptic plasticity at dendritic spines.

ZUSAMMENFASSUNG

Die Möglichkeit der Herstellung sogenannter „Knock-out"-Mäuse, in denen eine Genfunktion im gesamten Organismus inaktivert ist, hatte einen enormen Einfluß auf die Funktionsanalyse von Genen in vivo. Die ursprüngliche Technologie hat jedoch entscheidene Limitationen. Zum einen kann die komplette Geninaktivierung zur embryonalen oder frühen postnatalen Tod der Tiere führen. Zum anderen kann die fehlende Genfunktion während der Embryonalentwicklung von anderen Genprodukten kompensiert werden, was eine Funktionsanalyse massiv erschwert. Gewebs- und zelltypspezifische Geninaktivierungen haben diese Nachteile größtenteils nicht, jedoch war die Anwendung von modernen konditionalen Genregulationssysteme bisher auf die Spezie der Maus beschränkt. Die Entwicklung der Technologien zur konditionalen Gen-Inaktivierung für Ratten liegt sehr weit hinter der von Mäusen zurück, obwohl die Ratte als Tiermodell mehrere entscheidende Vorteile hat. Ihre Körpergröße vereinfacht die Anwendung besonders feiner Operationstechniken und ermöglicht die Implantation von Multielektroden-Sonden für elektrophysiologische Messungen in vivo. Desweiteren sind die höheren kognitiven Funktionen in Ratten wesentlich besser ausgeprägt als in Mäusen. Auch ähneln Ratten in ihrem Sozialverhalten eher den Menschen als die einzeln lebenden Mäuse. Alle wichtigen verhaltensbiologischen Paradigmen wurden in Ratten entwickelt und sind dort viel besser durchführbar, wie z.B. neuropsychiatrische Tests zur Bestimmung der Negativsymptomatik von schizophrenen Erkrankungen.

In der vorliegenden Arbeit wurden zwei transgene „knockdown" Rattenmodelle verhaltensbiologisch charakterisiert, in denen die Produktion der entsprechenden Gene durch die Expression einer artifiziellen miRNA unterdrückt wurde.

Im ersten Rattenmodell wurde die Produktion des Proteins Nogo-A im gesamten Körper inhibiert. Im zentralen Nervensystem wird Nogo-A in Oligodendrozyten und in Subpopulationen von Neuronen produziert und unterdrückt dort das Neuritenwachstum und die neuronale Regeneration. Studien mit Ratten in vivo

konnten zeigen, dass sich Axone von corticospinale Neuronen sich über eine lange Distanz regenerieren können und dadurch funktionelle Wiederherstellung signifikant verbessert wird, wenn die Tiere entweder mit einem neutralisierender Antikörper gegen Nogo-A oder mit Peptiden behandelt werden, die den Nogo-Receptor Ngr blockieren. Jedoch ist über die Funktion von Nogo-A auf das Verhalten der Tiere sehr wenig bekannt. Hier konnten wir zeigen, dass die funktionelle Inaktivierung von Nogo-A zu schizophrenie-ähnlichen Verhaltensveränderungen führt. Diese beinhalten eine Verminderung der kognitiven Flexibilität sowie ein reduziertes soziales Kontaktverhalten. Mit dieser Studie konnten wir die Verhaltensänderung, die durch eine Nogo-A defizienz ausgelöst werden, in Hinblick auf die Nogo-A knockout Mäuse erweitern.

Mit dem zweiten transgenen Modell konnten wir die erste induzierbare, gewebsspezifische Geninaktivierung in der Ratte realisieren, indem durch eine artifizielle miRNA die Produktion des Proteins Staufen2 (Stau2) in den exzitatorischen Neuronen des Großhirns unterdrückt wird. Stau2 ist ein Protein, das an doppelsträngige RNA bindet und essentiell am Transport von mRNAs in verschiedenen Zelltypen beteiligt ist. In Neuronen wird durch Stau2 der Transport bestimmte mRNAs in die dendritischen Fortsätze reguliert, die an der Ausbildung der synaptischen Plastizität beteiligt sind. Auch ist Stau2 an der lokalen Translation dieser mRNAs beteiligt. In vitro Experimente konnten zeigen, dass Stau2 die Morphologie von dendritischen Fortsätzen sowie ihre synaptische Plastizität reguliert. Dies konnte jedoch bisher nie am lebendigen Tier gezeigt werden. Mit den transgenen Tieren konnten wir zeigen, dass die Inaktiverung von Stau2 zwar zu keiner Veränderung des räumlichen Referenzgedächtnisses führt und auch die Furchtkonditionierung unverändert ist, aber zu einem signifikanten Verschlechterung des räumlichen Arbeitsgedächtnisses führt. Zudem haben die transgenen Ratten Schwierigkeiten, neue räumliche Umgebungen zu erkennen. Diese Verhaltensänderungen passen sehr gut zu den elektrophysiologischen Daten unserer Kollaborationspartner, die zeigen konnten, dass die Stau2 Inaktiverung zu einer Verstärkung der Langzeitpotenzierung und einer Inhibition der Langzeitdepression führt. Diese Daten zusammen demonstrieren die wichtige

Rolle von Stau2 bei der Ausbildung von synaptischer Plastizität in den dendritischen Fortsätzen.

2. INTRODUCTION

2.1 HISTORY OF RESEARCH IN COGNITIVE NEUROSCIENCE

The modern era of memory research is the result of three different approach based one on experimental analyses of learning and memory referring to ancient times, a second reflecting the investigation of damaged brain from patient, and the third that is based on use of animal models to study memory phenomena at both the cellular and system levels.

Already in the nineteenth century, empirical studies of memory established fundamental idea about learning capacity and forgetting mechanism with the hypothesis of multiple forms of memory existences expressed already in 1890 by James together with the idea that memories took time to 'consolidate' after learning (Lechner, Squire et al. 1999; McGaugh 1999; Nadel and Hardt 2011).

Even going back to 1804, the first idea that memory is not a single faculty of the mind could be found in notes that Maine de Biran wrote about mechanical memory, sensitive memory, and representative memory (Maine de Biran, 1804/1929).

Very frequently in the earlier literature is possible to finds the idea of two distinct kinds of memory. In fact, McDougall in 1923 differentiate between explicit and implicit recognition, and Tolman in 1948 wrote that there is more than one kind of learning (Tolman 1948). Indeed Ryle in 1949 distinguished between "knowing how" and "knowing that", followed by Bruner that in 1969 propose the idea of "memory without record" and "memory with record". The idea in the artificial intelligence literature as procedural and declarative knowledge was discussed in 1970s by Winograd.

The most important input for the memory understanding came in 1949 when Hebb in his "The Organization of Behavior: A neuropsychological theory" distinguished between the two form of memory asserting his seminal cell assembly theory. He defines a short-term memory (STM) as continuous and

coordinated activity in cell assemblies (a set of interconnected neurons that encode a stimulus). It means that neurons recurrently excite each other for some time after the original stimulation. At some point the interactionstop, maybe due to depletion of neurotransmitter, which marks the end of STM. A long-term memory (LTM) can arise out of STM, if the reverberatory activity leads to structural changes in the synapses connecting the neurons of the cell assembly.
Hebb proposed that an association could not be localized to a single synapse. Instead, neurons were grouped in "cell assemblies" and an association was distributed over their synaptic connections. Hebb, also following an idea of Lorente de Nó, proposed that sensory stimulation could initiate patterns of neural activity that were centrally maintained by activity in synaptic feedback loops. This so called 'reverberatory activity' made possible a trigged response after a delay.

2.2 TYPES OF MEMORY: DECLARATIVE AND NON-DECLARATIVE MEMORY SYSTEM

Declarative memory is involved in the acquisition, retention, and retrieval of knowledge that can be intentionally and consciously recollected (Cohen and Squire 1980). Such knowledge includes memory for events (episodic memory) or facts (semantic memory) (Tulving, E. 1983). The difference between the two memory systems lies in the differential mechanisms to recall stored information. Indeed, non-declarative memory is expressed through performance and is not accessible through conscious faculties, declarative memory is subject to conscious recollection (Squire and Zola 1996). The declarative memory has the ability to detect and encode what is unique about a single event, which occurs at a particular place and time. In the case of non-declarative memory can gradually extract the common elements from a series of separate events(Squire 2004).
It is now generally accepted that declarative (also known as explicit) memory requires for storage the medial temporal lobe and the hippocampus, whereas procedural (also known as implicit) memory does not. Nadel and O'Keefe wee the first to propose a distinction between episodic and semantic memory and use it to help to understand what amnesic patients could and could not learn and

recall (Gaffan 1974, O'Keefe J, Nadel L. 1978, Kinsbourne and Wood, 1975). They proposed that the hippocampus is involved in representation of spatial contexts with a major role in episodic memory. However for them the hippocampus was not important to semantic memory, which represents information without necessary links to context.

The most strong evidence of multiple memory systems theory came from the double dissociation studies, performed by creating in experimental animals irreversible and reversible lesions of the hippocampus and striatum. The different effects of lesions to the hippocampus and caudate nucleus of the striatum were analyzed while performing two radial maze tasks in order to behaviorally demonstrate a double dissociation of the mnemonic functions of the two memory systems (Packard, Hirsh et al. 1989).

Other works in animals added pieces to the theory of different memory systems. For example in 1982, the cerebellum was discovered to be essential for delay eyeblink conditioning (McCormick, Lavond et al. 1982), a form of learning that was entirely preserved both in animals with hippocampal lesions and in severely amnesic patients (Gabrieli, McGlinchey-Berroth et al. 1995; Clark and Squire 1998). In addition it was shown that still other types of learning, which involve the attachment of positive or negative relation of a stimulus (i.e. in fear conditioning or conditioned place preference) have an essential dependence on the amygdale (Fanselow MS 1994, Debiec and Ledoux 2004).

2.3. BRAIN STRUCTURES FOR DECLARATIVE MEMORY

As already discussed declarative memory refers to the acquisition, retention, and retrieval of knowledge that can be consciously and intentionally recollected (Cohen and Squire 1980) and includes memory for events or facts (Tulving, E. 1983). Episodic memories are measured by direct or explicit tests of memory, such as free recall, cued recall, or recognition, that refer to a prior episode (Clark and Squire 1998). This kind of memory is strictly bound with a specific brain structure defined as medial temporal lobe. The medial temporal-lobe memory system consists of multiple structures. The perirhinal and

postrhinal/parahippocampal cortices are the recipients of differing combinations of sensory information and provide the major polysensory input to the hippocampus through their entorhinal connections (Suzuki &Amaral 1994).provide the major input to the hippocampus through their entorhinal connections and receives different combinations of sensory information (Suzuki and Amaral 1994). The parahippocampal region provides major inputs to the hippocampal region, which is composed by several subregions such as of the subiculum, the Cornus Amonis (CA), and the dentate gyrus (DG).

Case of human amnesia after surgery as in patient R.B (Zola-Morgan, Squire et al. 1986), G.D, L.M and W.H (Rempel-Clower, Zola et al. 1996) helped in undercover the regions involved in learning and memory formation. It has been known for nearly 100 years that declarative memory is impaired by bilateral damage to either the medial aspect of the temporal lobe or the midline of the diencephalon. Damage to these areas makes it difficult to establish new memories (anterograde amnesia) as well as to retrieve some memories formed before the onset of amnesia (retrograde amnesia). General intellectual property is intact, as is immediate memory, language and social skills, personality, and memory for the remote, past, especially childhood.

The most famous case appeared in literature with Brenda Milner in 1957. She reported the effect on memory abilities caused by bilateral medial temporal lobe resection in a patient who became known as H.M. (1926–2008) (Scoville and Milner 1957). H.M. had frequent seizures that could not be correctly resolved or controlled by anticonvulsant drugs. The surgery was a new radical approach. H.M.'s bilateral medial temporal lobe resection included the hippocampal formation and adjacent structures including most of the amygdaloid complex and entorhinal cortex.

After surgery, H.M showed a normal general intellect and perceptual ability but with a profound forgetfulness. He could not form new memories (anterograde amnesia) and also could not access some memories acquired before his surgery (retrograde amnesia). His impairment extended to both verbal and non-verbal material, and it involved information acquired through all sensory modalities. The descriptions of H.M suggested some principles about how memory is

organized in the brain. It was clear that for the declarative memory storage the medial temporal system appears to have only a temporary role.
Several cases of circumscribed human amnesia have become available in recent years, which confirm and extend the findings that bilateral damage limited to the particular region of the hippocampal formation is sufficient to produce moderately severe anterograde memory impairment. Moreover bilateral damage beyond these regions, but still limited to the hippocampal formation, can produce more severe anterograde memory impairment (Squire 2004).

2.4 THE HIPPOCAMPUS

The circuitry structure of the hippocampus has been known since the time of Ramon y Cajal (1911). It appears grossly as an elongated, banana-shaped structure with its long axis extending in a "C"-shaped form extended from the septal nuclei rostrally, over and behind the diencephalon, into the temporal lobe caudally and ventrally. Distinct subregions can be distinguished: the hippocampus proper (consisting of CA3, CA2 and CA1), the dentate gyrus (DG), and the subiculum (Figure 1 A). Through a coronal sections of hippocampus (Figure 2 B) is possible to evidence the cortex that forms the hippocampus with itsthree-layered structure. The first layer is a deep layer, composed by a mixture of afferent and efferent fibres and interneurons. In the CA this part regions it is called stratum oriens whereas in the DG this layer is called the hilus. The cell layer extended superficial to this polymorph layer and it is composed of principal cells and interneurons. In the CA regions and the subiculum it is referred to as the pyramidal cell layer whereas in the DG this layer is called the granule layer. The most superficial layer is the molecular layer in the DG and the subiculum (Figure 1 B).

2.4.1 DG: ANATOMY, ROLE AND CONNECTIVITY

The principal layer of the DG is the granule cell layer; it contains the cell bodies of the granule cells, with a soma of approximately 7 μm. The granule cell dendrites extend into the overlying molecular layer where they receive synaptic connections from several sources. Granule cells have dendrites emerging only from the top or apical portion of the cell body. The axons of the granule cells are called mossy fibers and they originate from the basal portion of the cell body and extend into the hilus. The mossy fibers synapse pass through mossy cells in the polymorphic cell layer before merge into a bundle of fibers that exits the hilus and enters stratum lucidum of CA3. There are various types of polymorphic cells but they only project to other parts of the DG. The dendrites receive different types of synaptic contacts in each of these strata. The basal dendrites extend from the base of the pyramidal cell body into stratum oriens. (from Gordon M. Shepherd. The Synaptic Organization of the Brain).

The DG combined the medial and lateral perforant path inputs to generate a spatial representation. However only the lateral perforant path input is used for the visual objects detection involving the spatial locations. In CA3 the medial and lateral perforant path inputs are combined to generate a spatial representation that contains within a representation of the visual objects occupying the spatial locations. In CA1, it appears that the medial and lateral perforant path inputs do not mix much because only the medial perforant path appears to be involved in generating a representation of space, whereas the lateral perforant path is used to identify visual objects. The DG (as CA3, and CA1) receives information from both the medial and lateral entorhinal cortex that provides information necessary for proper hippocampal function (Witter, Naber et al. 2000). This supportsthe concept that the dentate gyrus, CA3, and CA1 can be dissociated from each other using behavioral tasks (Gilbert and Kesner 2003; Kesner, Lee et al. 2004; Rolls and Kesner 2006). Converging evidences show that the DG is involved in binding animal's internal spatial representation with the sensory information on external landmarks (Hunsaker, Mooy et al. 2007).

2.4.2 CA1 AND CA3:ANATOMY, ROLE AND CONNECTIVITY

The main layer of the CA region is the pyramidal cell layer and contains the cell bodies of the pyramidal cells. The CA1 region is adjacent to the subiculum. The CA3 region is adjacent to the fimbria/fornix and choroid plexus. The CA2 region is a small boundary between CA1 and CA3, and CA4 is located in the hilus of the DG. The somas of the pyramidal cells have a triangular shape and they are smaller than those in CA3. They measure 40 to 60 μm at their base versus 20 to 40 μm for CA1 pyramidal cells. The stratum oriens contains the basal dendrites of the pyramidal cells. The stratum moleculare contains the apical dendrites of the pyramidal cells. The dendrites of CA3 pyramidal neurons are also shorter and thicker than those of pyramidal cellsinCA1. The axons of the granule cells called MFs, project to the CA3 region and establish synaptic contacts with CA3 pyramidal cells in the stratum lucidum. The stratum lucidum is characterized by a postsynaptic components of synapses between the MF terminals and the apical dendrites of CA3 pyramidal cells called “thorny excrescences”.

Hippocampal region CA1 play a role in matching of CA3 output with afferent input from entorhinal cortex (Eichenbaum & Buckingham, 1990, Hasselmo and Wyble 1997; Lisman and Grace 2005).

Moreover NMDA dependent plasticity in the CA1 may be critical for intermediate, but not short-term memory. One suggestion is that the CA1 region is directly involved in a part of the information across time, generating specific units or duration of events based on specific order of occurrence of events in different epochs of time made (Rolls and Treves 1998). CA1 region of the dorsal hippocampus is also important in supporting some association where the stimuli can be spatial or non-spatial only if there is a temporal interval interposed between the two stimuli (Gilbert and Kesner 2003). it is possible to state that the hippocampus is involved in supporting a large number of arbitrary associations, but if CA3 requires the presence of a spatial component to facilitate the association, CA1 requires instead the presence of a temporal component for any arbitrary association (Kesner, Hunsaker et al. 2005). Converging evidence suggests that the projection from the entorhinal cortex to the CA1 subregion of

the hippocampus called perforant pathway (pp), may provide necessary environmental input to support specific computational resources underlying delay-dependent retention and retrieval of spatial information. On the other hand, the dentate/mossy fiber system within the trisynaptic circuitry provides the necessary framework for optimal storage or encoding of new information in the CA3 subregion (Colbert and Levy 1992; Hasselmo 1995; Brun, Otnass et al. 2002). The CA1 subregion play a role in comparing information from the two afferent inputs, and the CA3 input contains the contents of processed forms of memory, while the perforant pathway supplies unprocessed sensory information. The comparison of converging inputs may lead to the mismatch from expectations, (Hasselmo and Schnell 1994) detection of novelty (Vinogradova 2001), and/or a facilitation of retrieval mechanisms (Lee and Kesner 2002; Vago, Bevan et al. 2007).

CA3 is involved in the memory processes within short-term memory with the acquisition of novel information through NMDA receptor-mediated plasticity mechanisms (Lee and Kesner 2002). Contextual fear-conditioning experiment with subregion-specific lesions provide further evidence for supporting this role of CA3 in rapid acquisition of novel information (Lee and Kesner 2004). However, short-term memory for distinct places depends on CA3 but also on CA1. It has been shown that the plasticity mechanism in CA3 was activated only when animals encountered novel configurations of familiar cues for the first time(Lee and Kesner 2004). Finally, during the delay period in rats in a spatial position short-term memory task, single unit activity has been recorded in CA3 (Hampson and Deadwyler 2000) and in monkeys with experiment involving object–place and a location–scene association short-term memory (Wirth, Yanike et al. 2003). It is possible to affirm that the plastic changes in the CA3 network are essential in encoding novel information involving associations between objects and places, odors and places, or between landmark visual cues and spatial locations, and that the mechanism is NMDA receptor-mediated.

The CA3 subregion of the hippocampus is also necessary in tasks that require multiple trials to acquire the task. Indeed, lesions of the CA3 (but not the CA1 or DG) impair the acquisition of object–place and odor–place paired associate learning, a task that requires multiple trials to learn (Gilbert and Kesner 2003). It

has been suggested that the hippocampus and its subregions support the formation of arbitrary associations, including paired-associate learning (Eichenbaum H, Cohen NJ. 2001, McNaughton BL, Morris R. 1987, Hasselmo and Wyble 1997).

The CA3 subregion is also essential in supporting the retrieval of information from when a short-term delay is introduced (Kesner and Rolls 2001; Kesner 2007) As the information circulates through the recurrent network in CA3, buffering of information within the network is likely to occur. In summary, CA3 may play a key role in short-term memory tasks, especially when the nature of the tasks entails encoding of novel information or pattern completion.

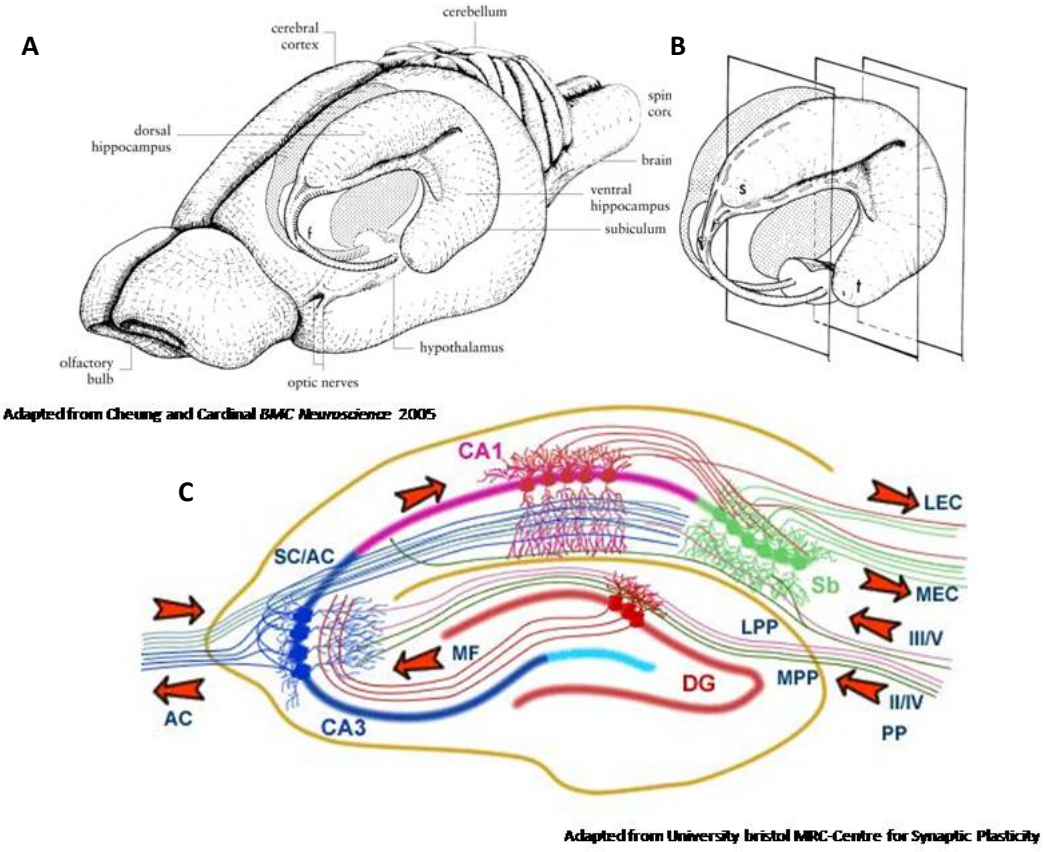


Figure 1. The hippocampus: (A) 3D Rat brain organization of the hippocampus and related structures. (B) left hippocampus coronal sections. (C) Hippocampus network. Perforant Path: The perforant path (pp) is the major input to the hippocampus. The axons of the perforant path arise principally in layers II and III of the entorhinal cortex (EC). Axons from layers II/IV project to the granule cells of the dentate gyrus (DG) and pyramidal cells of the CA3 region, while those from layers III/V project to the pyramidal cells of the CA1 and the subiculum. The pp can be segregated into lateral and medial pathways (LPP and MPP, respectively), depending on whether the fibres arise from the lateral or medial entorhinal cortex. Mossy Fibre Pathway: are the axons of DG granule cells. They extend from the dendategyrus to CA3 pyramidal cells, forming their major input. MF synapses on CA neurons are large aggregations of termini, with multiple transmitter release sites and post-synaptic densities. Multiple granule cells can synapse onto a single CA3 pyramidal cell. Schaffer Collateral/Associational Commissural Pathway: This pathway is derived from axons that project from the CA3 region of the hippocampus to the CA1 region (Figure C and Description from Hippocampal Pathways, MRC Centre for Synaptic Plasticity, University of Bristol).

2.5 SYNAPTIC PLASTICITY

2.5.1 SYNAPTIC PLASTICITY AND MEMORY

Memory formation dependents on changes in synaptic activity that triggers strengthening of associations between neurons; indeed, activity-dependent synaptic plasticity at appropriate synapses during memory formation is believed to be necessary and sufficient for storage of information.

Within the hippocampus, synaptic efficacy is regulated in a bidirectional manner to prevent individual neurons and the underlying neural network from saturation. This regulation is mirrored through the processes of long-term potentiation (LTP) and long-term depression (LTD) (Martin and Morris 2002; Malenka and Bear 2004), two models that help understanding the mechanism by which strengthening of synaptic connections can be achieved. Whereas hippocampal LTP has been shown to be important for spatial learning and memory (Tsien, Huerta et al. 1996), hippocampal LTD seems to be essential for cognitive flexibility and working memory (Zeng, Chattarji et al. 2001; Nicholls, Alarcon et al. 2008).

2.5.2 LONG-TERM POTENTIATION (LTP)

LTP results from synchronized pre- and post-synaptic activity, causes a facilitation of chemical transmission that lasts for hours in vitro, and in vivo also for weeks or months (Bliss and Gardner-Medwin 1973; Abraham, Logan et al. 2002).

The first description of LTP made by Bliss and Lomo in 1973 reported that trains of high-frequency stimulation to the rabbit perforant path caused a sustained increase in efficiency of synaptic transmission in the granule cells of the dentate gyrus (Bliss and Lomo 1973). Together with others studies which followed during the 1970s, this hypothesis confirmed the Hebbian nature of this form of synaptic plasticity, and it was immediately recognized that the synaptic changes that underpin certain forms of learning and memory might be similar to those upon which expression of LTP relied.

Numerous variants of the synaptic plasticity and memory hypothesis have been advanced over the years (Kandel and Schwartz 1982; Lynch and Baudry 1984; McNaughton and Morris 1987; Siegelbaum and Kandel 1991; Izquierdo and Medina 1995; Morris and Frey 1997; Baudry 1998). However, the common theme of the hypothesis is represented by the idea that activity-dependent synaptic plasticity is appear in specific synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed (Martin, Grimwood et al. 2000; Kesner 2007)

The mechanisms by which LTP consolidation occurred involve NMDA receptors activation during high-frequency stimulation. The reaction consists of glutamate molecules released from the pre synaptic terminal diffuse across the synaptic cleft and bind to both sub-types of receptor, opening AMPA receptor channels. The resulting inward current flow carried by Na^+ ions depolarizes the post-synaptic membrane to produce an excitatory post-synaptic potential (EPSP). Low concentrations of released glutamate do not depolarize the postsynaptic membrane sufficiently to relieve the Mg2+ -block of the NMDA receptor channel (Nowak et al. 1984). High concentrations of glutamate released at a strongly active synapse produce strong depolarization of the post-synaptic membrane, resulting in the expulsion of magnesium ions from the NMDA receptor channel, and allowing influx of Na+ and Ca2+ ions. It is this calcium influx that induce LTP (Lynch, Larson et al. 1983, Malenka et al. 1988). The consequence of the increase in intracellular calcium concentration is the increased calmodulin kinase II (CaMKII) and protein kinase C activity (Lisman, Schulman et al. 2002) resulting in a significant effect is increased AMPA conductance as a result of AMPA receptor (AMPA-R) phosphorylation and increased recycling of AMPA-R, which is due to CaMKII-induced changes in cytoskeletal proteins. Numerous evidences suggested that to lead LTP expression the increased expression of AMPA receptors on the postsynaptic membrane is the primary requirement (Geiger, Melcher et al. 1995; Lynch 2004).

Another important point is that the molecular mechanisms of LTP induction and maintenance do not remain the same in all synapses. For instance at the mossy fibre–CA3 pyramidal cell synapse, the NMDA receptor is not required for LTP

induction (Harris and Cotman 1986) and the site of LTP expression is primarily pre-synaptic (Weisskopf and Nicoll 1995). In contrast, at both medial perforant path–dentate gyrus granule cell (Morris, Anderson et al. 1986) and Schaffer collateral–CA1 pyramidal cell synapses (Collingridge et al. 1983) LTP induction is mediated by the NMDA receptor. Moreover, there is a major post-synaptic component to LTP expression at both the latter connections (Nicoll and Malenka 1995, Cooke and Bliss 2006)

2.5.3 LONG-TERM DEPRESSION (LTD)

LTD results in a long-lasting decrease in synaptic efficacy. To induce LTD two principal protocol has been used over the time: a long trains of low-frequency (1 Hz) stimulation (Dudek and Bear 1992), or mismatching of pre- and post-synaptic action potentials (Markram 1997). It has been proposed that there are two mechanistically distinct forms of LTD. One consisting in a depotentiation, which refers to the reversal of LTP, and the other a 'de novo' LTD, which refers to depression from an unpotentiated baseline. Some forms of LTD are dependent upon the NMDA receptor and are triggered by low concentrations of post-synaptic calcium (Nishiyama, Hong et al. 2000). Calcium-responsive phosphatases such as calcineurin and protein phosphatase 1 (PP1) are implicated as effector molecules in the mechanisms of LTD. These phosphatases dephosphorylate kinase targets such as glutamate receptors (Morishita, Marie et al. 2005) and the kinases themselves (Blitzer, Connor et al. 1998). LTD-like processes have been suggested to be equally effective at storing information that is essential for learning and memory (Kemp and Manahan-Vaughan 2007; Massey and Bashir 2007). For example, exist correlations between depotentiation, LTD and spatial exploration (Abraham, Logan et al. 2002, Cooke and Bliss 2006).

The importance of hippocampal CA1 LTD has been shown in the formation of long-term spatial memory during Morris water maze tasks. (Ge, Dong et al. 2010). Anyway Hippocampal LTD has been implicated in forms of learning and memory other than spatial memory. For example, LTD induction in behaving animals can be facilitated by exposure to novel objects (Manahan-Vaughan and

Braunewell 1999) and novelty exposure could reverse LTP in the hippocampus (Xu, Anwyl et al. 1998).

2.6 CELLULAR AND MOLECULAR MECHANISMS: FROM SHORT TO LONG-TERM MEMORY

Long-term memory differs from short-term memory not only in time course but also in molecular mechanisms. Long-term memory, but not short-term memory, requires the synthesis of new proteins (Yin and Tully 1996; Kandel and Pittenger 1999). Studies during the past decade in Drosophila, Aplysia, and mice suggest that cAMP, PKA, and the cAMP-responsive transcription factor CREB are critically involved in the conversion of short-term to long-term memory for both declarative and non-declarative memory. Several experiments indicated that LTP itself has stages, as long-term facilitation in Aplysia. It has been proposed that LTP can be distinguished in an early-stage LTP (E-LTP) that requires modification induced by Ca2+/calmodulin-dependent protein kinase II α (CaMKII α) and the tyrosine kinase fyn (Nicoll, Kauer et al. 1988), and a late-phase LTP (L-LTP) that requires the kinase PKA and protein synthesis (Frey, Huang et al. 1993). Moreover, there is increasing evidence for rapid receptor insertion and remodeling during E-LTP and for actual structural changes during L-LTP (Luscher, Nicoll et al. 2000).

Some of these ideas were tested using mice with targeted knockout of CaMKII and the tyrosine kinase fyn, kinases that had previously been implicated in LTP in pharmacological studies (Nicoll, Kauer et al. 1988). Those experiment revealed that mice lacking CaMKII α displayed a partial loss of E-LTP in CA1 and impairment on spatial memory tasks (Silva, Paylor et al. 1992; Malinow 1998).

Other studies had similar conclusion showing how mice with targeted deletions of the tyrosine kinase fyn also displayed deficits in E-LTP and spatial memory. On the contrary mice with non-receptor tyrosine kinases src and yes deficit were normal (Grant, O'Dell et al. 1992). Other studies demonstrated that expression of a dominant-negative inhibitor of PKA in neurons of the forebrain using the CaMKIIα promoter caused an attenuated L-LTP but a normal E-LTP (Abel, Nguyen et al. 1997). These mice have no differences in a contextual task

compared to wild-type animals, with a intact good short-term memory but they were impaired in selective long-term memory. Therefore the PKA signaling pathway in mice (as in Aplysia and Drosophila)seems to be important for maintaining both LTP and memory for prolonged periods of time.

Studies in Drosophila suggest that CREB-mediated induction of transcription is necessary to produce the long-lasting changes in synaptic strength required for the long-term storage of memories (Yin and Tully 1996). In Aplysia, modifications of existing proteins create a short-lasting increase of synaptic strength. CREB switch functions from this short lasting increase of synaptic strength to one that is long-lasting and produced by the synthesis of new proteins (Martin, Casadio et al. 1997). The CREB target genes, whose transcription is regulated during consolidation, include a set of immediate- early genes (such as C/EBP or zif268) that affect transcription of downstream genes. These results both increase and decrease of protein expression involved in protein synthesis, axon growth, synaptic structure and function.

2.7 SPINES AND MEMORY

2.7.1 MORPHOLOGY OF SPINES

A morphological change that has been reported by several researches involves a rapid expansion of spine heads after tetanic stimulation in hippocampal slices or cultures. Most excitatory synapses in the brain terminate on dendritic spines. They are specialized locus on dendrites that contain a postsynaptic density (PSD) including receptors, channels and signaling molecules that couple synaptic activity with postsynaptic biochemistry (Sheng and Kim 2002). Spines provide a closed compartment that allows rapid changes in the concentrations of signaling molecules, such as calcium, and therefore make possible rapid and efficient responses to inputs (Koch and Zador 1993). Spines density is thought to be around 1–10 spines per micrometer of dendrite length, but some neurons, as the ones in hippocampus contain thousands of spines throughout the dendritic arbors. They come in a wide range of sizes and shapes, their lengths varying from

0.2 to 2 μm and volumes from 0.001 to 1 μm3. Using electron microscopy was possible to identified three main morphological categories of spines: thin, filopodia-like protrusions ("thin spines"), short without a well-defined spine neck ("stubby spines") and large with a large bulbous head ("mushroom spines") (Bourne and Harris 2008; Hotulainen and Hoogenraad 2010).

2.7.2 FUNCTION OF SPINES

It has been suggested already from Ramon y Cajal and Hebb that strengthening or weakening particular synaptic connections in response to experience, meaning modulating the number of dendritic spines and/or their morphology it could help to understand the storage of memories mechanism. Evidence of alterations in memory process and storage has been found in several species of animal underwent experiment using complex versus simple environments, or exposed to various training or stimulation regimens (Bailey and Kandel 1993). Indeed, there is evidence that induction of synaptic plasticity (LTP induction or memory formation) leads to changes in the number or shape of spines(Chang and Greenough 1984; Yuste and Bonhoeffer 2001; De Roo, Klauser et al. 2008). However changes in spine morphology have also been seen after LTP induction (Fifkova and Anderson 1981; Malinow and Malenka 2002).

Synaptic transmission that occurs after learning or LTP Long-term can be modulated by different spine morphology (Volfovsky, Parnas et al. 1999). Large spines receive input from large pre-synaptic terminals and more vesicles as well as polyribosomes are preferentially translocated into those large spines during synaptic plasticity. This event probably facilitates the incorporation of local protein synthesis machinery (Ostroff, Fiala et al. 2002). Also morphological change as shortening or widening the neck of a spine affects calcium influx into the dendrite and therefore might affect biochemical events in spines (Majewska, Brown et al. 2000). It has also been shown that glutamate sensitivity correlates with spine shape. The ratio AMPA /NMDA receptors in Schaffer collateral synapses increases linearly with the diameter of the postsynaptic density (Takumi, Ramirez-Leon et al. 1999). Transmission could be enhanced with an

increase of spines number since more connections would be made with the presynaptic neuron (Lamprecht and LeDoux 2004).

2.7.3 CHANGES IN SPINES AFTER A LEARNING EXPERIENCE

The architecture of spines depends on the specialized structure of cytoskeletal filaments (Matus 2000). These microfilaments are composed of actin, which is present in the spine cytoplasm in and connected with event in postsynaptic density. Developmental studies have shown that structural plasticity of spines after LTP induction and memory acquisition depend on reorganization of actin (Fischer, Kaech et al. 1998).

Therefore a reduction in actin based spine motility, could results in LTP and memory consolidation leading to spine stabilization (Fischer, Kaech et al. 1998; Matus 2000).

It has been hypnotized that the AMPA glutamate receptors could have a stabilizing effect on spine morphology(Fischer, Kaech et al. 2000). Actin-based spine motility is suppressed when AMPA is applied to hippocampal neurons, and this suppression is completely blocked by AMPA antagonists. While NMDA receptors might be important in the initial phase of spine motility, a stabilization phase is mediated by AMPA receptors contributing to the long-lasting spine stability (Lamprecht and LeDoux 2004).

At present, the evidence for spine involvement in memory is still debated, and the main issue is still the nature of the changes that take place in dendritic spines after a learning experience. Although all reported an increase in spine volume after tetanic stimulation, there is also heterogeneity of observations. For instance, some researchers found a threefold increase in spine volume within 2–4 min of stimulation (Matsuzaki, Honkura et al. 2004). A similar but smaller and slower change after the induction of LTP was also reported (Otmakhov, Tao-Cheng et al. 2004). The authors affirmed to have produced a persistent change in the spine volume independently of a marked increase in the expression of calcium/calmodulin-dependent protein kinase II.

It has been shown that LTP was associated with an increase in the amount of F-actin relative to G-actin in the spine head, and with a parallel increase in the size of the spine head (Okamoto, Nagai et al. 2004). They also found that LTD was associated with spine shrinkage and an increase in the relative amount of G-actin in the spine head. Additionally, it has been reported a slow and small increase in the diameter of the spine heads after high frequency (Zhou, Homma et al. 2004). This study also showed, for the first time, symmetry between changes in spines and synaptic responses, in that spine heads shrunk after the induction of LTD. It has been demonstrated that, after the induction of LTP, enlargement of the spine head is correlated with the expansion of postsynaptic density (Geinisman, Berry et al. 2001; Segal 2005).

2.8 BRAIN NETWORK DISCONNECTIVITY

As previously discussed, dendritic spines and memory formation are linked together. Memory formation is thought to lead to an increase in spine density. Vice versa , increase in spine density, for example after exposure to an enriched environment may enhance learning ability (Moser, Trommald et al. 1997). Synaptic plasticity activity is thought to be involved also in some diffuse mental diseases. For example a large number of neurophysiological and neuroimaging studies of patients with schizophrenia have furnished in vivo evidence for disconnectivity resulted from aberrant wiring of connections during development or from aberrant synaptic plasticity(Stephan, Friston et al. 2009).

Two possible explanations for how disconnection arises have been proposed. The first refer to synaptic disconnection, for example due to impaired modulation of NMDAR-dependent synaptic plasticity, for abnormalities of GABAergic and DAergic function resulting in NMDAR dysfunction (Laruelle, Frankle et al. 2005). Postmortem studies on schizophrenia showed that the disconnection derives from a reduction in dendritic field size and dendritic spines of cortical neurons (Sullivan, Kendler et al. 2003).

The other possible disconnection involve the interregional or local functional coupling that in schizophrenia are abnormal because of impairments of

structural, anatomical connectivity, due to aberrant axonal wiring association (Davis, Stewart et al. 2003). In this context, white matter, myelin, and oligodendrocytes have received increasing attention for their potential role in the pathophysiology of schizophrenia. In fact, various functional and microstructural changes have been detected in white matter of schizophrenic patients, including reductions in volume and decreased coherence along white matter tracts, the latter being consistent with misalignments of axons (Kubicki, McCarley et al. 2007; Carletti, Woolley et al. 2012). Moreover post-mortem brains analysis of schizophrenic patient and healthy subjects has identified differential expression levels of a number of oligodendrocyte- and myelin-related genes in multiple brain regions (Kerns, Vong et al. 2010; Roussos, Katsel et al. 2012), pointing to disruption of oligodendrocyte function and abnormalities in myelin maintenance and repair. Myelin-related genes represent only one part of the whole genetic contribution to schizophrenia, however a relatively large number of genes found to be associated with schizophrenia belong to this category (Rietkerk, Boks et al. 2009)clearly indicating a myelin-related function to schizophrenia pathogenesis. Among all the candidate genes potentially involved in schizophrenia, Nogo-A received particular attention in the last few years (Willi and Schwab 2013).

2.8.1 Axonal plasticity and Nogo-A protein

The molecular mechanisms by which axon regeneration is restricted in the adult mammalian CNS are poorly understood. The lack of growth-promoting molecules together with the presence of negative extracellular cues is thought to provide a non-permissive environment for re-growing fibers. In particular, one myelin component present in the central nervous system(CNS) have been characterized as potent inhibitors of axonal growth: Nogo-A, the largest transcript of the recently identified Nogo gene (Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000).

Nogo-A is a protein with potent neurite growth inhibitory activity and is present in CNS myelin (Caroni and Schwab 1988). Studies in rats (Schwab 2004)showed

a successful regeneration of corticospinal tract axons over long distances and significant enhancement of functional recovery using neutralizing antibodies against Nogo-A (Schnell and Schwab 1990; Liebscher, Schnell et al. 2005), or peptides blocking the Nogo receptor subunit NgR (GrandPre, Li et al. 2002).

Neuronal Nogo-A is highly expressed in the fetal and early postnatal brain. On the contrary is down-regulated in most anatomical structures in adulthood, except in some regions of high plasticity (e.g., hippocampus), in which neuronal Nogo-A expression remains high (Huber, Weinmann et al. 2002). Nogo-A play a major role in controlling neurodevelopmental processes and neural plasticity mechanism. Nogo-A is highly expressed in pre- and postsynaptic neurons including pyramidal cells of CA3 and CA1 in adult hippocampus known to show synaptic plasticity eventsthroughout life, (Lee, Raiker et al. 2008). Nogo-A thus appears here again as a stabilizer of synapses, the suppression of which leads to growth and higher plasticity in axons and dendrites. Studies using anti-Nogo-A antibodies or Nogo-A KO models showed an induction of marked changes in the complexity of the basal and apical dendritic arbors of CA1 and CA3 neurons and leads to massive sprouting of the CA3 axons (Zagrebelsky, Schweigreiter et al. 2010). Silencing NgR1 expression can reproduce these phenotypes at the dendritic and spine level. Moreover, electrophysiological recordings in acute hippocampal slices have revealed that the inactivation of Nogo-A or NgR1 with function-blocking antibodies or KOs increases LTP (Raiker, Lee et al. 2010; Delekate, Zagrebelsky et al. 2011).

Interestingly NgR1 (also known as Nogo66 receptor and Reticulon 4 receptor) is encoded by a gene located on chromosome 22q11, a well-known hotspot for genetic predisposition in schizophrenia (Liu, Abecasis et al. 2002). Post-mortem brain tissue Examination in patients with psychiatric disorders together with human genetic linkage studies and suggested a link between Nogo signalling and bipolar disorder and schizophrenia (Novak, Kim et al. 2002; Sinibaldi, De Luca et al. 2004; Budel, Padukkavidana et al. 2008). One study in individuals affected by schizophrenia from different families, described several point mutations inNgR1,some of which directly affected Nogo binding (Budel, Padukkavidana et al. 2008). Moreover, translational behavioral test relevant for assessing face validity of animal models of schizophrenia support the hypothesis that there is a

link between Nogo-A/NgR signaling and schizophrenia. Indeed, mice lacking NgR1 showed mild behavioral alterations that mimic some symptoms of schizophrenia. Moreover mice KO for Nogo-A protein showed deficit in Nogo-A and subsequent deficient sensorimotor gating, disrupted latent inhibition, perseverative behavior, and increased sensitivity to the locomotor stimulating effects of amphetamine (Willi R. et al, 2010) aspect related to the symptoms of schizophrenia (Baruch, Hemsley et al. 1988).

2.9 DENDRITIC MRNA

2.9.1 IMPORTANCE OF DENDRITIC MRNAS IN LEARNING AND MEMORY

The biological basis of learning and memory are thought to be trigged at the synapse through molecular mechanisms known as LTP and LTD. Moreover, lasting activity-dependent changes in synaptic strength depend on new protein synthesis and the growth or remodeling of synapses. Increasing evidence suggests that, in the dendrite, protein synthesis is carried out on a small, localized scale directly adjacent to remodeling synapses.

In neurons mRNA it is used in transport during development to regulate growth cone turning (Lin and Holt 2007), axon determination (Morita and Sobue 2009) and neurite outgrowth (Hengst, Deglincerti et al. 2009), and synapse maturation (Miniaci, Kim et al. 2008). Also after maturation neurons use dendritic local translation so that proteins are available at specific sites ready to respond to local inputs with changes in the proteome that regulate synaptic strength (Sutton and Schuman 2006; Sanchez-Carbente Mdel and Desgroseillers 2008; Lebeau, Miller et al. 2011).

In particular, the mRNAs localization at the synapse has been proposed as a important mechanism for synaptic plasticity and memory consolidation (Kiebler and DesGroseillers 2000; Klann and Dever 2004).

Several studies showed the presence of specific mRNAs encoding protein involved in cytosolic or cytoskeletal events in dendritic layers of the hippocampus and at postsynaptic densities of hippocampal neurons. These

proteins include mRNAs encoding microtubule-associated protein 2 (MAP2) (Garner, Tucker et al. 1988), the -subunit of Ca 2+ /calmodulindependent protein kinase II (CaMKII) (Miyashiro, Dichter et al. 1994), brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (Arc) (Lyford, Yamagata et al. 1995), tyrosine-related kinase B (TrkB) receptor, IP3 receptor, the atypical protein kinase M, the NMDA receptor (NMDAR) NR1 subunit and glycine receptor subunit (Blichenberg, Schwanke et al. 1999; Steward and Schuman 2003).

2.9.2 THE "SYNAPTIC TAGGING" HYPOTHESIS

Synaptic plasticity is modulated and maintained in individual dendritic spines over extended period of time. How this is achieved is explained bythe hypothesis of synaptic tagging and capture (Frey and Morris 1997; Redondo, Okuno et al. 2010). In this model, the activated synapse signals to the nucleus to activate transcription of plasticity-related genes (Greer and Greenberg 2008). In order to target specific mRNAs in the dendrites, they must first be sequestered from the translational machinery in the cytoplasm and organized into ribonucleoproteinparticle(RNPs). Later, mRNA translocation in live neurons involve large granules containing mRNAs, RNA-binding proteins, ribosomes, and translational factors (RNA-containing granules) in a rapid, bidirectional, and microtubule dependent manner (Knowles, Sabry et al. 1996; Kiebler and DesGroseillers 2000), (Kiebler and Bassell 2006). Upon a yet to be identified "synaptic tag(s)",those RNPs are internalized into dendritic spines, where local translation within the synapse maintains the initiated plasticity(Martin and Zukin 2006; Sutton and Schuman 2006). This hypothesis is supported by several facts. First, specific mRNAs have been identified in neuronal dendrites (Steward and Schuman 2003). Second, mRNA sequence elements that are responsible for the dendritic localization of specific mRNAs could be identified(Doyle and Kiebler 2011). Third, both polyribosomes and translation factors can be detected in dendritic spines (Ostroff, Fiala et al. 2002; Tang, Reis et al. 2002). Forth, local translation has been shown to be essential for the maintenance of long-lasting

forms of synaptic plasticity, e.g. late phase LTP and mGluR-dependent LTD (Huber, Kayser et al. 2000; Vickers, Dickson et al. 2005).

Several studies on composition of neuronal transport RNPs reported an heterogeneity composition, including microtubule motor proteins that transport RNPs in dendrites,aswellas numerous other proteins, including well known regulators of mRNA transport as purine-rich-element-binding protein α (Pur-α) and Staufen1(Ohashi, Koike et al. 2002) or translation related protein such as eukaryotic translation initiation factor 2 α (eIF2 α) and eukaryotic translation elongation factor 1A (eEF1A) (Klann and Dever 2004). RNPs can also contain mRNA-binding proteins that have been implicated in mRNA stabilization as synaptotagmin-bindingcytoplasmic-RNA-interacting protein (SYNCRIP), or protein involved in translocation and translation as FMRP or protein involved in fragile-X mental retardation as the autosomal homolog 1 (FXR1) and 2 (FXR2) (Bannai, Fukatsu et al. 2004; Bagni and Greenough 2005; Bramham and Wells 2007).

2.9.3. mRNA TRAFFICKING: ROLE OF STAUFEN2

The double-stranded RNA-binding protein family Staufen (Stau) is essential for the localization of mRNAs in different cell types in Drosophila and mammals. It has been implicated both in dendritic RNA transport and cell body translational regulation. In invertebrates, Stau is required for the proper localization of bicoid and oskar mRNA to either the anterior or the posterior pole of the Drosophila oocyte and in the asymmetric localization of mRNAs, such as prospero in Drosophila neuroblasts (St Johnston 1995; Campos-Ortega 1997; Li, Yang et al. 1997; Broadus, Fuerstenberg et al. 1998; Kiebler, Hemraj et al. 1999). It has been show that Stau is also involved in the translation of oskar message at the posterior pole of the Drosophila oocyte (Breitwieser, Markussen et al. 1996). Two Staufen isoforms (Stau1 and Stau2) are present in the n Xenopus oocytes and move to the vegetal cytoplasm(Allison, Czaplinski et al. 2004; Goetze, Tuebing et al. 2006).

So farin mammals two homologus of Staufen proteins, that are encoded by distinct genes, Staufen1 (Stau1) and Staufen2 (Stau2) have been identified

(DesGroseillers and Lemieux 1996). At least two (Stau163 and Stau155) and four (Stau262, Stau259, Stau256and Stau252) splice isoforms exist for Stau1 and Stau2, respectively (Monshausen, Gehring et al. 2004). All the isoform of Stau1 and Stau2 conserved dsRNA-binding domains. To note that that Stau1 is ubiquitously expressed, while Stau2 is mainly expressed in the brain (Duchaine, Hemraj et al. 2002).

Stau1 and 2 proteins play a role in the transport of RNAs along microtubule to dendrites of polarized neurons (Kiebler, Hemraj et al. 1999).In neurons, Stau1 and Stau2 are located in the somatodendritic compartment and associate with RNA granules (Kiebler et al., 1999; (Tang, Meulemans et al. 2001). However, it seems that they do not colocalize, suggesting that they are located in distinct RNA granules and involved in different RNA regulation (Duchaine et al., 2002). Although Stau2is predominantly localized in the cytoplasm, it was recently shown that is able to shuttle between the nucleus and the cytoplasm(Macchi, Brownawell et al. 2004).The export of Stau2 depends on exportin-5, which is known to be responsible also for the export of tRNAs, microRNAs and maybe for dsRNA-binding proteins (Yi, Qin et al. 2003). Interestingly, only the largest isoform of Stau2 (Stau2 62, which contains five dsRNA-binding domain like in Drosophila), but not the other two differentially spliced isoforms (Stau2 59 and Stau2 52), changes its intracellular localization upon down-regulation of exp-5 (Bohnsack, Czaplinski et al. 2004). However also Stau2 59 isoform is able to leave the nuclear compartment, but in a CRM1-dependent manner (Lund, Guttinger et al. 2004).

Stau2 mediate the coordinate post-transcriptional expression of bound mRNAs depending on cell signaling.Stau2 is associated with hnRNP H1, or with protein and mRNA chaperone also involved in nuclear import/export (as hsc70). Moreover it is also associated with proteins that regulate translation initiation as PABPC1 and YB1 suggesting that Stau2- containing mRNPs may be involved in mRNP formation in the nucleus and/or in post-transcriptionally (Maher-Laporte, Berthiaume et al. 2010). Other mRNAs identified in Stau2-containing mRNPs in brains play a role in protein modifications such as phosphorylation, dephosphorylation and ubiquitination. Inhibition of Stau2 protein production in cultured hippocampal neurons, led to a reduction in the number of dendritic

spines and an increase of filopodia-like structures (Goetze, Tuebing et al. 2006). Other major Stau2-associative proteins include intracellular transport, translation, RNA metabolism, neurogenesis and synaptic functions. All these evidences suggests that Stau2 mRNPs may carry mRNAs whose translation generates proteins involved in the regulation of their own transport, translation and degradation allowing an auto-stimulatory process after an initial signalling event.

However a fundamental question is how mRNA translation is repressed during transport and reactivated in response to cell needs. It is believed that mRNA transport particles are translationally repressed at the level of initiation whereas ribosome-associated granules are kept silent during elongation. Proteomic results on Stau2-containing RNPs identified YB1 and PABPC1, two proteins that modulate translation and that may play a role translational regulation (Maher-Laporte, Berthiaume et al. 2010).

An important role for Stau2 was shown when down-regulation of Stau2 resulted in a reorganization of the actin cytoskeleton in dendrites and also affected the levels of β-actin mRNA in both the cell body and the dendrites (Goetze, Tuebing et al. 2006). Stau2 may bind to β-actin RNA influencing its stability and/or its dendritic transport. It has been proposed that the down-regulation of Stau2 may cause a reorganization of the dendritic actin cytoskeleton by affecting the stability of Stau2-interacting transcripts or by controlling the translation of transcripts, which code for key players in the observed actin dynamics. Another theory about Stau2 role in cognition regards impairment in chemically induced mGluR-dependent LTD after Stau2 knockdown in organotypic, hippocampal slice culture (Lebeau, Miller et al. 2011). While Stau1 specifically down regulates only Late-LTP, Stau2 knockdown alters only mGluR-LTD, demonstrating distinct roles for these two proteins in distinct forms of plasticity.

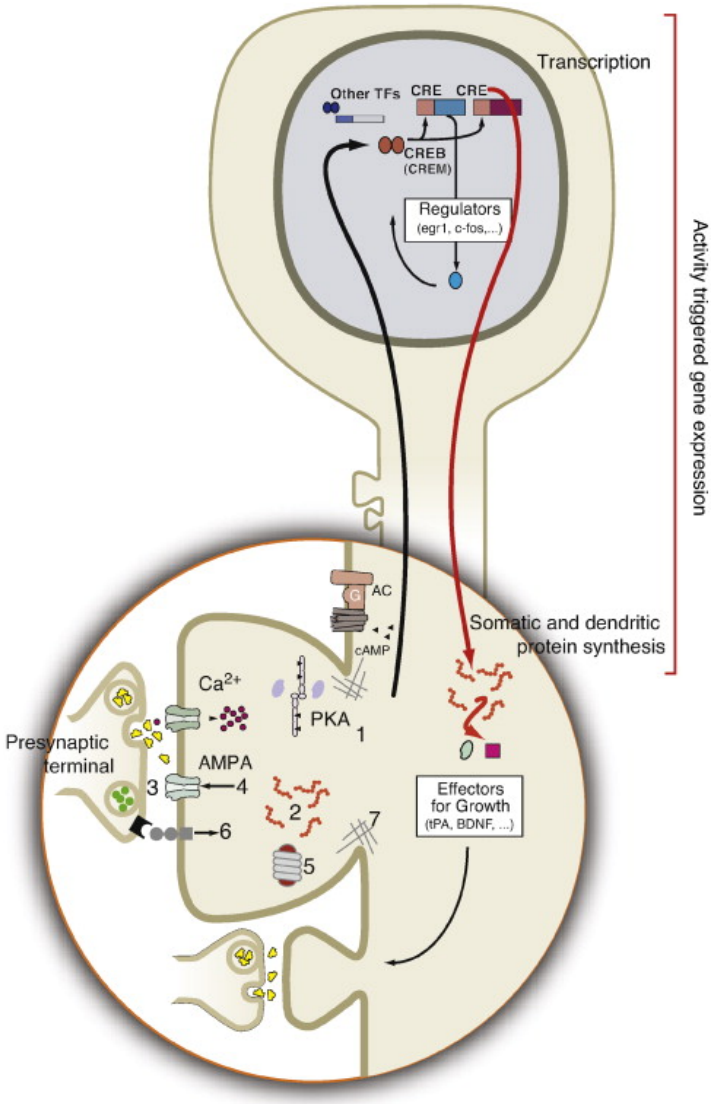


Figure 2. Synaptic tagging: molecular mechanisms for synaptic tagging in Aplysia sensory neurons or rodent hyppocampal neurons: (1) activation of protein kinases, (2) activation of local protein synthesis, (3) release of neurotrophic factors, (4) changes in ion channels, (5) local degradation of protein, (6) internalization of adhesion molecules, and (7) structural changes. Synaptic capture might result from the interaction of these mechanisms with the burst of gene expression achieved through the activation of transcription and/or translation (figure and description from Barco A., et al. 2008)

AIM OF THE STUDY

Gene targeting technologies are powerful tools for the study of gene functions in vivo. Moreover, animal models with cognitive deficits or animal models, that resemble particular diseases, are indispensabletools during the drug discovery processes and optimization.

Our laboratory generated novel transgenic rat modelsbased onthe RNA interference technology and used it to study the role of two particular proteins on synaptic plasticity and cognition.

The first transgenic rat model is a constitutive knockdown ofNogo-A protein within the entire animal. A large amount of studies have already characterized the role of this protein on axonal and spinal cord regeneration but poor information are available concerning the role of Nogo-A protein in cognitive disease such as schizophrenia. Webehaviorally analyzed the effect of Nogo-A protein depletion within the frame of this disease, focusing on the three major classes of symptoms characteristic for the schizophrenia: positive, cognitive and negative symptoms.

The second transgenic rat modelis the first inducible tissue-specific gene inactivation rats described so far.Stau2 protein is knocked down within excitatory neurons of the forebrain, thanks to the use of an artificial miRNA targeting the respective protein. Stau2 function has been very well characterized with in-vitro studies and in non-mammalian models, but few in known about its role in cognition in mammalian models. After validationof the gene inactivation technology,wecharacterized the phenotype of this rat model to understandthe role of Staufen2 in learning and memory processes.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

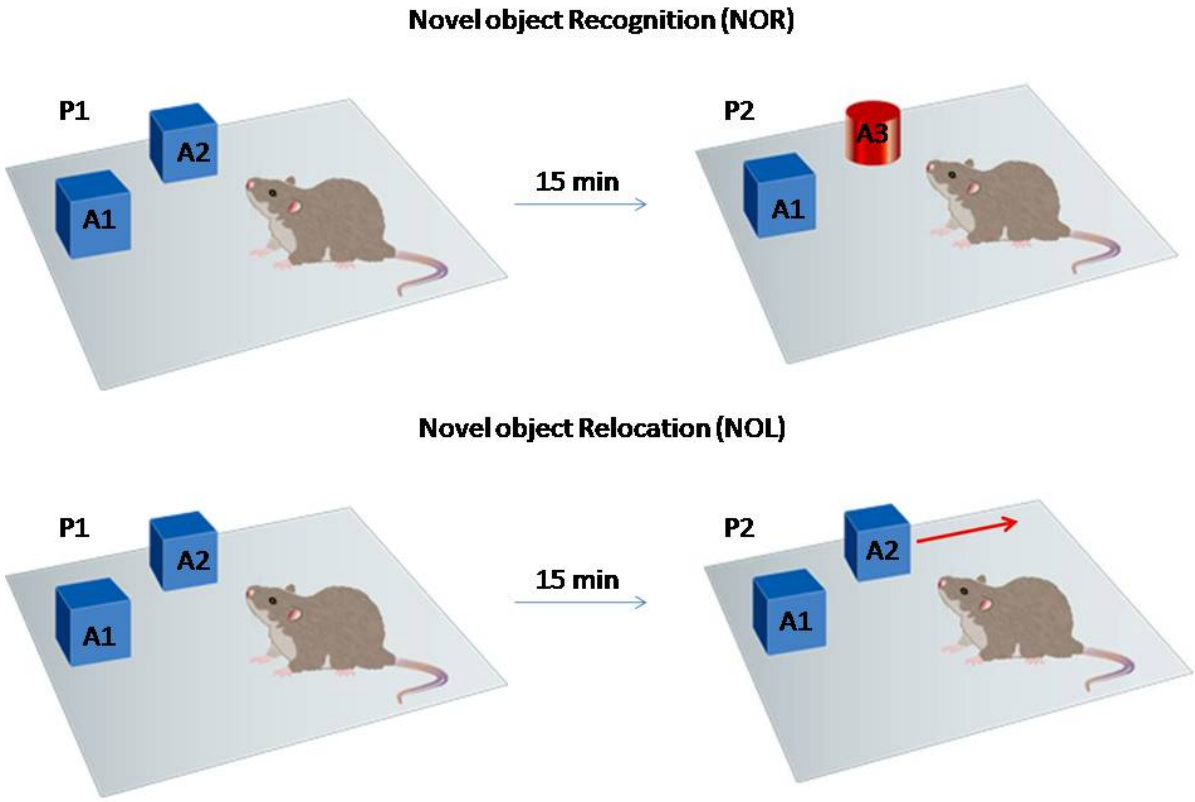
For the behavioral assessment of Nogo-A Knockdown rats, six months old male (L2,n=10) or WT littermate (n=10) rats were used. Effect of tamoxifen on rat behaviour was investigated with 8 male vehicle injected rats and 8 male tamoxifen injected rats. The role of Staufen 2 conditional knockdown was studied using male Stau2-/- rats and with a single transgenic controlrat line: CaMK-CreERT2. All animals were hosted and provided by the Animal House of the Central Institute of Mental Health (Mannheim, Germany). All Animals were kept in collective cages (3-5 animals/cage) on a 12-h light/dark cycle with constant temperature (21 ± 1 ºC) and humidity (50 ± 7%) and were allowed ad libitum access to commercial rat chow and water. The experimental protocols used were in line with national and international ethical guidelines and performed in compliance with the European Union Council (2003/65/EU), the German Animal Welfare Act. All experiments were approved by the Animal Welfare Comission of the Regierungspräsidium Karlsruhe, Germany.

3.2 OPEN FIELD

Basal locomotor activity was assessed in an open field (four equal areas, 51 x 51 x 50 cm). Distance travelled [cm] in the open field apparatus was digitally recorded for 30 minutes at a light intensity of 50 lx. The test was started by placing the rats in the center of the box and locomotor activity was analysed by the observation program Viewer^2 (Biobserve).

3.3 NOVEL OBJECT RECOGNITION: MEMORY WITHOUT STRESS

Object recognition testing was performed in an open arena (four equal areas, 51 x 51 x 50 cm) at a light intensity of 50 lx. The rats were habituated to the open field for 20 min, 1 day before testing. All stimulus objects used in the test were made out of ceramic or glass and were applied in duplicates. The test consisted of an initial three minutes sample phase (P1) and a three minutes discrimination phase (P2),the two phases separated by an inter-trial interval of 15 min. During P1, the rats were placed in the center of the open field and exposed to two objects (A1 and A2). After cessation of P1 the rats were returned to the homecage and the objects were removed. The rat was placed back in the open field after 15 min for object discrimination in P2 and was now exposed to the familiar object (A1, an identical copy of the object presented in P1) and a novel test object A3 (B). For the novel object relocation test, the animal was exposed to A1 and A2 objects. Fifteen min after, the discrimination phase (P2) was performed: the A1 object was place in the same position respect to P1 phase and the A2 object was instead placed in a different position respect to P1 phase. Both object were newly used and equal to the object used in P1, this to avid smell bias. Exploration of the objects (sniffing, licking) was recorded during P1 and P2. Sitting beside or standing on top of the objects was not scored as object investigation. Cleaning was done with 70 % alcohol followed by a drying phase before and during testing. Animals were videotaped during P1 and P2, and an observer blind to the genotype analyzed videos. For the calculation of percentage object discrimination, the exploration time of the novel object was expressed as percentage of the total exploration time of both objects during P2 [100/(A1 + A2(or A3)) * A2 (orA3)] (Figure adapted from Buckley F. et al., 2007).



3.4 PREPULSE INHIBITION (PPI) OF THE ACOUSTIC STARTLE RESPONSE (ASR)

Prepulse inhibition (PPI) was measured using the Startle Response System (SR-LAB™ Startle Response System, San Diego Instruments). As a startle stimulus a white noise pulse (intensity of 110, duration of 40 ms dB) was used. Four different white noise intensities (66, 70, 74 and 78 dB SPL) each of 20 ms, were used as prepulses. An acclimatization time of 5 min to the background noise (60 dB) was followed by the presentation of five initial startle stimuli. After this habituation program the test program was started with six different trial types presented in a pseudorandom order: (1) pulse alone, (2) no stimulus, (3)-(6) pulse with preceding prepulse (prepulse 66, 70, 74 or 78 dB SPL 100 ms before pulse). A total of 10 presentations of each trial type was given with an inter-trial interval randomized between 10 and 20 s. PPI was calculated as the percent decrease of the acoustic startle response (ASR) magnitude in trials when the

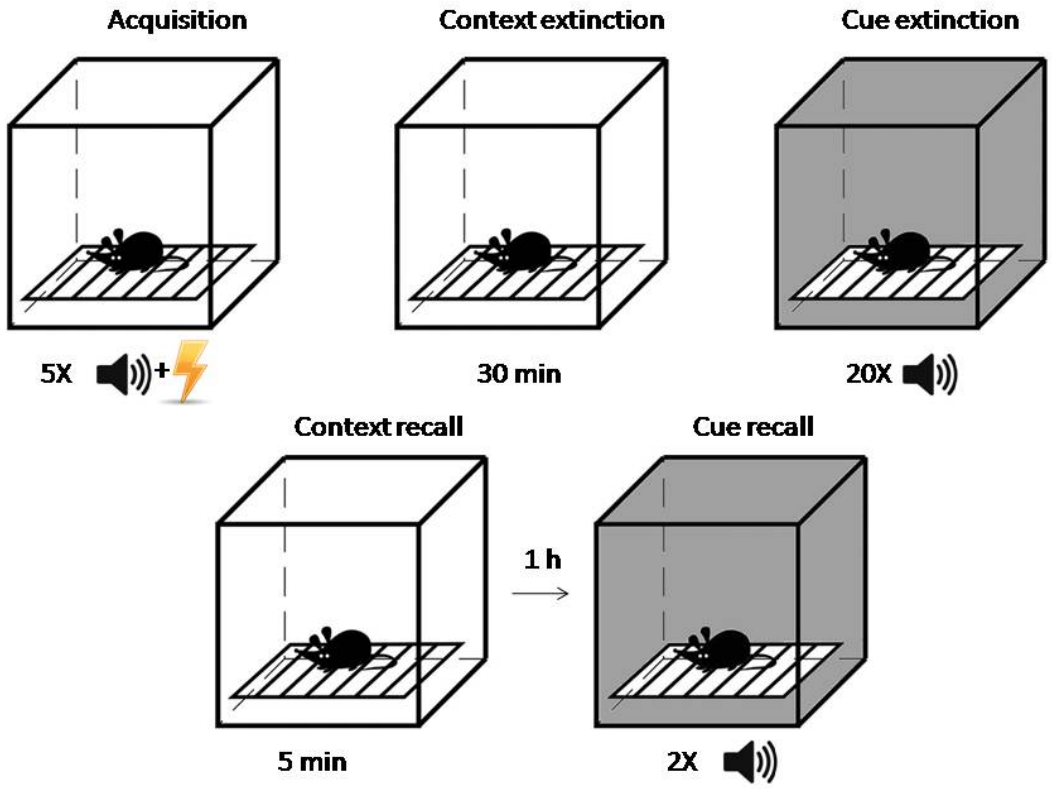
startle stimulus was preceded by a prepulse [100 x (mean ASR amplitude on pulse alone trials – mean ASR amplitude on prepulse-pulse trials)/mean ASR amplitude on pulse alone trials].

3.5 FEAR CONDITIONING

Auditory fear conditioning and extinction were performed in an operant chamber (Coulbourn Instruments, Allentown, PA) located in sound-attenuating cubicles (Med Associates, Burlington, VT) throughout all phases of the experiment. The floor of the chambers consisted of stainless steel bars that delivered a scrambled electric footshock. Between experiments, shock grids and floor trays were cleaned with soap and water, and chamber walls were cleaned with wet paper towels. The Footshock was delivered from a precision animal shocker (H13-15, Coulbourn Instruments) and the sound stimulus was generated with a conventional sound card, amplified with a HiFi amplifier (PR530A, Pyramid) and delivered via speakers in the chamber walls. Different chambers were used (contexts A and B). Context A measured 17cm ×18 cm×32cm in size (H10-11M-TC, Coulbourn Instruments) with two transparent walls and stainless steel grid floors. For the Context B, the same box was modified with the insertion of a plastic dark panel to cover one of the transparent wall and the metal grid. On the other transparent wall some stripes of different shapes were fixed. After each use context B was cleaned with alcohol. All the apparatus was controlled by a personal computer equipped with FreezeFrame software (Actimetrics Software) and an IMAQ-A6822 interface card (National Instruments). The movements of the tested animal were recorded with a digital video camera mounted at the ceiling of the cubicle and analyzed for the percentage of freezing using FreezeView software (Actimetrics Software). In all experiments, the virtual threshold for freezing was set to value of 3. For Staufen study, on Day 1, rats received five conditioning (30 s, 4 kHz, 77 dB; 3 min average intertrial interval (ITI)) that co-terminated with foot shocks (1 s, 0.5 mA). On Day 2, rats were returned to the chambers A for context extinction training, which consisted of 30min in absence of foot-shock. On Day 3, rats were returned to the chamber B for cue extinction and presented with 20 tones in absence of

footshock. On day 4 rats were tested for context recall in the box A for 5min without any tone and after 1h in the context B for the cue recall presenting 30s tone twice. For the tamoxifen study, rats were tested only with training phase, a context and cue recall.

Scheme of fear conditioning test



3.6 Morris Water maze

Animals were trained in a black circular water maze (1.5 m diameter, 50 cm in height; 25 °C water temperature). The apparatus was located in a room containing extra maze visual cues consisting of various geometric shapes, placed on the wall surrounding the maze. During training, an invisible escape platform

(14x11cm) was located in the water in a particular quadrant, 1 cm below the water level. Swim behaviors were recorded using the tracking software NoldusEthovision® v.3. On Staufen study, the water maze behavioral procedures consisted of four-days training. Each training session consisted of 6 swim trials, during which rats were placed in a starting point (changed randomly during each trials), and were allowed to swim to the escape platform, which was consistently located in the same position. Rats remained on it for 10 s before being returned to their cages and being replaced in the maze after an inter-trial interval of 30 s. Rats that failed to find the escape platform within 60 s were manually guided to it. On the day 4, a learning trial was performed. Rats were placed into the maze but the escape platform was removed from the water maze. Behavioral parameter as distanced moved and distance to zone were measured for a total of 2 minutes.

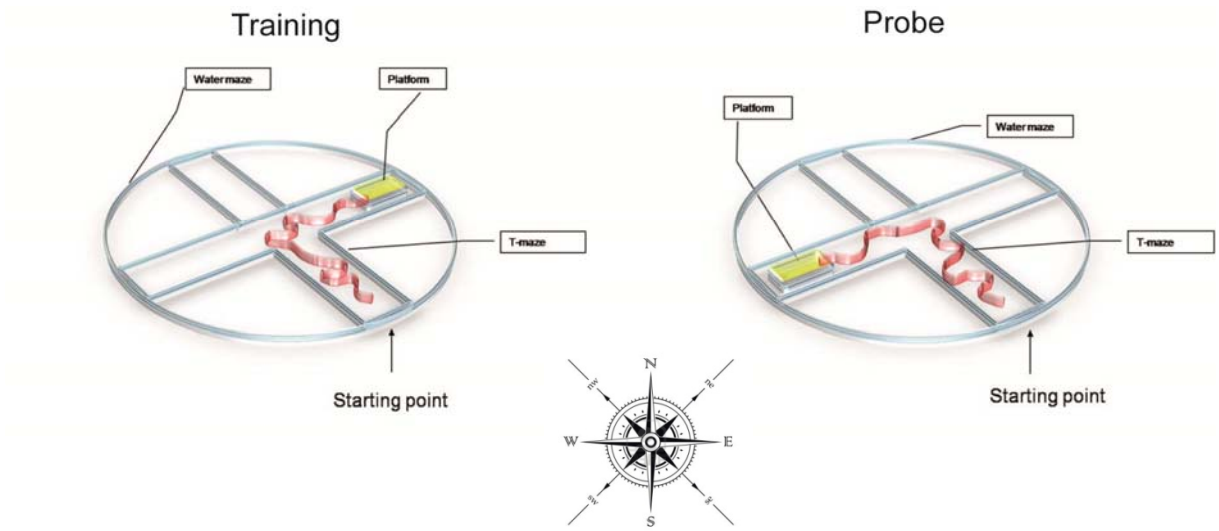
For the tamoxifen study the protocol consisted of 3 days of training including 3 trials per day and a probe trial on day 4.

3.7 WATER T-MAZE TEST

For reversal learning capability of Nogo-A knockdown rats, animals were trained in a black circular water maze (1.5 m diameter, 50 cm in height; 25 °C water temperature) into which a plastic plus-maze (arms wall 38 cm height, 15 cm arm-width, and 51 cm arm-length) was inserted. The plus-maze was elevated 5 cm from the bottom of the water maze. Final water level in the tank was 35 cm. The maze was surrounded by visual cues consisting of various geometric shapes. None of these cues were placed in proximity to the ends of the plus-maze arms to avoid spatial bias. During training, an invisible escape platform (14x11cm) was located at the end of one arm of the maze (e.g. East), 1 cm below water level. The arm opposite to the start arm was blocked by a guillotine door, resulting in a “T” configuration of the maze. Swimming behaviour was recorded using the tracking software Ethovision v.3 (Noldus). Rats were trained in the plus-maze for a total of four days. Each training session consisted of five swim trials, during which rats were placed in the start arm of the maze (South), and were allowed to swim

to the escape platform, which was consistently located in one arm of the maze (East). Rats remained on it for 10 s before being returned to their cages and being replaced in the maze after an inter-trial interval of 30 s. Rats that failed to find the escape platform within 60 s were manually guided to it. On the fifth day, a reversal learning trial was performed. Rats were placed into the same start arm (South), but the location of the escape platform was switched to the West arm. Each other arms-entering during test trial were scored as an error.

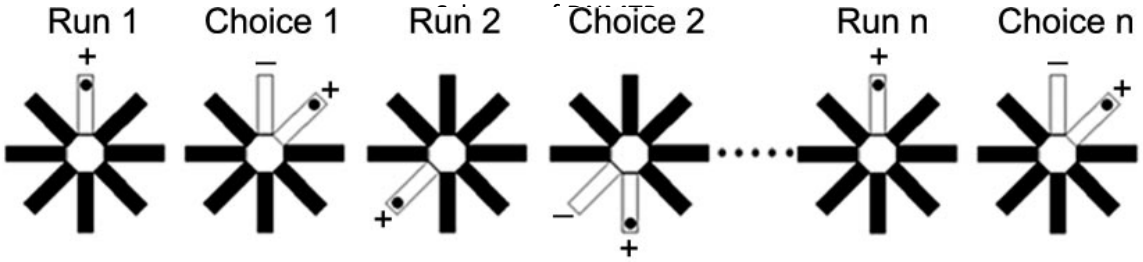
Scheme of reversal water maze



3.8 DELAYED NON-MATCHING TO PLACE TASK ON AN 8-ARM RADIAL MAZE (DNMTP)

The apparatus used for behavioural testing was an elevated eight-arm radial maze (arms wall 38cm height, 15cm arm-width, and 51cm arm-length) made of grey Plexiglas and located in a testing room enriched with distal partial cues. Guillotines doors were located at the entrances of each arm and they were controlled manually using a wire system that did not allowed the operator to interfere with the maze area remaining in a hidden position. Rats were put on a food restriction diet so that their body weights were reduced to and maintained

at 85%of the ad libitum weights. Before behavioral testing, they were habituated to the apparatus over 2 days by allowing them the free exploration until they collected condensed milk (MilchMädchen, Nestlè®)in food wells of each arm. The working Memory Tasks consisted on a first part (Run) during which rats were forced to visit one arm and a second one (choice)in which they visited an adjacent arm, randomly chosen between the Left and Right side. The arm presentation was delayed of 1minin which the rats were held on the central platform of the maze. To avoid a door strategy choice, a black cylinder was lowered covering the view on the arms. Each daily sessions were composed by a total of 4 trials. Once the rats maintained a criterion of around 85% of correct choice over 6 consecutive days, the delay was increased to 5 min for 3 days of test and to 10 min for further 3 days of cognition task.



3.9 ELEVATED PLUS MAZE

To measure anxiety-like responses, the elevated plus-maze test was used. The apparatus consisted of two dark gray PVC with two open arms (50 cm × 12 cm) and two closed arms(with 50 cm high walls), which were arranged so that the similar arms were opposite to each other. The open arms were also equipped with 0.5 × 0.5 cm edges to ensure that no animals would fall off the maze. The maze, elevated 50 cm above the floor, was cleaned with 30% ethanol solution and dried afterward. It was placed in a room under 40lux light conditions. The 5-min test procedure began when the animal was placed in the centre of the maze, facing a closed arm. The trials were video recorded and computer analyzed with the ethological software viewer2 (Biobserve GmbH, Bonn, Germany). The percent of time spent in open arms and the percent of open arm entries were

used as measures of anxiety-like behaviour, while the number of entries into the closed arms was used as an indicator of general motor activity (Cruz et al. 1994).

3.10 IMMUNOHISTOCHEMISTRY

After behavioural studies, rats were perfused with 4% PFA and brains were dissected. Dissected brains from perfused animals were further postfixed with 4% paraformaldehyde/PBS at 4°C for 24 to 48 hrs and brain sections (50 µm) yielded by using a vibratome (Leica, Nussloch, Germany). Double immonofluorescence staining was performed in order to characterize the cellular expression pattern of Stau2 and to provide evidence for an efficient knockdown of Stau2 in eGFP positive neurons. The following primary antibodies were used: rabbit polyclonal anti-Stau2 (kindly provided by Michael Kiebler, 1:750), chicken polyclonal anti-eGFP (Millipore, Germany, 1:1000), mouse monoclonal anti-CaMKIIa (Acris, Germany, 1:500), mouse monoclonal anti-NeuN (Millipore, Germany, 1:4000), mouse monoclonal anti-GAD67 (Millipore, Germany, 1:1000). Secondary antibodies used were AF488 donkey anti-mouse IgG (Invitrogen, Germany, 1:200), AF488 donkey anti-chicken IgG (Invitrogen, Germany, 1:200), Cy3 donkey anti-mouse IgG (Jackson Immuno, Germany, 1:200), AF555 donkey anti-rabbit IgG (Invitrogen, Germany, 1:1000) and Cy2 donkey anti-rabbit IgG (Jackson Immuno, Germany, 1:200). Stained sections were examined using a Leica SP5 confocal laser-scanning microscope.

3.11 DAB STAINING

DAB staining was used to characterize Staufen2 expression in wildtype rats and eGFP expression in tamoxifen-treated and non-treated Stau2 -/- rats (slightly modified from(Spergel, Kruth et al. 1999). For DAB staining, brain tissue sections were permeabilized in 1% H2O2 in phosphate-buffered saline (PBS) (150 mMNaCl,10mM Na-phosphate, pH 7.4) for 10 min at room temperature and than three times washed with PBS for 10 min each. Sections were incubated in 2% goat serum in 1% BSA/0.3% Triton X-100/PBS for 1 h at room Temperature to block unspecific binding sites. The primary antibody anti-GFP (Millipore,

Germany, 1:1000) in blocking solution and slices were incubated at 48°C overnight. Slices were twice washes in 0.3% BSA/0.1% Triton X-100/PBS, and incubated for 1 h at room temperature with the biotinylated secondary antibody in 0.3%BSA/0.1% Triton X-100/PBS. Slices were than washed twice in the same buffer without antibody. Sections were then incubated in DAB solution in 20 mMTris–HCl with 0.012% H2O2 until staining raised. The reaction was stopped by washingwith PBS. Stained slices were mounted on glass slides and cover slipped with Eukitt (Sigma Aldrich, Taufkirchen, Germany). Slices were imaged with a stereoscope.

3.11 Quantification of mRNA concentrations by real time RT-PCR

Tissue from hippocampal subregions was yielded from 200 µm thick frozen brain slices by manual microdissection using self-made punching needles. Total RNA was subsequently isolated using TRIzol Reagent (Invitrogen, Germany) according to the manufacturer's recommendations. Next, 1 µg of total RNA was used for reverse transcription with SuperScript III and oligo(dT)20 primer (Invitrogen, Germany) according to manufacturer's protocol. Resulting undiluted cDNA solutions were subjected to real time PCR analysis as triplicates.

Real-time PCR reactions were run on an Applied Biosystems 7900 HT fast real-time PCR system. For the quantification of Stau1, Stau2 and the housekeeping control gene CycA, real time PCR was performed in a total volume of 20 µl using the Taqman Universal PCR master mix (Applied Biosystems, Germany) according to the manufacturer's protocol with the following primers and probes: Stau1: forward primer 5'-ttccagagcccagggatt-3'; reverse primer 5'-gagagatacacactcgttcttgttg-3', probe #60 from universal probe library (Roche Applied Science, Germany); Stau2: forward primer 5'-aggatcagctcgacaagacc-3'; reverse primer 5'-ggaaatccaggctttggac-3', probe #58 from universal probe library (Roche Applied Science, Germany); CycA: forward primer 5'-CTTCCCAAAGACCACATGCT-3'; reverse primer 5'-TGCTGGACCAAACACAAATG-3', probe #42 from universal probe library (Roche Applied Science, Germany). For the quantification of eGFP and the housekeeping control gene PPIA, real time PCR was performed in a total volume of 20 µl using the Taqman Power SYBR-

Green PCR master mix (Applied Biosystems, Germany) according to the manufacturer's protocol with the following primers: eGFP: forward primer 5'-ACCCAGTCCGCCCTGAGCAA-3', reverse primer 5'-GCGGCGGTCACGAACTCCAG-3'; PPIA: forward primer 5'-GTCAACCCCACCGTGTTCTT-3'; reverse primer 5'-CTGCTGTCTTTGGAACTTTG-3'. Final concentrations of primer were 300 nM.

3.12 QUANTIFICATION OF MATURE MIRNA

Detection of processed miRNA was performed using a Custom Taqman Small RNA Assay (AssayID: CS70K4Q, Applied Biosystems) according to the manufacturer's protocol. Briefly, 10 ng of total RNA was dissolved in 5 µl RNAse free water and then mixed with 7 µl of recommended RT-master mix (containing Superscript III reverse transcriptase) and 3µl of the 5 x RT primer provided with the Taqman Small RNA Assay. RT-reaction was run in a thermocycler, programmed for 30 min at 16 °C, 30 min at 42 °C and 5 min at 80 °C. Real time PCR reactions were run on an Applied Biosystems 7900 HT fast real-time PCR system in accordance with the recommended protocol. Each PCR-reaction was performed in triplicates and consisted of 1 µl undiluted RT reaction, 1 µl Custom Taqman Small RNA Assay and 10 µl Taqman Universal PCR Master Mix (Applied Biosystems) in a total volume of 20 µl.

4. RESULTS

4.1 CONSTITUTIVE NOGO-A KNOCKDOWN IN RAT BRAIN

4.1.1 MOLECULAR DATA

To investigate the role of Nogo-A in the central nervous system and more precisely during learning and memory, our lab created a Nogo-A (Rtn4) knockdown. The transgenic rat was generated by using microRNAs (miRNA) against Nogo-A, expressed by Pol II promoters. This model consists of a Nogo-A-targeting miRNA in an intronic sequence preceding the open reading frame of the reporter gene EGFPin the vector pCAG-Intron-EGFP. This construct enables the labelling of miRNA-expressing cells and a quantitative measure of the amount of miRNA produce since the miRNA is spliced from the EGFP mRNA (Tews, Schonig et al. 2013).

Quantitative PCR revealed significant reduction of Nogo-A mRNA in several CNS regions in knock-downrats to about 50% of WT levels. Western blots revealed a reduction of Nogo-A expression in the cortex to about 30% of WT mean, and in hippocampal regions the expression was reduced to 30% (Tews, Schonig et al. 2013). Importantly, Nogo-B expression remained unchanged. Maximum synaptic strength (LTP saturation) was significantly increased in Nogo-A knockdown rats compared to WT rat suggesting that Nogo-A is a repressor of synaptic plasticity also in the motor cortex (Tews, Schonig et al. 2013).

4.1.2 BEHAVIORAL CHARACTERIZATION OF NOGO-A KNOCK DOWN RATS

Those transgenic rats (n=10) were analysed to investigate the consequences of reduced Nogo-A expression on behavior compared to WT animals (n=10). We focused on the analysis of distinct neuropsychiatric intermediate phenotypes, some of which have been associated with Nogo-A function in a KO mouse model (Willi, Weinmann et al. 2010).

Basal locomotor activity was assessed in an open field(Figure3A). No genotype differences were observed between the groups for the distance travelled during the 30 min test session(Student's t-test; $p > 0.05$). The effect of Nogo-a knockdown in anxiety related behavior was tested with an elevated plus maze (Figure 3B). Analysis of the time spent in closed arms was equal between WT and knockdown rats. Also the time spent in the closed and in the central part of the maze did not show any difference, from which one can deduce an absence of anxiety related phenotype derived from genetic manipulation of Nogo-A.

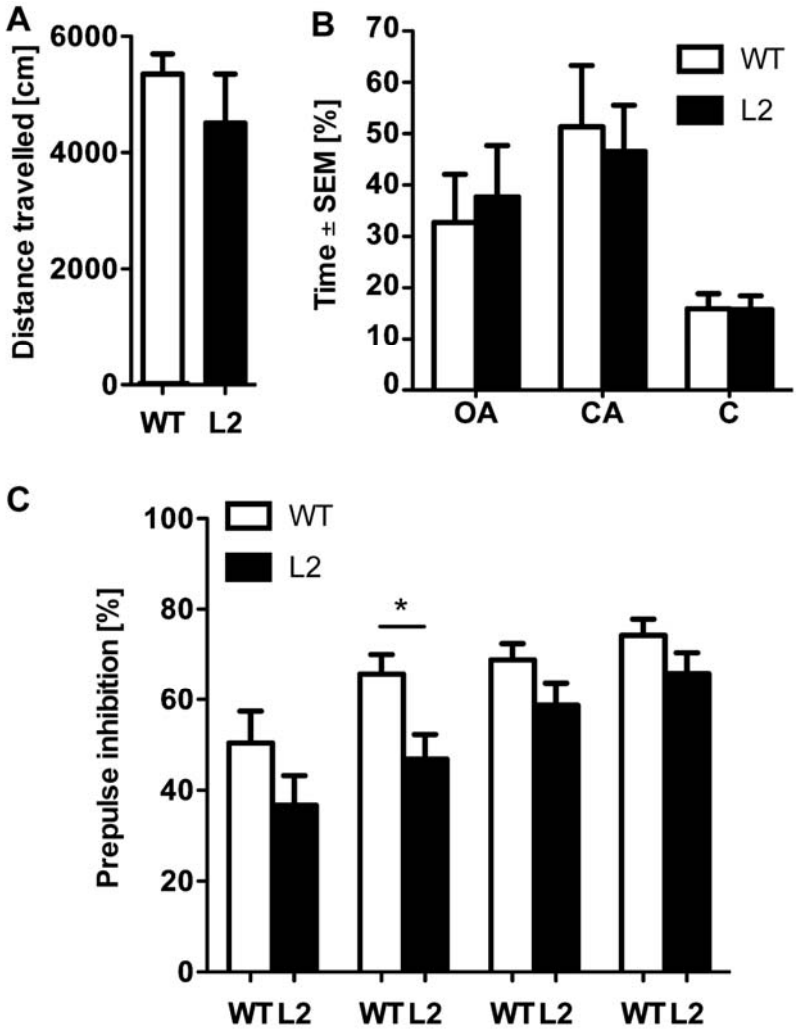


Figure 3: ***Behavioral characterizationby basal locomotor activity and anxiety.*** *(A) Locomotor activity in the open field arena showed no difference on distance travelled between wild-type (WT) and Nogo-A knockdown rats (L2). (B) On elevate plus maze transgenic rats show an anxiety tract similar to WT animals. The time spent in the closed arms (CO) open arms (OA) and in the centre (C) are comparable between the two groups of animals (C) Prepulse inhibition (PPI) revealed a significant reduction in knock-down rats compared to WT rats ($F_{Genotype}$ (1,54)=4.97; Two way ANOVA p=0.039). However, Bonferroni post hoc testing revealed that startle amplitudes of L2 rats were significantly lower than those of WT rats only at a prepulse intensity of 70 dB (PPI-70) (*: p<0.05; **: p<0.01; § p=0.039).*

However, when animals were tested for their sensorimotoric gating, a marked deficit in prepulse inhibition (PPI) could be observed in knockdown rats compared to WT controls ($F_{Genotype}$ (1,54)=4.97; p=0.039, Figure 3C).

Cognitive functions of the animals were evaluated using the novel object recognition and the object relocation paradigms (Figure 4). In these tasks evaluating short-term memory capacity, the animal had to identify a novel or a relocated object from the familiar objects, memorized before. During object recognition and object location testing, Nogo-A knockdown animals were found to show decreased short-term memory capabilities compared to WT controls. Percentage discrimination between the novel/familiar object (Student's t-test; p= 0,0015) (Figure 4 A) and the object location (Student`s t-test; p = 0.0004) (Figure 4 B) were significantly reduced compared to WT rats. However no significant differences were seen between the genotypes in effective object exploration time during the sample phase P1 of novel object recognition (Exploration values [s]: WT: 13.4 (± 0.65 S.E.M); Nogo-A: 11.3 (± 1.73 S.E.M)) and during novel object relocation test (Exploration values [s]: WT: (14.9 (± 4.7 S.E.M); Nogo-A: 13.2 (± 4.2 S.E.M))indicating the absence of any bias for a special position or object (Figure 4).

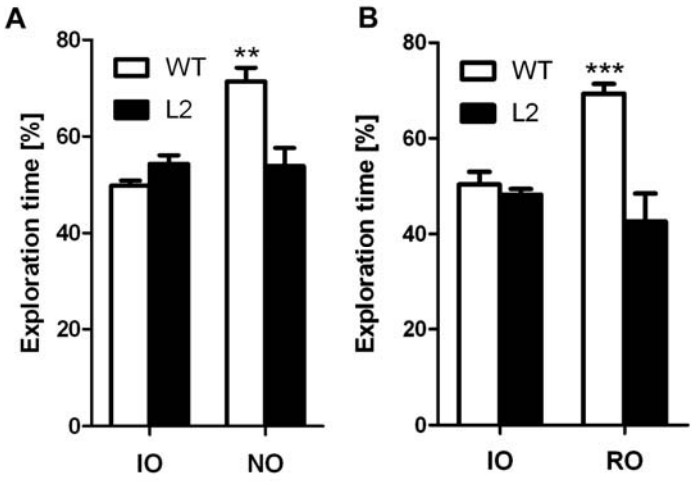


Figure 4: ***Behavioral characterization with novel object recognition and relocation*** *(A) Effects of Nogo-A knockdown on novel object recognition memory showed no significant differences in percentage of time spent in exploration of the identical objects (IO) between Nogo knock-down (L2) and WT rats, during the training phase of the test. In contrast, knock-down rats have a significant impairment in discriminating between the novel and the familiar object during testing (NO)*t-tests.*(B) Novel object relocation tasks showed no significant differences in exploration of the identical objects (IO) between knock-down and WT rats, during the training phase of the test. During the test phase L2 rats had a significant impairment in discriminating between the familiar and the relocated object (RO) t-test: *: p<0.05; **: p<0.01.*

Behavioral differences between knock-down rats and controls were also observed during tests of social interaction (Figure 5). A significant decrease in total social interaction was observed in Nogo-A rats compared to WT (p=0.009, Figure 5 B). These differences originated only from a highly significant decrease in non-anogenital exploration (p=0.0006; non-ag), since no significant differences between the groups were detected for anogenital exploration and approach/following during interaction with the unfamiliar social partner ((p=0.136; Figure 5 A). Furthermore, knock-down rats showed a strong tendency for lower social contact behaviors compared to WT animals (p=0.054; Figure 5 D). Finally, knock-down rats were found to withdraw significantly more often from social contact if initiated by the social partner (social evade, p=0.0037; Figure 5 E).

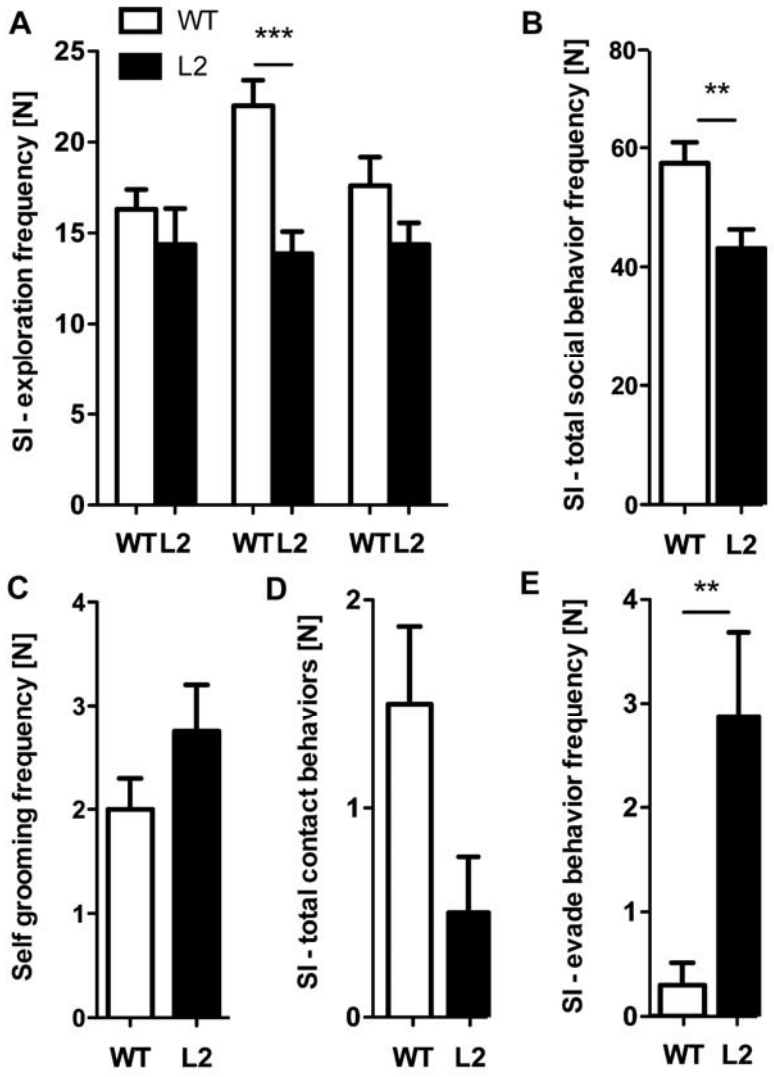


*Figure 5**: Behavioral performance during social interaction with an unknown social partner (social interaction).** (A) Significant differences between WT and knock-down rats (L2) were found for non-anogenital exploration (non-AG, while no differences were observed for anogenital exploration (AG) and following/approach (FA). (B) The total frequency of all these social behaviors resulted higher in WT rats compare to Nogo-A animals. (C) Self grooming activity did not change between the two groups of rats. (D) A strong trend was found for a decrease in number of social contact behavior (grooming/crawling). (E) Nogo-A knock-down rats show significantly more social withdrawal behavior than WT littermates. t-test: *: p<0.05; **: p<0.01.*

No significant differences between the groups were detected for self-grooming behavior (Figure 5 C), which could be considered another source of evidence for non-anxiety phenotype event. This is supported also by the strong tendency to do not create new social contacts (Figure 5 D). This behavior is correlated also to

the social evade frequency, that is significant higher in knock-down rats (Figure 5 E). Behavioral flexibility was assessed in the discrimination reversal learning task using a water T-maze (Figure 6). A learning deficit occurring during the reversal phase of the task without any deficit at the initial acquisition is an indicator of preservative behavior, a factor known to be implicated in schizophrenia and autism (Ridley 1994). Performance during the initial acquisition phase did not differ between both genotypes, indicating that there was no general learning deficit in Nogo-A knock-down animals(Figure 6 A). During the reversal phase, knock-down rats showed similar learning compared to WT rats (correct choice %; F value $_{(1,126)}$ = 0.86; p= 0.365, Figure 6 B). However, during the second trial they showed a much lower accuracy in searching the platform in the correct arm (Bonferroni post-hoc test; $p < 0,05$)(Figure 6 B), meaning that they have more difficulty in adapting to reverse contingency. The same was found for the latency of escape (F value $_{(1,126)}$ = 1.47; p= 0.24) (Figure 6 D) that on the second trial was significantly different (Bonferroni post-hoc test; $p < 0,05$) and on the total number of errors (F value $_{(1,126)}$ = 1.00; p= 0.329) with a significant difference on the second trial (Bonferroni post-hoc test; $p < 0,05$) (Figure 6 E).

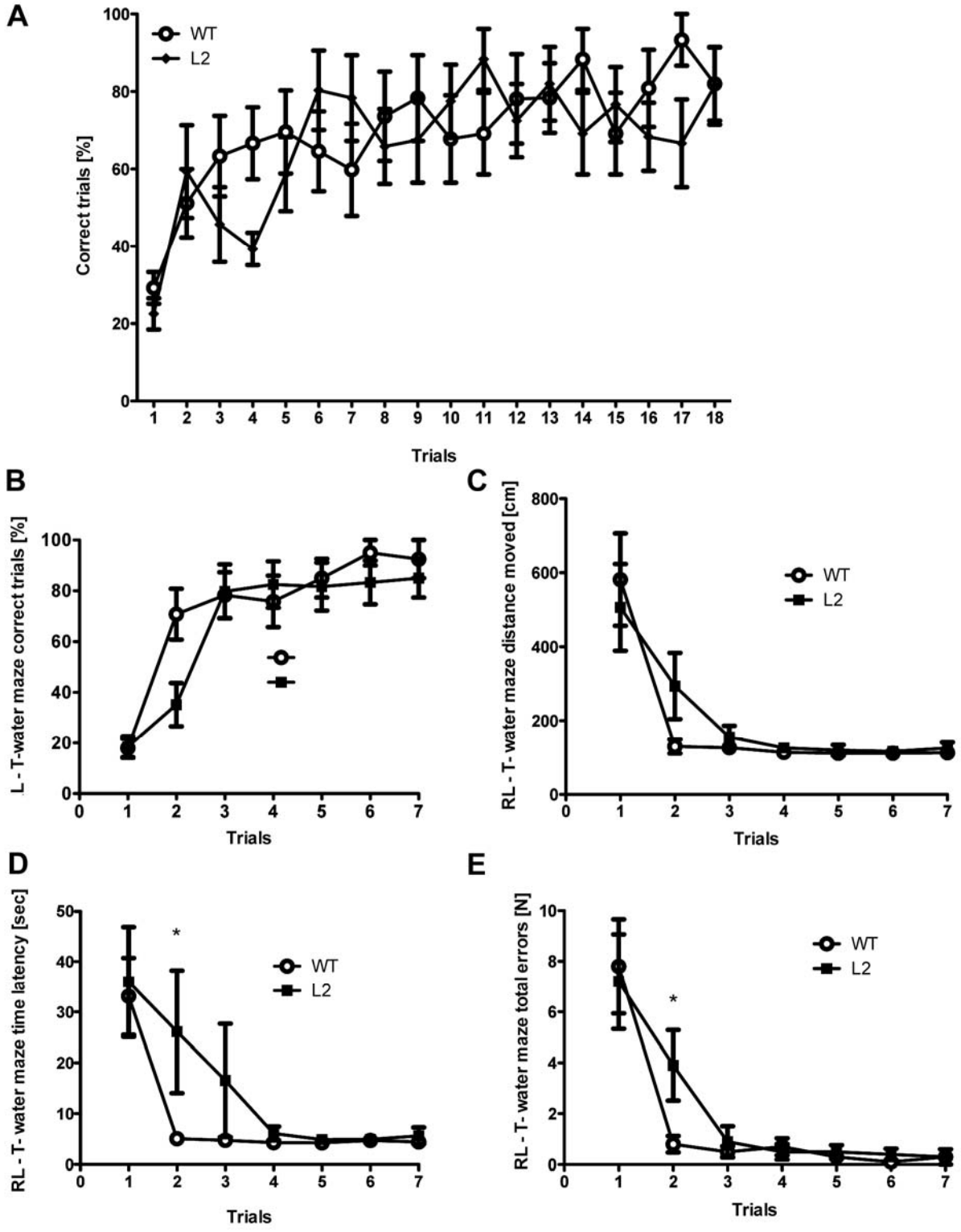


Figure 6: ***Reversal learning in the water T-maze****. (A) Two way ANOVA revealed that there was no significant difference between animals of the two genotypes in the reversal of their escape strategy in the water T-maze, as seen from their percentage of correct trails within the task ($F_{Genotype}$ (1.108)=1.22; p=0.284). In contrast, Bonferroni post hoc testing revealed that in trial two, Nogo-A knock-down (L2) rats have a significant impairment in finding the escape platform. All data are mean*

*values ± SEM. Asterisks represent p-values obtained by comparing L2 and WT rats with either unpaired t-test or Bonferroni post-hoc test following two way ANOVA of repeated measures: *: p<0.05; **: p<0.01; ***: p<0.001*

In conclusion, Nogo-A knockdown rats showed deficit in gating property, associated with difficulty in reversal learning and lower exploration and reduced social contact behavior, correlated to higher withdrawal from social interaction initiated by the social partner. The presence of these symptoms in the rat model is of particular significance since they describe a schizophrenia-like phenotype (Marcotte, Pearson et al. 2001).

4.2 INDUCIBLE STAUFEN 2 KNOCKDOWN IN RAT BRAIN

4.2.1 STAUFEN2 EXPRESSION IN ADULT RAT BRAIN

As previously described by using Northern blot analysis and in situ hybridisation, Staufen2 (Stau2) is expressed throughout the entire brain (Monshausen, Putz et al. 2001).We further characterized Stau2 expression within the adult rat brain by using a rat polyclonal Stau2 antibody for immunohistochemistry on respective brain sections(Figure.7). Particularly high expression of Stau2 was observed in the bed nucleus of striaterminalis, the hippocampus, the central nucleus of the amygdale and the hypothalamus (Figure 7 A). Within the hippocampus, Stau2 protein is present in the pyramidal cell layers of all CA subregions, the granule cell layer (GCL), the hilus (Hi) and the subgranular zone of the dentate gyrus (DG) as well as in individual cells located in the stratum oriens and stratum radiatum (Figure 7 B).

Quantitative RT-PCR analyses of Stau2 expression performed with tissue samples from respective hippocampal subregions, demonstrate that Stau2 mRNA concentration is similar in CA1 and CA3 and slightly elevated in the DG, an information that can also be deduced from immunohistochemical data (Figure 7 C).

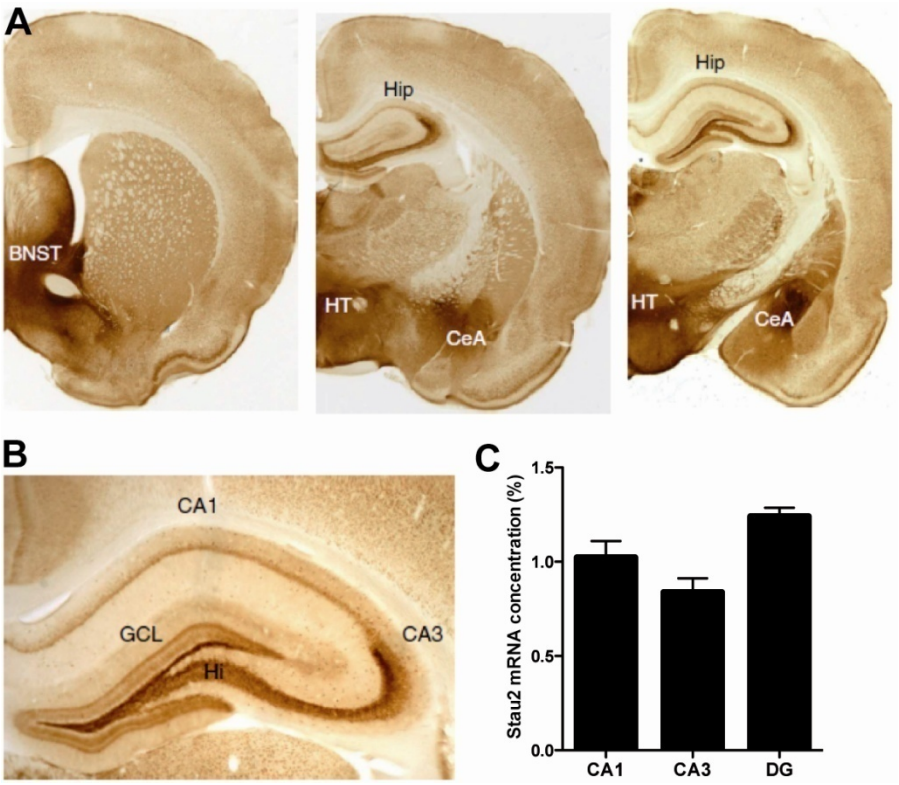


Figure 7: ***Staufen2 expression in the adult rat brain***. *(A) Immunohistochemical staining of coronal sections of WT Sprague Dawley rats using a Staufen2 (Stau2) antibody. Most intense Stau2 signals can be detected in the bed nucleus of thestriaterminalis (BNST), the hippocampus (Hip), the central nucleus of the amygdala (CeA) and the hypothalamus (HT).(B) Higher magnification of the hippocampus demonstrates substantial Stau2 signals in CA1, CA3, the dentate gyrus-hilar region (Hi) and the dentate gyrus granule cell layer (GCL). (C) Quantification of Stau2 expression in hippocampal subregions by qRT-PCR. In comparison to CA1 (100%), percentage of Stau2 expression is 84% in CA3 and 124% in DG.*

Immunofluorescent visualization of Stau2 protein further characterized its distribution. Co-immunostaining of Stau2 and neuronal marker protein NeuN in hippocampus shows that Stau2-positiv cells are all neurons with the exception of some individual cells found in the subgranular zone of the DG. In addition, a substantially lower Stau2 protein concentration was observed in neurons of the granule cell layer compared to other DG and CA subregions (Figure 8).

In order to specify the types of neurons expressing Stau2 in hippocampal subregions, double staining of Stau2 with respective marker proteins was performed. In CA1 and CA3, Stau2 protein is found not only in CaMKII-positive

excitatory pyramidal neurons (Figure 8 C), but also in GAD67-positive GABAergic interneurons, which are located in the stratum oriens or stratum radiatum as well as in the pyramidal neuronal layer (Figure 8 B). Here, interneurons also display higher Stau2 protein concentration compared to excitatory pyramidal neurons, even if the observation remains qualitative. Within the DG, Stau2 is present in four different neuronal populations: CaMKII-positive excitatory neurons granular neurons, CaMKII-positive neurons within the hilus, GAD67-positive interneurons found in the subgranular, granular and molecular layers and CaMKII negative neurons located in the subgranular zone. Stau2 protein concentration seems to be similar in subgranular and hilar neurons excitatory neurons and interneurons, but much lower in CaMK-II positive neurons of the granule cell layer. However quantitative analysis with different methods should be done to confirm this result.

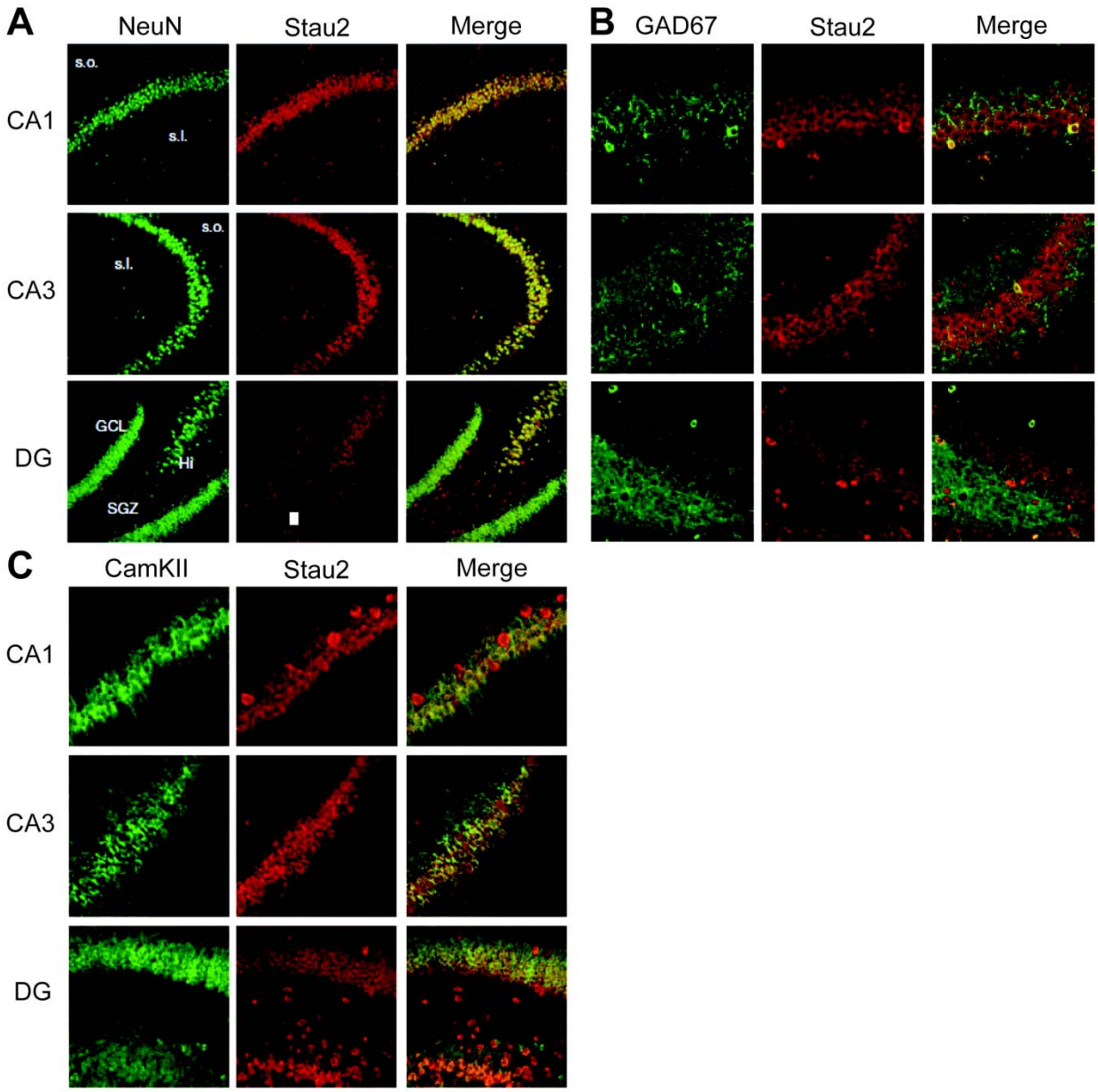


Figure 8: Analysis of Staufen2 expression in hippocampus and cortex (*A) Double immunofluorescence staining of hippocampal sagittal section of wildtype Sprague Dawley rats with antibodies specific for Stau2 (red) and the neuronal marker protein NeuN (green). In CA1 and CA3, Stau2 positive neurons can be detected in the respective pyramidal layers and in attached strata (s.o.: stratum oriens; s.r.: stratum radiatum). In dentate gyrus (DG), Stau2 can be detected in NeuN-positive neurons of the hillus (Hi) and the granule cell layer (GLC) as well as in NeuN negative cells within the subgranular zone (SGZ). Stau2 protein concentration of granule cell layer neurons is much lower compared to Stau2 positive cells in all other dentate gyrussubregions. (B) Dual immunofluorescence stainings of hippocampal sagittal section with antibodies specific for Stau2 (red) and the molecular marker for ínterneurons GAD67 (green). Neurons with particular strong Stau2 signal could be*

identified as interneurons located either within the pyramidal layers (see CA1 or CA3), the granule cell layer (see DG) or the hippocampal strata (for stratum moleculare see DG). (C) Double immunofluorescence staining of hippocampal sagittal section with antibodies specific for Stau2 (red) and the neuronal marker for excitatory pyramidal neurons alpha calmodulin-dependent kinase II (CaMKII) (green). Here, in CA1 and CA3 approx. 90% of Stau2 expressing neurons are CaMKII-positive and located within the respective pyramidal layer. In addition, CaMKII-negative neurons with a particular high Stau2 concentration can be detected within the pyramidal layers (see CA1) or hippocampal strata (for stratum oriens see CA3).

4.2.2 Generation of transgenic rats for Cre-dependent conditional Stau2 inactivation

In order to study the contribution of Stau2 protein within forebrain CaMKII-positive pyramidal neurons to synaptic plasticity and learning and memory processes, we decided to generate genetically modified rats with cell-type specific Stau2 inactivation. In Bartsch's laboratory an inducible Stau2 knock-down rat line was generated. The Cre-recombinase and RNAi technologies were combined. Rationally designed miRNA, able to lead to gene inactivation, were used in combination with co-expression of reporter eGFP to visualize the affected neurons.

Efficient Stau2 knockdown was achieved by using the sequence of an shRNA that inhibited Stau2 protein expression in cell culture very potently (Goetze, Tuebing et al. 2006). This sequence was incorporated into the miRNA design miRNA3 (Berger, Pesold et al. 2010) yielding miR(Stau2). After required in vitro tests, the final construct (pCAG-STOP-Intron-miRStau2-EGFP) was microinjected into fertilized Sprague Dawley oocytes, resulting in 6 founder animals of transgenic linespCAG-loxP.STOP.loxP-miR(Stau2)-EGFP (CAGS-Stau2).

4.3 SUITABILITY OF TAMOXIFEN-INDUCIBLE GENE KNOCKDOWN IN THE ADULT RAT BRAIN.

The obtained floxed Stau2 rat line was mated with CaMK-CreERT2 animals, where CreERT2 recombinase is expressed under the control of the αCaMKII promoter and is tamoxifen-inducible. To obtain the transgenic Stau2 rat line,CaMK-CreERT2 x CAGS-Stau2 double transgenic animals were injected with tamoxifen Tamoxifen (Tx, Sigma-Aldrich, Munich, Germany, T5648) previously dissolved in a pH neutral medium-chain triglyceride (Neutralöl, Euro OTC Pharma, Bönen, Germany) at a final concentration of 20 mg/mL. Rats between two- to three-month-old were injected intraperitoneally with 40 mg/kg body weight of tamoxifen alternating once or twice per day for five consecutive days (starting with a single injection on day 1). Experimental animals for immunohistochemistry and Cre reporter analysis were analysed 10 days after the last injection (for protocol see Schönig et al., 2012).

Before behaviorally characterize our Stau2 (-/-) rats model, the effect of tamoxifen injection was investigated, to determine its eventual role or effect on onset of some behavioral tracts to avoid influences to our further investigations. Indeed to date this is the first tamoxifen inducible rat line generated.

Food availability was maintained *ad libitum* for all the experimental period for both groups of animals. Body weight variance was recorded under the whole time during the tests (Figure 9A). During 3days of non tamoxifen administration, body weight was similar between the two groups with a natural and constant increase. At day4 tamoxifen injection protocol started and a significant reduction on the averaged body weight was registered for the treated animal(Figure 9 A; $P<0.001$).

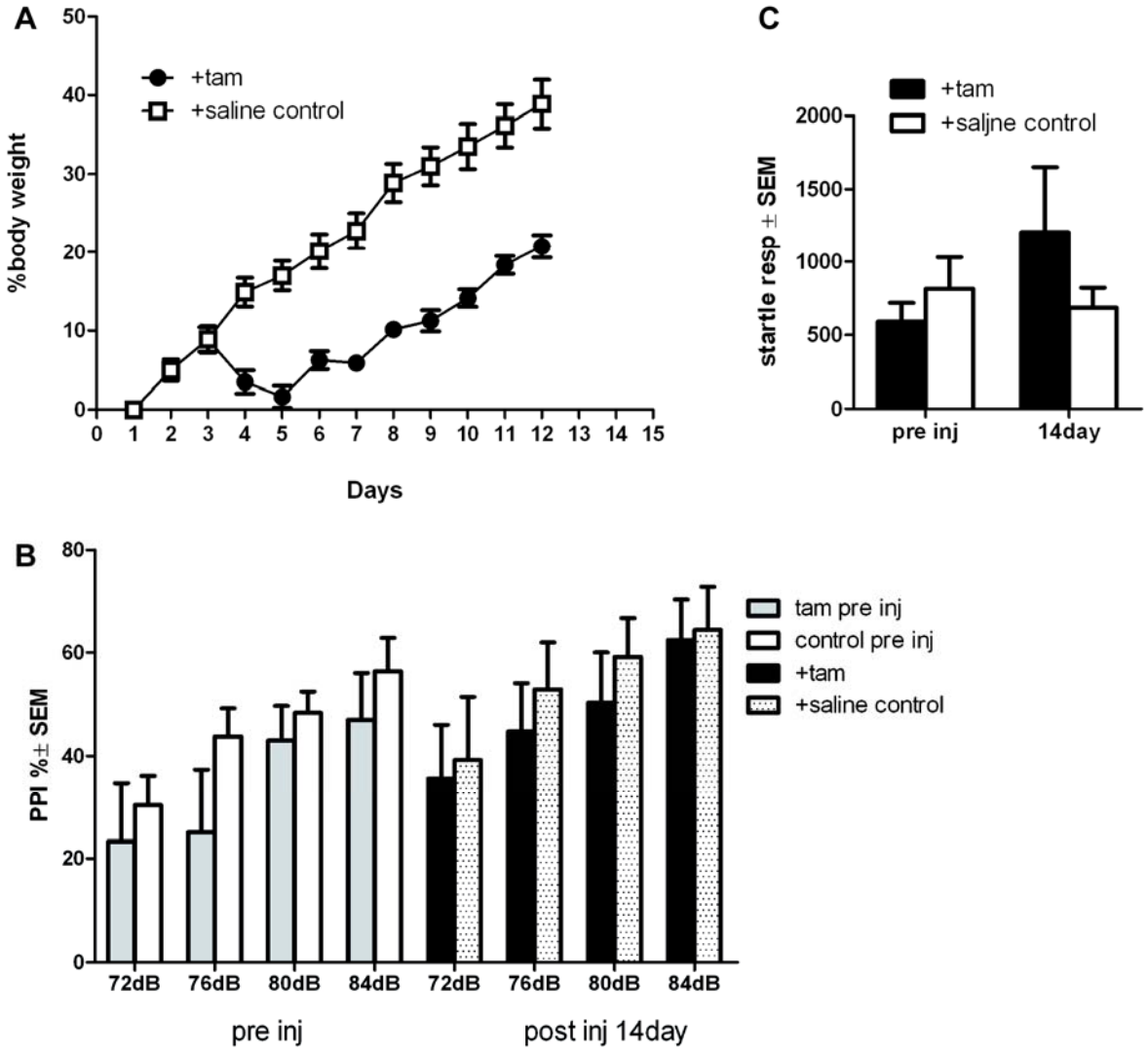


Figure 9: ***Tamoxifen injections protocol effect*** *(A) Body weight curve. All animals had a similar average weight value at the beginning of the experiment. At day 4, tamoxifen was administered and treated rats showed a drop in body weight. (B) Prepulse inhibition (PPI) measured at 72, 76, 80 and 84 db before and 14 day after tamoxifen administration revealed no significant differences. (C) Startle response before and 14 days after tamoxifen injection led to similar startle amplitudes in both groups.*

As a further characterization, sensomotor gating properties were tested in tamoxifen treated rats using a Pre Pulse inhibition task (PPI, Figure 9 B). Pre injection stage show a normal value with no difference between tamoxifen injected rats and control group. Chronic treatment with Tamoxifen for two weeks revealed no significant effect on PPI and no significant differences

between the amplitude of startle response (ASR; pulse-alone trials) of saline controls and the tamoxifen injected group neither in time ($F_{(1,14)}$ = 1,908; P > 0.05) or interaction ($F_{(1,14)}$ = 4,468; P > 0.05) (Figure 9C).

The next step was to assess the effect of tamoxifen on anxiety-like behavior. For this purpose elevated plus maze was carried out. The test showed a significant preference in both group for closed arms ($F_{(1,14)}$ = 54,90) with no significant changes in time spent on the open arms (treatment saline vs. + Tam p > 0.05)(Figure 10 A). Similarly the closed arm visits remained statistically non-significant for both analyzed groups (treatment: $F_{(1,18)}$ = 0.07, P = 0.79; Figure 10 B).

Next, rats were tested to assess antidepressant-like behavior effect of tamoxifen administration. The effects of Tamoxifen during the forced swimming test are illustrated in Figure 10 C. Both control and tamoxifen injected animals showed no significant difference in immobility, latency to immobility and climbing. On the contrary, control group display a swimming action significant higher (P=0,0194).

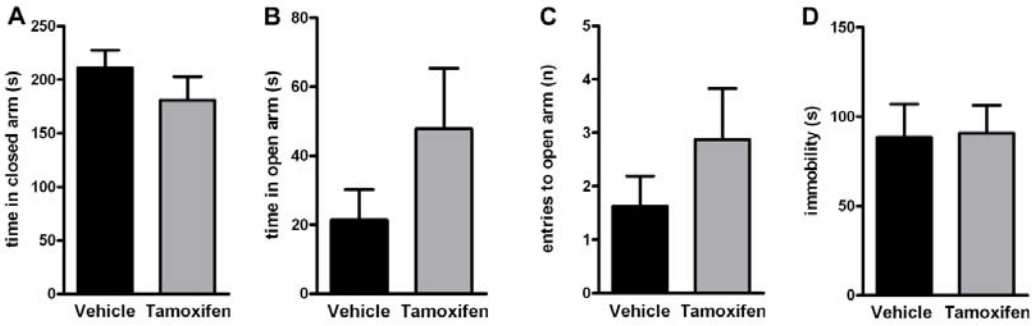


Figure 10: ***Anxiety related behavior.*** *Time spent in closed arms (A) and in open arms (B) in the elevated plus maze did not reveal differences as well as number of entries in open arms (C). (D) In the forced swimming test, tamoxifen treated rats and vehicle rats has the same performance in terms of latency to reach immobility, the time spent immobile, the swimming time and climb activity.*

Most important assessment was the study of tamoxifen administration on memory capability. The first test performed previously in our lab was a novel object recognition test (Figure 11 B). The pre-injection phase revealed the same ability for tamoxifen treated rats and control group in distinguish a new object from the familiar object. Following tamoxifen administration, already at day 4, treated animals displayed a deficit in memory recognition (t-test P=0,0063). From day 4 to day 46,no more significant impairment was found between the two groups, with a gradual recovery of memory recognition for tamoxifen treated rats.

To deeply study the effect of tamoxifen administration on learning and memory abilities, we also performed a Morris water maze (Figure 11 B-F). Three days of training (each one consisting of 3 trials) revealed that rats receiving tamoxifen perform as well as the rats receiving vehicle in term of distance moved and latency to find the hidden platform (Figure 11 C, D). During the probe trial on the fourth day, tamoxifen injected animal showed no memory deficit compared to vehicle rats. For both groups, the times spent in the target quadrant and the distance moved in it, are above the time and distance of the others quadrant of the maze, revealing an intact memory ability (Figure 11 E, F).

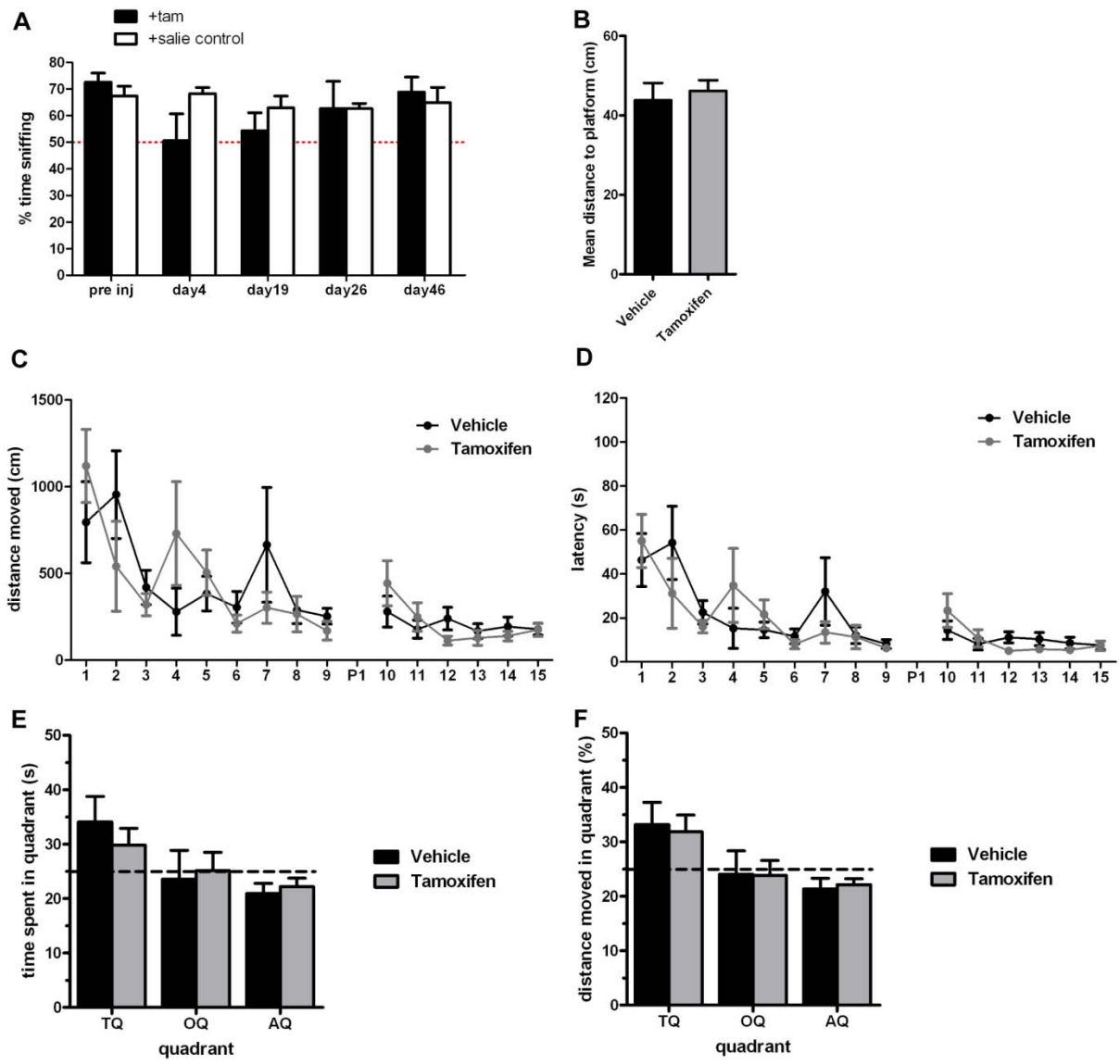


Figure 11: ***Learning and memory abilities****. (A) Rats were tested before the treatment (pre-inj) in a novel object recognition test. The test was repeated at day 4, 15, 26 and 46 after starting with vehicle or tamoxifen administration, but no differences were observed. (B-F) In the Morris water maze test tamoxifen or vehicle treated rat did not differ in their performance. (B) Mean of the distance to the hidden platform during the probe test. (C) Distance moved during the training phases. (D) Time spent to reach the hidden platform during the training phases. (E) Time spent in the target quadrant (TQ) in the opposite quadrant (OQ) or in the 2 lateral quadrants (AQ) of the tank during the probe test.*

Also the memory capability in stressful situation was tested using a fear conditioning paradigm. During the acquisition phase both groups showed a similar curve of learning (Figure 12 A). The freezing times during tones/shocks

presentation were equal for tamoxifen injected rats and for vehicle animals (Figure 12 B). During the recall phase for the context, both groups showed the same level of freezing (p=0,2319). The same happened with the cue recall phase when the rats were in a new environment with a freezing close to 0 that increase significantly and to the same level for tamoxifen injected rats and vehicle rats (Figure 12 C).

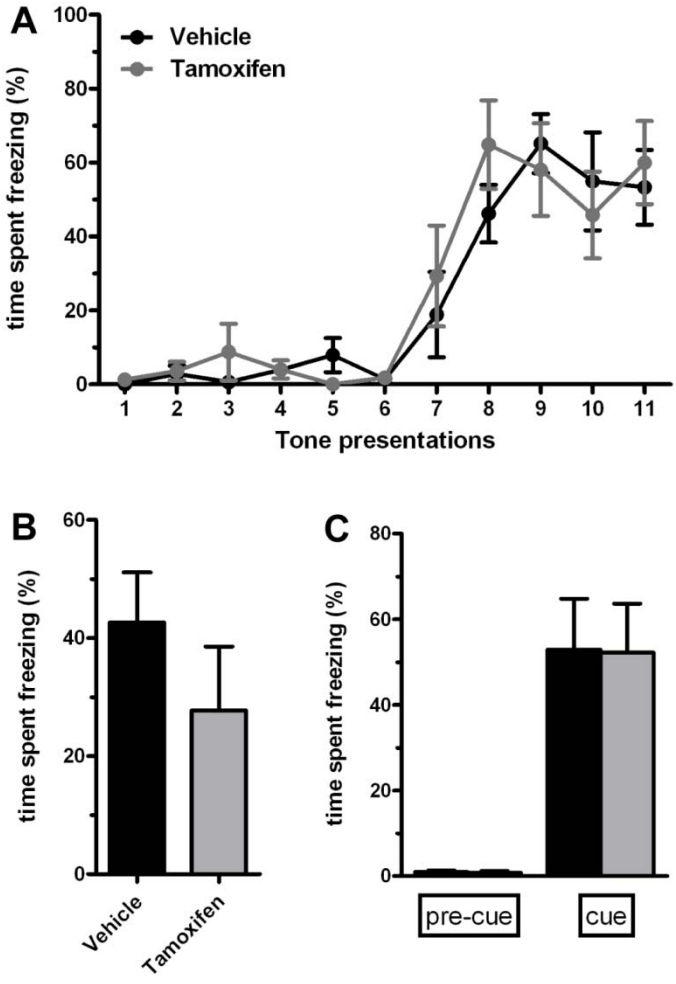


Figure 12: ***Fear conditioning****(A) Percentage of freezing time during the training phase where a sound stimulus is paired with foot shook indicating learn of predict aversive events. The first 5 points are baseline freezing before the start of the tone presentation protocol at 6days which show no arousal behavioral in bal condition. (B) Percentage of freezing time during the context recall phase in the same chamber without any sound presentation. (C) Percentage of freezing time during cue recall in a new*

chamber with tone presentation (cue). Exposure to the new chamber without any cue as baseline is indicated by pre-cue bars.

In conclusion, the activation of the transgenic system based on administration of exogenous antagonist of the estrogens receptor with tamoxifen, do not alter the behavioral tract of experimental animals at protocol dose. However a slightly impairment in novel object recognition was detected but limited to the first weeks after treatment starts. Therefore, further behavioral test were performed after 6 weeks from the tamoxifen protocol administration, a time point when the substance does not interfere with any behavioral aspect.

4.4 CHARACTERIZATION OF TRANSGENIC RATS WITH FOREBRAIN-SPECIFIC CONDITIONAL STAU2 DEFICIENCY

4.4.1 MOLECULAR CHARACTERIZATION

CAGS-Stau2 line was crossed with the rat transgenic line CaMKIIα-CreERT2, in which the tamoxifen-inducible CreERT2 recombinase has been shown to provide widespread Cre-mediated recombination within the forebrain upon tamoxifen induction (Schönig et al., 2012).For functional characterisation, CaMK-CreERT2 x CAGS-Stau2 double transgenic animals (Stau2-/-) were injected with tamoxifen (Tx) for 1 week and sagittal brain sections were analyzed by immunohistochemistry for eGFP expression. Intense eGFP positive neurons could be detected across all layers of cortical regions (Figure 13 A),in accordance to the expression pattern of endogenous αCaMKII (Ouimet, McGuinness et al. 1984). Within the hippocampus, the focus of our study, eGFP positive neurons were abundantly present in all CA subregions and in the hilus of the DG (Figure 13 B). However, only sparse eGFP production was observed in the DG granule cell layer, which was unexpected considering the endogenous αCaMKII expression pattern (Figure 13 B). In the brain of non-induced Stau2-/- animals (-Tx), eGFP positive cells were absent. (Figure 13).

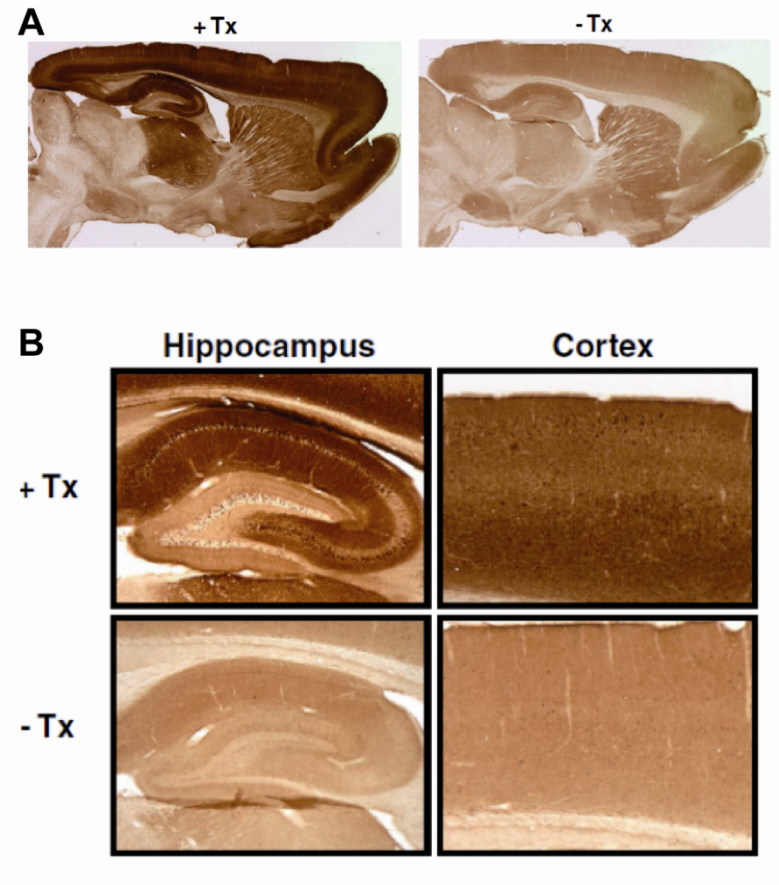


Figure 13: **Tamoxifen induced Stau2 deletion**. *(A) Immunohistochemical analysis of CreERT2 mediated recombination using an antibody specific for GFP. In uninduced double transgenic Stau2 -/- animals (-Tx), no eGFP containing neurons could be identified in hippocampus and cortex on sagittal brain sections. Several weeks following tamoxifen injection of Stau2 -/- rats (+Tx), substantial eGFP production is observed on respective brain sections in both hippocampus and cortex.(B) Higher magnifications ofuninduced(-Tx) and induced (+Tx) double transgenic Stau2 -/- animals in hippocampus (left panel) and cortex (right panel) on sagittal brain sections.*

Stau2 knockout neurons (eGFP positive) were further characterized. Co-immunostaining with an αCaMKII antibody revealed that 80% of CaMKII-positive neurons were labeled with eGFP in CA1 and 60% in CA3. In the DG, 60% of CaMKII-positive hilar neurons expressed eGFP, while in the granule cell layer only 5-10% of all neurons were eGFP-positive (Figure 14). Dual label immuno-fluorescence of Stau2 and eGFP performed on brain slices from Tx-induced

Stau2-/- rats demonstrate that Stau2 protein expression is highly reduced in eGFP-positive neurons in comparison adjacent eGFP negative cells in all hippocampal subregions (Figure 14 B).

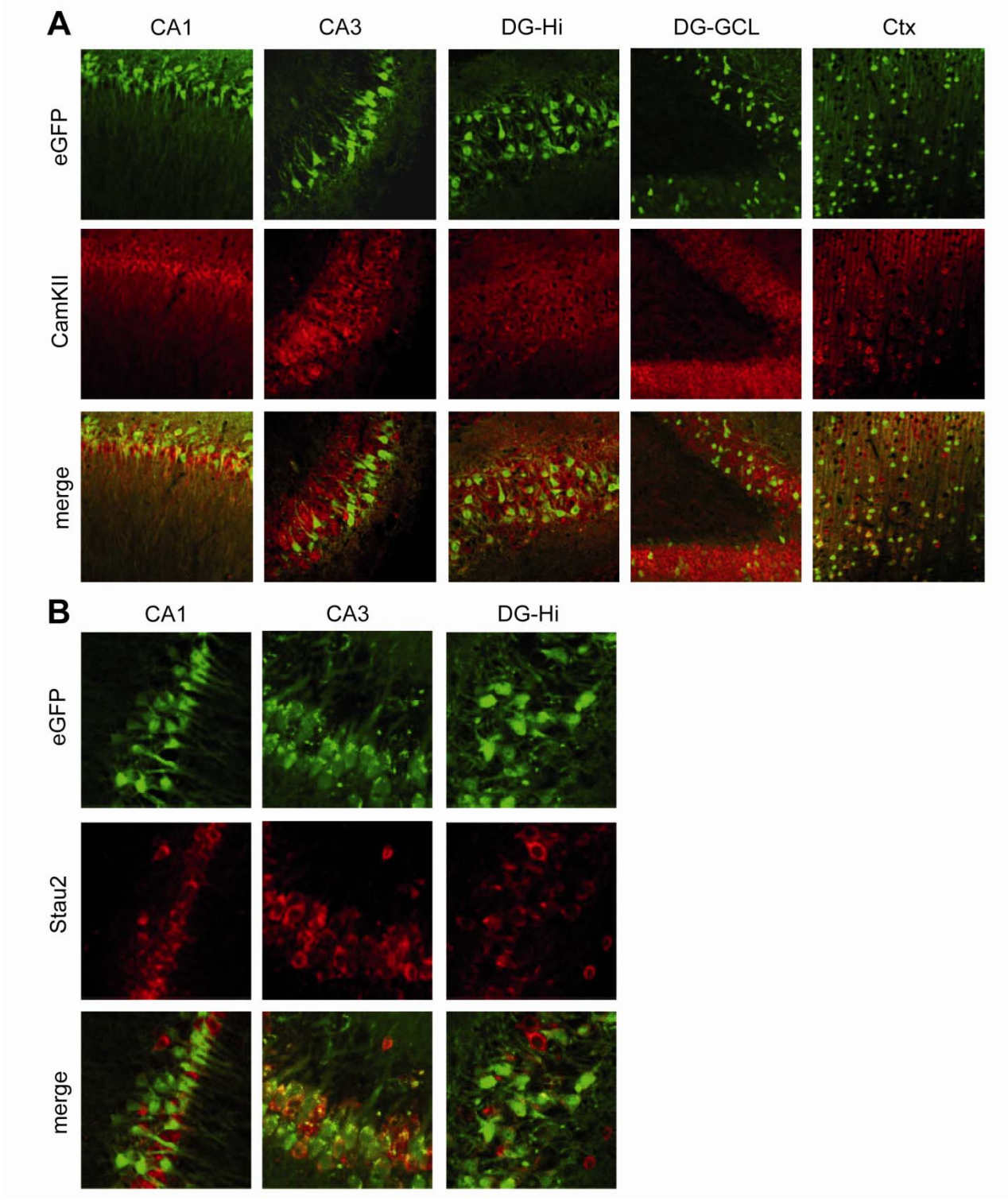


*Figure 14:**Cells type specific analysis of eGFP and Staufen 2 proteins** (A) Double immunofluorescence staining of hippocampal sagittal sections with antibodies specific for GFP (green) and the neuronal marker for excitatory pyramidal neurons alpha calmodulin-dependent kinase II (CaMKII) (red). In all investigated hippocampal subregions (CA1, CA3, hilus of the dentate gyrus (DG-Hi), granule cell layer of the dentate gyrus (DG-GCL)) and the cerebral cortex (Ctx), eGFP producing neurons are CaMKII positive. (B) Dual immunofluorescence visualisation of eGFP (green) and Stau2 (red) using specific antibodies. In hippocampal subregions CA1, CA3 and the hillus of the dentate gyrus (DG-Hi) Stau2 concentration is considerably reduced in eGFP positive neurons compared to adjacent eGFP negative neurons.*

The described qualitative analysis was supported by quantitative qRT-PCR data. We collected tissue samples and isolated total RNA from CA1, CA3 and DG regions of the hippocampus of both single transgenic CaMK-CreERT2 and Tx-induced double transgenic Stau2-/- animals. Quantification of eGFP mRNA by qRT-PCR in hippocampal subregions showed that eGFP expression and Stau2 knock down was different in different hippocampal subregions. eGFP was2.4-fold lower in CA3 and 6.1–fold lower in DG compared to CA1 (Figure 15 A), mirroring the immunohistochemical qualitative analysis (Figure 14).

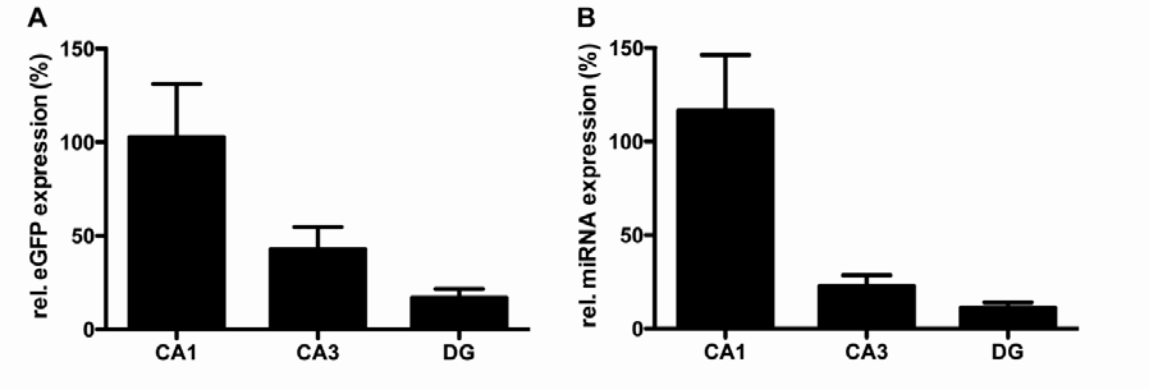


*Figure 15: **Quantification of eGFP and miR (Stau2) after Tamoxifen induction** (A) Quantification of eGFP expression in hippocampal subregions by RT-PCR. Data are normalized to CA1 expression and relative eGFP mRNA concentrations are 42% in CA3 and 17% in DG. (B) Quantification of miR(against Stau2) expression in hippocampal subregions by RT-PCR. In comparison to CA1 (to which the data are normalized), relative eGFP mRNA concentrations are 22% in CA3 and 11% in DG.*

After determining the regional distribution of eGFP, which should mirror the miR(Stau2) expression, we next measured the inhibition of Stau2 expression by miR(Stau2). qRT-PCR analysisof processed miR(Stau2) showed that eGFP mRNA and miRNA expression parallel each other in all hippocampal subregions of Stau2-/- animals (Figure 15 B).Furthermore Stau2 downregulation was confirmed with the same technique (Figure 16). In comparison to control animals, Stau2 mRNA expression was reduced by 69% in CA1, by 55% in CA3 and by 59% in DG tissue samples of Tx-induced Stau2-/- rats (Figure 16 A). This Stau2 knockdown is not compensated by the other rat staufen homologue of *Drosophila staufen* Staufen1 (Stau1) since its expression is unchanged between Stau2-/- and CaMK-CreERT2 rats (Figure 16 B).

Dual label immuno-fluorescence of Stau2 and eGFP performed on brain slices from Tx-induced Stau2-/- rats demonstrate that Stau2 protein expression is highly reduced in eGFP-positive neurons in comparison to adjacent eGFP negative cells in all hippocampal subregions (Figure 14 B). Taking into account that in analysed tissue samples the RNA from identified eGFP-negative neurons faithfully expressing Stau2 is present along with RNA from neurons with Stau2 knockdown, one might suggest that knockdown efficiency achieved in individual eGFP positive neurons is underestimated in qRT-PCR analysis and should be more than 90 %.

From these data we conclude that Stau2-/- rats represent a valid animal model to study the contribution of Stau2 protein in CaMKII-positive excitatory pyramidal and hilar neurons to hippocampal synaptic plasticity and related behaviors.

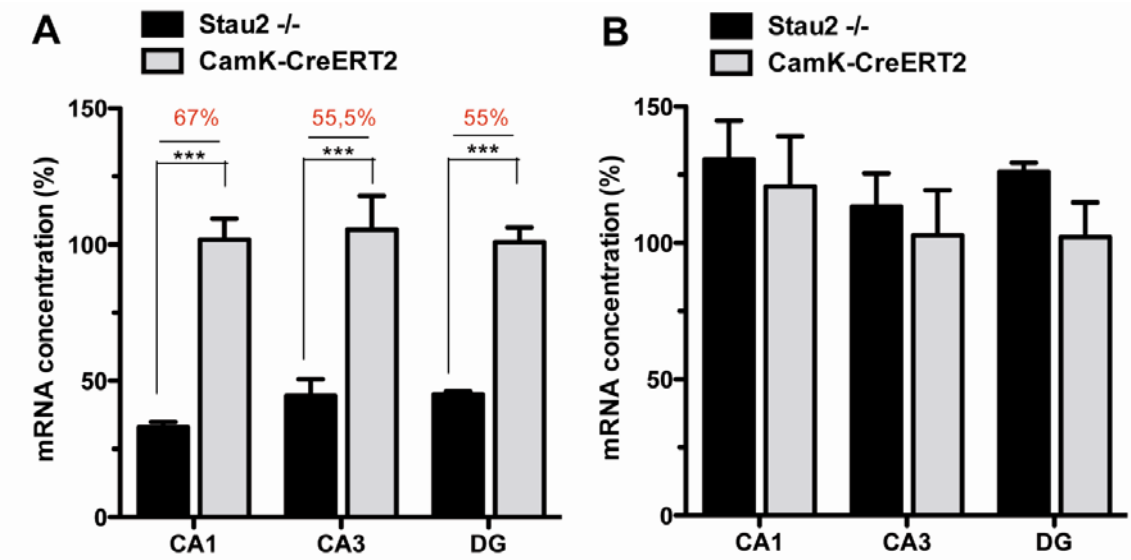


Figure 16: ***Quantitative RT-PCR analysis of Staufen1 (Stau1) and Staufen2 (Stau2)****. (A)qRT-PCR analysis confirmed that the expression of Stau2 in hippocampal subregions displays a profound reduction in CA1 (69%), CA3 (55%) and dentate gyrus (DG; 59%) in Stau2-/- rats in comparison to controls. (B)On the contrary the expression of Staufen1 (Stau1) was unaltered between Stau2 -/- and control rats, excluding any compensatory effect.*

4.4.2 Behavior characterization of Staufen2 knockdown rats

Stau2-/- rats have normal motor activity, spatial memory and learning o n fear conditioning.

In collaboration with others laboratories it was showed that in the hippocampal subregion CA1, the spine density was significantly lower (-12 %) in apical, but not in basal CA1 dendrites of Stau2-/- rats. In addition, length of spines from apical CA1 dendrites was significantly reduced but not in basal CA1 dendrites (Oliver von Bohlen und Halbach, Institut für Anatomie und Zellbiologie, Ernst-Moritz-Arndt-Universität Greifswald, Germany). However, alteration in dendritic spine morphology did not have any functional consequence on short-term plastic processes, and they are not affected in behaving Stau2-/- rats. Beside this founding, it was shown that Stau2-/- rats generated larger and longer-lasting LTP than their respective controls. In contrast, Stau2-/- rats seem devoid of a proper synaptic mechanism to generate LTD, showing a noticeable imbalance between LTP and LTD processes(Agnes Gruart, Division of Neurosciences, Pablo de Olavide University, Seville, Spain, data not shown).

Changes in long-term synaptic plasticity in the hippocampus similar to those findings were often correlated with behavioral deficits in spatial learning and memory tasks (Zeng, Chattarji et al. 2001; Nicholls, Alarcon et al. 2008). Therefore, Tx-induced Stau2 -/- rats and Tx-injected single transgenic CaMK-CreERT2, serving as control animals, were subjected to behavior paradigms, in order to assess various forms of spatial learning and memory. Before starting with respective learning and memory task, the open field test showed that Stau2 -/- rats were indifferent in their distance moved, basal horizontal and vertical locomotor activity, speed and rearings when compared to CaMK-CreERT2 animals (Figure 17, for all t-test : $p > 0.05$).

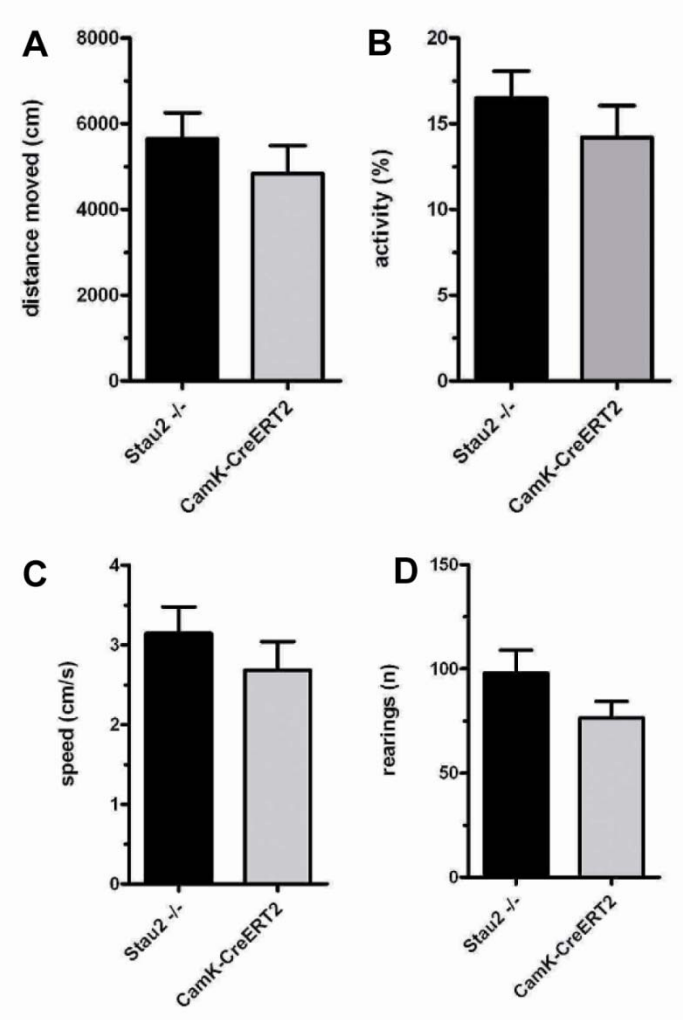


Figure 17: ***Locomotor behavior measurement.*** *Stau2-/- (black) were compared with CaMK-CreERT2 rats (gray)in an open field test. Distance moved (A), activities (B), speed (C) and rearing behaviors (D) were automatically recorded and did not show significant differences.*

The first learning and memory task performed was the hidden platform version of the Morris water maze, assessing spatial reference learning and memory (Figure 18). Here, both Stau2-/- and CaMK-CreERT2 rats learned to escape the water maze with the same efficiency as deduced from the similar decay of latencies to find the escape platform (Figure 18A) and distances moved in the maze (Figure 18 C). When challenged in the probe trial, both groups of animals spent significantly more time in the quadrant of the water maze, where the platform had been located during learning trials (Figure 18B). This suggests that Stau2-/- encode spatial reference memory with same efficiency as controls.

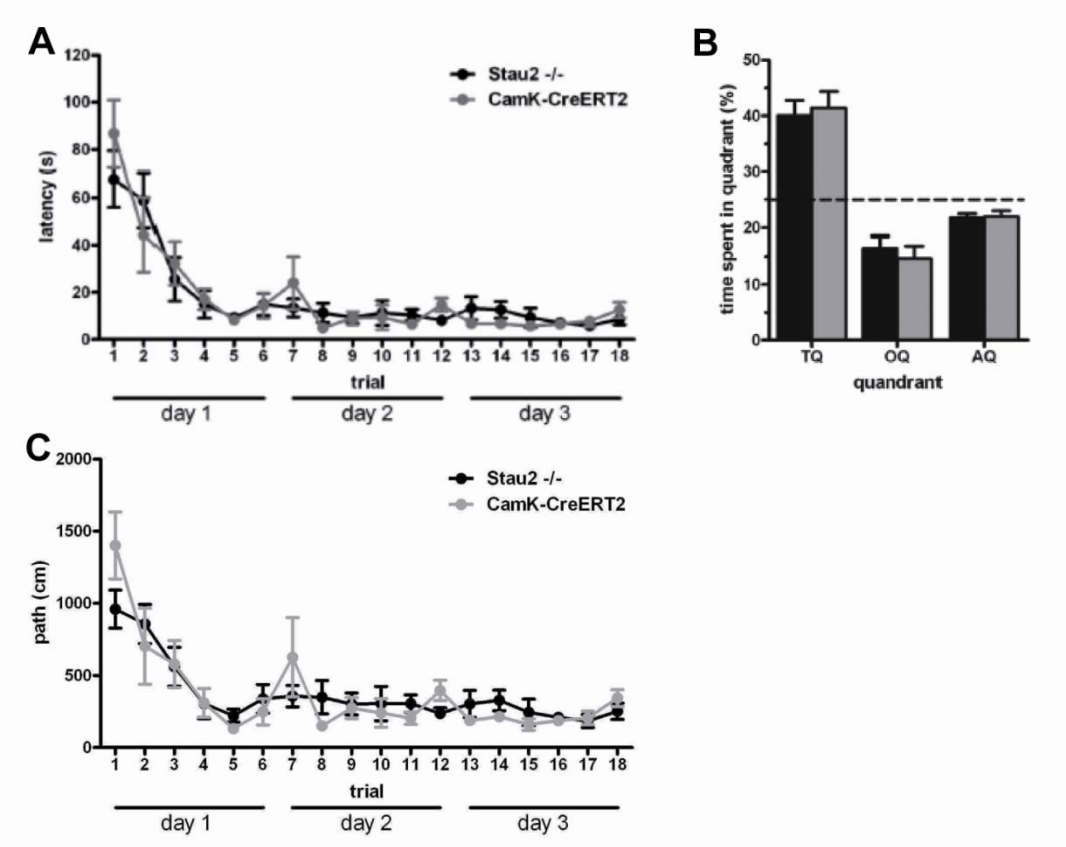


Figure 18: ***The Hidden Platform Morris Water Maze Task.*** *(A) Individual Stau2-/- (black) or CaMK-CreERT2 (gray)rats were trained for 3 days with 6 training trials per day. No significant difference was observed between rats of both genotypes ($F_{Genotype}(1,306)=0.02$; $p = 0.894$), sinceboth learned to find the location of the hidden platform in the Morris Water Maze ($F_{Trials}(17,306)=17.11$; $p < 0.001$), an observation deduced from their respective latencies. (B) Percentage of time spent in comparison to the*

located platform,inthe target quadrant (TQ), the opposite quadrant (OQ), and the average of the two adjacent quadrants (AQ) during the probe trial on day 4. No significant difference could be detected between Stau2-/- (black bars) and CaMK-CreERT2 rats (gray bars) ($F_{Genotype}(1,36)=0.03$; $p = 0.860$), showing that both memorized the platform location as seen by the significantly higher occupancy for the target quadrant of both groups of animals ($F_{Quadrant}(2,36)=44.90$; $p < 0.001$). (C) The path length during the training phase (3 days with 6 trials per day) showed that both Stau2-/- (black bars) and CaMK-CreERT2 rats (gray bars) learned to find the location of the hidden platform ($F_{Trials}(17,306)=11.24$; $p < 0.001$) in the water maze. No significant difference was observed between rats of both genotypes ($F_{Genotype}(1,306)=0.005$; $p = 0.947$).

Next, Stau2-/- and CaMK-CreERT2 rats were subjected to a fear-conditioning paradigm (Figure 19). Both groups of animals learn the association between the tone and the shock equally well as seen in the highly similar increase of freezing behavior upon tone presentations during the fear acquisition trial (Figure 19 A, B). When respective animals were tested for their context and cue-dependent memory, Stau2 -/- rats showed again unaltered fear responses specific towards either the context, in which the tone-shock association was acquired (Figure 19 D) or the tone that was paired with shock presentations (Figure 19 C). This shows that Stau2 -/- have no deficit in both the hippocampus-dependent contextual fear memory and the hippocampus-independent fear memory towards the tone cue. Finally, the ability to extinguish learned fear association was tested in a fear extinction trial. Here, both groups of rats showed a significant decrease in fear response toward repeated tone cue presentations that are not paired with a foot shock in this test (Figure 19 D).

From these findings we conclude that the observed alteration in Stau2 expression has no impact on processes of hippocampus-dependent reference learning and memory.

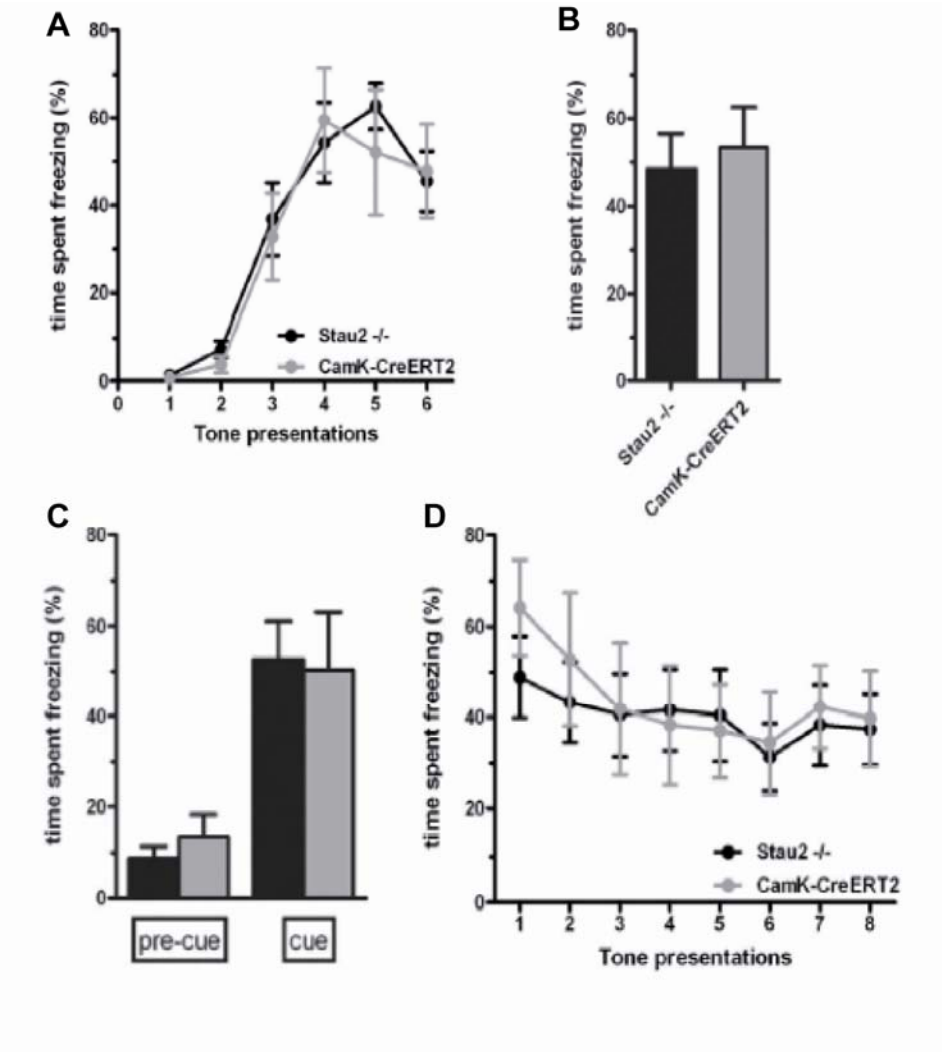


Figure 19: ***Fear Conditioning****. (A-B) In the acquisition of conditioned fear phase, the time spent freezing plotted over tone presentations shows that both Stau2-/- (black bars) and CaMK-CreERT2 rats (gray bars) learn the association of the tone with the electric shock ($F_{presentations}(5,95)=32.19$; $p < 0.001$). However, no significant difference was observed between rats of both genotypes ($F_{Genotype}(1,95)=0.06$; $p = 0.808$). (C) In the recall of contextual fear phase, 24 h after the fear acquisition trial, animals were placed into the conditioning chamber used for the acquisition of conditioned fear for 5 min. No significant difference ($p = 0.707$) was observed in the freezing response of Stau2-/- (black) and CaMK-CreERT2 (gray) rats toward the context in which fear conditioning took place. (E) In the recall of cued fear, that followed the contextual recall session, animals of both genotypes were introduced into an unknown conditioning chamber. After 2 min habituation (pre-cue), the tone associated with the shock was presented for 2 min (cue). Freezing response recorded during both time frames show that both Stau2-/- (black) and CaMK-CreERT2 rats (gray) displayed a highly specific fear response for the tone cue ($F_{cue}(1,19)=35.07$; $p < 0.001$). No significant difference was observed between rats of both genotypes ($F_{Genotype}(1,95)=0.02$; $p = 0.899$). (D) In the extinction of conditioned fear phase, the time spent freezing plotted over tone presentations shows that both Stau2-/- (black bars) and CaMK-CreERT2 rats (gray bars) reduced their fear response toward the cue upon tone presentations not*

paired with the electric shock ($F_{presentations}(7,126)=2.18$; $p = 0.04$) with no difference observed between genotypes ($F_{Genotype}(1,126)=0.41$; $p = 0.893$).

Stau2-/- rats are impaired in spatial working memory at intermediate, but not short-term delays

Impaired LTD in mice had been previously associated with deficits in spatial working memory tasks (Zeng, Chattarji et al. 2001; Nicholls, Alarcon et al. 2008). Therefore, a delayed non-matching to place task on an 8-arm radial maze (Lee and Kesner 2002; Lee and Kesner 2003) was conducted with both Tx-injected Stau2-/- and CaMK-CreERT2 rats (Figure 20). In this behavior paradigm, the animals were trained to choose an unvisited over a previously visited arm of the radial maze in order to be rewarded. Remembering the visited arm was made gradually more difficult by introducing a delay time between the initial visit and the choice phase, during which the animal had no possibility to explore the maze. At short-term delay times (1 min), control and Stau2 -/- rats displayed similar accuracies in selecting the unvisited arm as seen from the high correct choice rate (Figure 20 A) and the low number of unrewarded arm visits per animal. However, when delay times were increased to 5 or 10 min, control rats still conducted the task with a similar accuracy as observed with a short delay, whereas Stau2 -/- rats highly significantly dropped in their performance almost to the level of a randomly choice of the arm (Figure 20 A) increasing the total number of errors (Figure 20 C). Moreover, Stau2-/-rats significantly increased in their latencies to enter the arm during the choice phase with longer delay time compared to control animals (Figure 20 B). This suggests that the observed deficit in choosing the rewarded arm in Stau2 -/- rats is due to a working memory deficit and not caused by increased impulsivity of these animals.

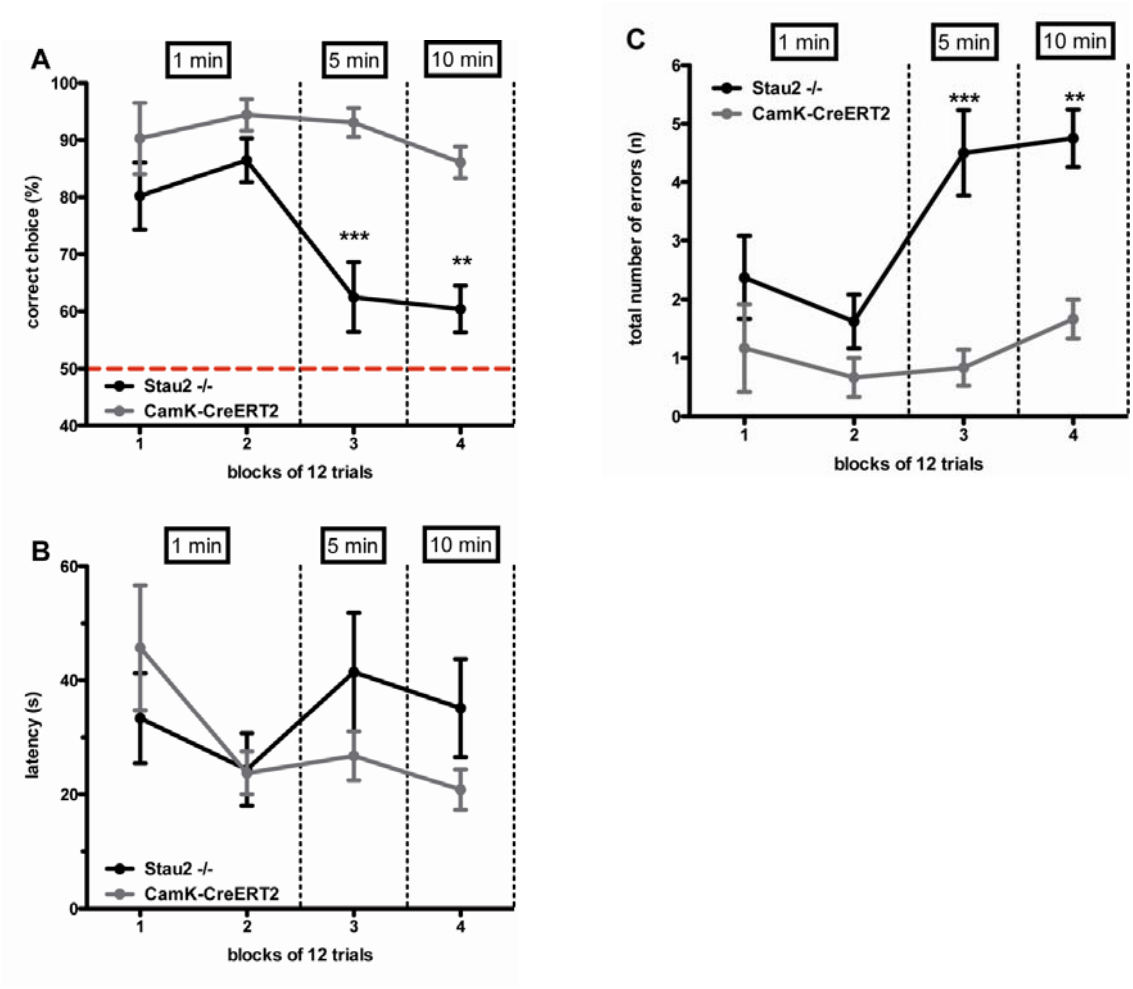


Figure 20: ***Delayed non-match to place (DNMP) 8 arms radial maze task****. Animals were trained in blocks of 12 trials (4 trials/day) at individual delay times. (A) Performances of Stau2-/- (black bars) and CaMK-CreERT2 control rats (gray bars) in the DNMP task with variable delays indicate a highly significant deficit in spatial working memory of Stau2 deficient animals ($F_{Genotype}(1,36)=21.12$; $p < 0.001$). Bonferrroni post hoc testing showed that Stau2-/- rats significantly drop in their task performance at intermediate (5 min, 10 min) but not at short time delays (1 min). (B) Latencies to enter the radial maze arm with the anticipated reward during the choice phase of the DNMP task displayed that Stau2 -/- animals (black bars) take significantly more time for their decision with increasing delay times compared to CaMK-CreERT2 control rats (gray bars) ($F_{Genotype}$*delay time $(3,36)=3.64$; $p = 0.022$). (C) Performances of Stau2-/- (black bars) and CaMK-CreERT2 rats (gray bars), here expressed as the total number of entrances into unrewarded arms (errors) in the choice phase of the DNMP task, also indicate a highly significant deficit in spatial working memory of Stau2 deficient animals ($F_{Genotype}(1,36)=21.13$; $p < 0.001$) at intermediate (5 min, 10 min) but not at short time delays (1 min). Data are presented either as mean values + SEM or mean values ± SEM. Stars represent p-*

*values of significances between genotypes obtained by Bonferroni post hoc analysis following two-way ANOVA of repeated measures: ** p<0.01; *** p<0.001.*

Stau2-/- rats have a deficit in detecting spatial novelty

Having identified a spatial working memory deficit, we next aimed to investigate spatial novelty detection abilities in Stau2-/- rats, since both behaviors have been recently associated with deficits in EC-CA1 synaptic transmission (Vago and Kesner 2008). Induced Stau2 -/- and control rats were tested in their abilities to encode hippocampus-independent object recognition memory (Figure 21). Here, both groups of animals were significantly able to identify the novel object (Figure 21A). However, when respective animals were subjected to the hippocampus-dependent novel object relocation paradigm, Stau2-/- rats significantly failed to recognize the novel position of a familiar object in contrast to CaMK-CreERT2 control rats (Figure 21B).While the control group showed significant increase in exploration for the relocate object to the choice level,Stau2-/- animals showed a significant reduction of investigation time to the second exposure of the same but relocated object, carried out 15 min later. During the sampling phase of novel object recognition and relocation both group had no difference in time of exploration maintaining a 50% value in time sniffing (Figure 21). Together this founding show the rodents' ability to recognize a novel object in the environment but impairment in detect a novel position of a familiar object.

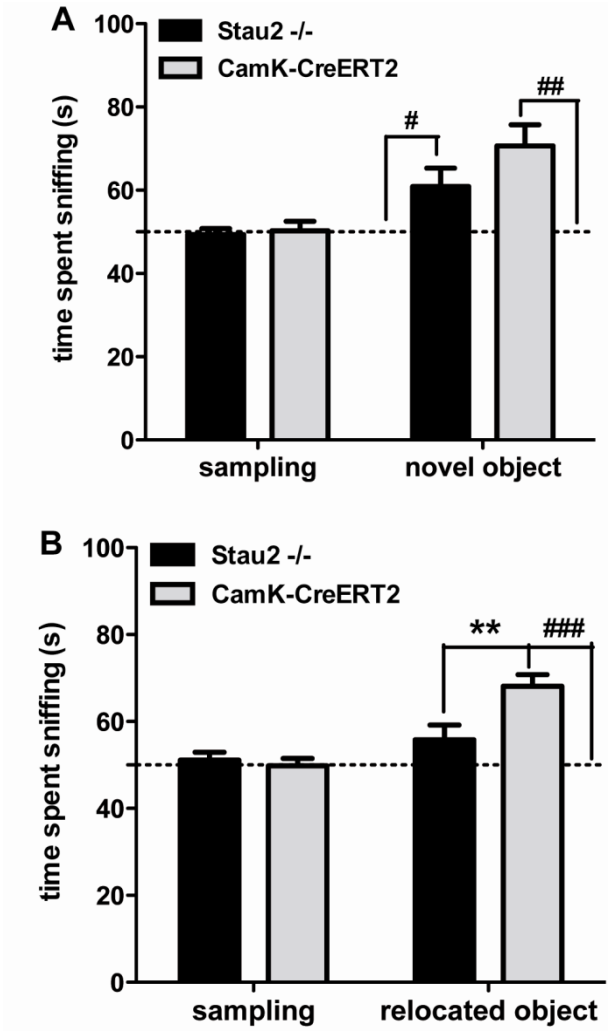


Figure 21: ***Memory ability on Novel objects tasks*** *(A) The novel object recognition task showed that both Stau2-/- (black) and CaMK-CreERT2 control rats (gray) spent more time investigating the novel object during the study phase of the task, an indication of an intact object recognition memory (*F_{Object} *(1,18)=16.34; p < 0.001)(*$F_{Genotype}$ *(1,18)=2.50; p = 0.131). One sample t-test indicates that exploratory behavior toward the novel object is beyond chance level in both groups of animals. (B) The novel object relocation task showed that Stau2-/- rats (black) display a significant deficit in recognizing the novel position of a familiar object as seen from Bonferroni post hoc testing of a 2 way ANOVA of repeated measures (*$F_{Genotype}$*(1,18)=4.47; p = 0.049). One sample t-test shows that only CaMK-CreERT2 control rats (gray) explore the relocated familiar object more than chance level.*

5. DISCUSSION

5.1 NOGO-A KNOCK-DOWN RAT MODEL UNCOVERED NOVEL PHENOTYPIC TRAITS, CORRELATED TO HUMAN SCHIZOPHRENIA.

Nogo-A is an important neurite growth-inhibitory protein that stabilizes the adult CNS wiring, restricts regeneration and also negatively regulates hippocampal plasticity (Schwab 2010). Moreover, a recent evidence suggests that aberrant Nogo-A signaling poses an increased risk for schizophrenia (Willi and Schwab 2013). The Nogo gene has been mapped to chromosome 2p16, which is a genetic regioninvolved in schizophrenia (Lewis, Levinson et al. 2003). Moreover Nogo mRNA was reported to be overexpressed in post-mortemsamples of frontal cerebral cortices from individuals with schizophrenia (Novak, Kim et al. 2002). There is also evidence for an association of the nogo receptor gene(NgR) since the gene is encoded by chromosome 22q11, a well-known hotspot locus for genetic linkage to schizophrenia risk (Karayiorgou, Morris et al. 1995)

Animal models of complex heterogeneous psychiatric disorders are clearly very valuable preclinical tools with which to investigate the neurobiological basis of the disorder. Animal models used to study schizophrenia can be classified into four major categories: developmental, drug-induced, lesion-induced or derived from genetic manipulation. There are several potential difficulties during the attempt to model schizophrenia in animals, including the standard caveat of faithfully reproducing what is generally perceived to be a cognitive disorder in less cognitively developed animals(Jones, Watson et al. 2011). Indeed, patients typically experience a combination of symptoms, often divided into positive (e.g., hallucinations, delusions, thought disorganizations), negative (e.g., loss of motivation, affective blunting, alogia, social withdrawal) and cognitive (e.g., deficits in attention, memory and executive functions) (Andreasen 1995).

Recently, a Nogo-A knock-out mice showed several behavioral phenotypes commonly used as correlates of schizophrenia symptoms. Those mice

constitutively lacking Nogo-A displayed deficiencies in both PPI and latent inhibition (Willi, Weinmann et al. 2010). Other tests showed that these mice were spontaneously hyperactive (Willi, Aloy et al. 2009), a trait of rodent models of schizophrenia (Gainetdinov, Mohn et al. 2001)that could correspond to psychomotor agitation present in schizophrenic patients. Nogo-A knock-out mice showed higher sensitivity to the motor stimulant effect of amphetamine (Willi, Aloy et al. 2009, (Willi, Weinmann et al. 2010), pointing to a psychotic-like phenotypic profile. Beyond the phenotypes just described, mice lacking Nogo-A also exhibited behavioral perseveration, resembling another clinical manifestation seen in schizophrenic patients (Meyer, Feldon et al. 2011).Compared to the existing animal model for schizophrenia, novel phenotypic traits could be identified in our transgenic Nogo-A knockdown rats resembling the negative symptoms of the disease, a difficult aspect to model in murine model. Indeed, Nogo-A knock down rats showed significantly lower exploration and reduced social contact behavior, as well as much higher withdrawal from social interaction initiated by the social partner compared to their WT littermates. The presence of these negative symptoms in the rat model is of particular significance. In free social interactions, knockdown rats showed normal exploratory behavior, but a marked attenuation and avoidance of social contact. We exclude that this social withdrawal behaviour might be related to increased anxiety, since the open field test with knockdown rats indicates no signs of anxiety and Nogo-A KO mice do not differ in anxiety-related behaviours from their WT controls (Willi, Aloy et al., 2009). Social withdrawal and isolation are among the key components of negative symptoms in schizophrenia and thus social withdrawal observed in Nogo-A knockdown rats supports a schizophrenia-like phenotype.

Elucidating schizophrenia-related gene functions in transgenic rats offers several important add-ons to the research. Although mice can be used for a broad variety of behavioral task, the performance of rats in comparison is still outstanding. Moreover, when it comes to more sophisticated operant procedure that requires recognition of complex changes to be rewarded, some of these tasks have been reported only for the rat (Eagle, Lehmann et al. 2009; Abbott 2010; Enkel, Gholizadeh et al. 2010). Further, rats show more human-like social

behaviors than solitary living mice (Ben-Ami Bartal, Decety et al. 2011); this is particularly important for modelling intermediate phenotypes for disorders such as autism and schizophrenia (Baker 2011). Their larger size make rats more conducive to study by instrumentation and facilitates manipulation such as blood sampling, performing surgeries, *in vivo* electrophysiological recordings with multiple electrodes (Colgin et al., 2009) and optogenetic methods (Royer, Zemelman et al. 2010; Witten, Steinberg et al. 2011). Rat models bridge the gap between basic research and drug development, since they are widely used in the pharmaceutical industry to predict how humans will metabolize drugs and to identify potential side effects. Modelling human conditions in rats, rather than in mice enables more predictive studies of complex neurobehavioral conditions. In fact, a model for drug addiction resembling the human situation has so far only developed in the rat (Deroche-Gamonet, Belin et al. 2004; Hermann, Weber-Fahr et al. 2012). Since, several behavioral tests are more advanced or validated for the rat species, especially regarding the behavioral assessment of complex neuropsychiatric disease phenotypes (e.g. negative symptoms in schizophrenia), the analyzed Nogo-A knock-down rat model provides a novel, promising tool to study a potential role for Nogo-A in cognitive and behavioral functions.

Furthermore, KO models are not the most suitable tools for a translational study, due to e.g. compensation and lethality that are frequent undesirable side effects of conventional KO mice. Indeed in Nogo-A KO mice was found a significant up-regulation of the small splice-isoform Nogo-B (Simonen, Pedersen et al. 2003).

During the design of rodent model must be considered that several genes of interest for neurobiological research, such as protein kinases, transcription factors, and growth factors, subserve critical functions throughout development. Chronic expression of a transgene could therefore cause a developmental abnormality or adaptation, leading to masking or distortion of the acute role of the protein of interest. In our knockdown model for Nogo-A protein, a miRNA-based approach targets splice-form specific mRNAs and leaves the endogenous genetic locus intact.

A more sophisticated method to avoid the complication of functional or developmental compensation or drastic developmental phenotypes, is represented by the CreERT2/loxP-recombination system that allows temporal

control and region specificity of conditional gene manipulation. Temporal control of recombination is a prerequisite for distinguishing the developmental role of a gene from its present function during adulthood. Although inducible Cre-recombinase mediated gene knockouts have been very successfully applied in mice (Branda and Dymecki 2004), development of gene inactivation technologies in rats lag behind those available for mice. Targeted, ubiquitous gene inactivation in the rats could be accomplished by using either RNAi technology (Dann, Alvarado et al. 2006; Lim, van den Brandt et al. 2008), embryonic injection of zinc finger nucleases (Geurts, Cost et al. 2009) and due to the recent availability of rat embryonic stem cells (Buehr, Meek et al. 2008; Li, Tong et al. 2008) through classical homologous recombination (Tong, Li et al. 2010). So far, no inducible tissue-specific gene inactivation has been described for the rat. However we took advantage of a fusion protein consisting of Cre-recombinase and a mutated ligand-binding domain of the human estrogen receptor (ER) that was developed to achieve tamoxifen dependent Cre activity (Feil, Wagner et al. 1997);With this method we generated a transgenic rat model, where we specifically inactivated the mRNA transport protein Staufen2 in forebrain excitatory neurons, by crossing the Stau2 floxed rat line with a CaMK-CreERT2 transgenic line.

5.2 KNOCK-DOWN OF THE MRNA TRANSPORT PROTEIN STAUFEN 2 LEADS TO A SPECIFIC DEFICIT IN SPATIAL WORKING MEMORY

In this study, we investigate the function of the mRNA transport protein Stau2 using the first transgenic rat model providing inducible tissue-specific gene inactivation. Using a conditional knockdown approach in transgenic rats, we specifically inactivated Stau2 in forebrain excitatory neurons of adult animals. We obtained variation in terms of spine morphology, LTD and LTP (data not shown) and behavior. In our recent study (manuscript in preparation; experiment in collaboration with Prof. Agnès Gruart , Sevilla) hippocampal neurons of Stau2 deficient rats show a significant reduction in spine density, however only by a factor of 10%. In addition a significant reduction of spine

length in both apical and basal dendrites of CA1 pyramidal neurons, conflicting with both previous studies has been shown. This found can add new information useful to clarify the controversial discussion about the role of Stau2 in spine morphology (Goetze, Tuebing et al. 2006; Lebeau, DesGroseillers et al. 2011). The other finding in our Sta2 (-/-) rats model (manuscript in preparation) is significantly enhanced LTP in Stau2-/- animals that maintained over up to 4 days. In addition, low frequency stimulation, instead of inducing LTD as seen from control animals, elicited LTP in respective hippocampal subregions obtaining an opposite electrophysiological phenotype with enhanced LTD combined with impaired LTP.

Parallel to the profound changes of long term synaptic plasticity in vivo, Stau2 deficient animals showed a specific deficit in spatial working memory, whereas spatial reference learning and memory and fear conditioning remained unaltered. This is in agreement with several studies using conditional mouse mutants that show that an LTD impairment in the hippocampus leads to this profile of learning and memory phenotypes. One example is represented by knockout mice for the dopamine transporter (DAT-KO), which show a selective and reversible impairment in hippocampal LTD, associated with a delayed in acquisition of place navigation but intact spatial memory in the Morris water maze (Morice, Billard et al. 2007). Another study supports our findings and describes a mouse model with a specific loss of NMDA receptor-dependent LTD that was accompanied by a deficit in behavioral flexibility. In the Morris water maze, these animals learn normally but exhibit both delayed acquisition of a new platform location and perseveration behavior. In the delayed non-match to place T-maze task, these animals also exhibit a flexibility deficit in the form of enhanced inter-trial interference, performing normally on the first trial of a day but exhibiting impaired performance on subsequent trials (Nicholls, Alarcon et al. 2008). Accordingly, our model of Stau2-/- rats, characterized by an impairment of a proper synaptic mechanism to generate LTD, showed a maintained spatial memory. However a deficit in spatial working memory was revealed. This is further sustained by another work, where hippocampal long-term depression was found to be an index of spatial working memory, with parameters of memory capability strongly correlated with the magnitude of LTD

(Nakao, Ikegaya et al. 2002). Moreover the same conclusion came from an experiment with acute exposure of exogenous cannabinoids in rats that impaired in vivo LTD of synaptic strength at hippocampal CA3-CA1 synapses and spatial working memory (Han, Kesner et al. 2012).

The normal ability during the fear conditioning of Stau2-/- rats could be explained taking in consideration the enhanced LTP that is thought to be correlated with an enhanced cued and contextual fear memory (DeAndrade, Zhang et al. 2012).

However, the genetic modifications applied in our case affect mainly excitatory neurons within the prefrontal cortex, which could have a significant contribution to the observed working memory deficit. In fact, the prefrontal cortex has long been proposed to be exclusively involved in cognitive flexibility and in working memory functions (Fuster 2000). Interestingly, a recent study provided evidence that within a spatial working memory task the prefrontal cortex and the hippocampus process short term memory in parallel, thereby able to compensate each other (Lee and Kesner 2003). By combining lesion and pharmacological inactivation techniques, Lee and Kesner observed that temporal inactivation of the hippocampus always led to a specific deficit at intermediate- but not short-term delay times in a delayed non-matching to place paradigm (DNMP) on the 8 arm radial maze when the prefrontal cortex was intact. Since in our study, we used exactly the same DNMP paradigm to investigate the spatial working memory, the fact that Stau2-/- rats display a specific deficit at intermediated but not short term delay times strongly suggests that the alterations observed in hippocampal synaptic plasticity account for the deficit in spatial working memory. Indeed, a mutant mouse model, having the opposite shift in bidirectional synaptic plasticity compared to our Stau2-/- rats (impaired LTP and enhanced LTD), displayed an increased performance in spatial working memory also only at intermediate delay times (Malleret, Alarcon et al. 2010), matching our observations. In the light of all these data, our study can provide further evidence that hippocampal LTD is an essential electrophysiological correlate for short term memory storage during spatial working memory.

Beside the well-documented role in the acquisition and storage of spatial memory, the hippocampus is known to perform mismatch predictions thereby

serving as a detector of spatial novelty (Lisman and Otmakhova 2001; Kemp and Manahan-Vaughan 2007). Whereas the recognition of a novel object is processed by the perirhinal cortex, the associations of an object to a place require the hippocampus (Brown and Aggleton 2001). In addition, LTD in CA1 is facilitated, when rats explore a new environment containing new and/or familiar objects, whereas the exploration of a new environment itself impairs LTD (Kemp and Manahan-Vaughan 2004). Other works are consistent with our finding. For example it has been shown that deficit of LTD impair the mechanisms of visual recognition memory (Griffiths S. et al. 2008). Consistent with these findings, the hippocampal LTD deficit in Stau2-/- rats occurs in parallel with the animal's inability to detect a novel position of a familiar object, although it is capable to recognize a novel object. Our study provides therefore strong evidences that LTD may encode the novel acquisition of object location, more than exploration of new space. Finally, our study demonstrates that Stau2 is essential for the form of LTD necessary for detection of spatial novelty.

Aberration in RNA-binding protein expression was already associate with spatial learning and memory deficits (Bolognani, Qiu et al. 2007) and patients affected by the Fragile X syndrome (FXS) are characterized by developmental and behavioral deficits, that include mild to severe mental retardation, autism, anxiety, aberrant attention, learning, and memory (Hagerman, 2002; Tsiouris and Brown, 2004; Jacquemont et al., 2007). Our work support the thesis that RNA-binding protein as Stau2 play a major role in synaptic plasticity and learning and memory, and therefore could be a good animal model to simulate such diseases.

5.3 IMPLICATIONS OF STAU2 ON THE SYNAPTIC TAGGING HYPOTHESIS

The synaptic tagging hypothesis elegantly explains the molecular and cellular mechanisms underlying learning and memory (Redondo, Okuno et al. 2010). This hypothesis allows us to think about the properties of LTP in a new way. It is based on the idea that the neural mechanisms of initial long-term potentiation (LTP) expression (potentiation and tagging) can be dissociated from those

regulating the availability of plasticity-related proteins (PRPs) that stabilize synaptic change (Wang et al., 2010). The synaptic tagging hypothesis suggests an early phase in which synapses are prepared, or "tagged," for protein capture, and a late phase in which those proteins are integrated into the synapses to achieve memory consolidation.

However, its major challenge remains the identification of a "synaptic tag" molecule necessary for positioning plasticity related proteins in activated synapses. Although a protein (Homer1a) has been shown to be specifically targeted by a synaptic tag (Okada, Ozawa et al. 2009), the majority of plasticity related proteins are synthesized within the "tagged" dendritic spine by local translation of mRNAs, brought to the synapse by RNA transport granules (Doyle and Kiebler 2011). Since so far no synaptic storage of mRNAs could be identified, Doyle and Kiebler proposed the "sushi belt model" for dendritic transport of mRNAs of plasticity related genes. In this model, different types of RNA transport granules transport mRNAs specific for a certain form of synaptic plasticity and circulate along the dendrite, where are incorporated on demand into a dendrite that underwent synaptic stimulation (Doyle and Kiebler 2011). Several findings support this idea. It has been shown that MAP2, αCaMKII and ß-actin localize in different RNA granules, which are transported fast along the dendrites of hippocampal neurons. In addition mRNAs are present in very few copies in a transport particle (Tubing, Vendra et al. 2010; Mikl, Vendra et al. 2011). It has been shown for the Stau2 analogue Stau 1, that its knockdown leads to an impairment of LTP and an enlargement of dendritic spines in organotypic slice cultures (Lebeau, Maher-Laporte et al. 2008; Lebeau, DesGroseillers et al. 2011). This is the exact opposite modification of synaptic plasticity in comparison to the one observed in our Stau2knock-down model (data not shown). Although much more investigations need to be done, Stau1 and Stau2 RNA transport granules might be sufficient for bidirectional regulation of synaptic plasticity.

Our findings, moreover, support an RNA transport function for Stau2 protein. This becomes evident when comparing the synaptic plasticity changes in Stau -/- rats with those obtained in fmr1 knockout mouse model, for Fragile X syndrome (Bakker, Verheij et al. 1994). Although FMRP, the product of *fmr1*gene, has been implicated in the regulation of mRNA transport to dendrites (Dictenberg,

Swanger et al. 2008), its major function is the repression of mRNA translation at a local synapse, which is relieved upon mGluR activation (Bassell and Warren 2008). Interestingly, *fmr1* knockout mice display enhanced LTD (Huber, Gallagher et al. 2002) and an enlargement of dendritic spines (Comery, Harris et al. 1997), which is the opposite of what we observe in Stau2 deficient rats. Since Stau proteins and FMRP colocalize in RNA transport granules (Kanai, Dohmae et al. 2004), these data suggest that Stau2 might be necessary to anchor mRNA of plasticity related genes to the transport granules, whereas FMPR actively represses their translation within the dendritic spines.

6. CONCLUSION

6.1 THE RAT AS A PREFERRED MODEL IN NEUROSCIENCE

Although mice can be used for a broad variety of behavioral tasks, the performance of rats in these tasks is still outstanding. One good example is the delayed non-matching to place radial arm maze task, applied here for investigating spatial working memory. Whereas mice already drop to chance level at delay time of 1 min (Saxe, Malleret et al. 2007; Malleret, Alarcon et al. 2010), wild-type Sprague Dawley rats still perform well at delay times of 10 min. When it comes to more sophisticated operant procedures that require recognition of complex changes to be rewarded, some of these tasks have been reported only for the rat (Eagle, Lehmann et al. 2009; Abbott 2010; Enkel, Gholizadeh et al. 2010). It will be very difficult to train mice for such a paradigm in reasonable time or may be not possible at all. Further, rats show more human-like social behaviors than solitary living mice (Ben-Ami Bartal, Decety et al. 2011). This is particularly important for modeling intermediate phenotypes in disorders such as autism and schizophrenia (Baker 2011). Their larger size make rats more conducive to study by instrumentation and facilitates manipulation such as blood sampling, surgeries, in vivo electrophysiological recordings with multiple electrodes (Colgin et al., 2009), and optogenetic methods (Royer,

Zemelman et al. 2010; Witten, Steinberg et al. 2011). Moreover, rat models bridge the gap between basic research and drug development, since they are widely used in the pharmaceutical industry to predict how humans will metabolize drugs and to identify potential side effects. Modeling human conditions in rats, rather than in mice enables more predictive studies of complex neurobehavioral conditions. In fact, a model for drug addiction resembling the human situation has so far only developed in the rat (Deroche-Gamonet, Belin et al. 2004; Hermann, Weber-Fahr et al. 2012). The two rat models, presented in this work, confirm these observations. Indeed only through the knock-down of Nogo-A in a rat but not in mouse (Willy et al., 2010), we could unravel Nogo-A-dependent lower exploration and reduced social contact behavior. These are features already shown in schizophrenic patients but difficult to recreate in a murine model. Until now, in rats most of the schizophrenic-like behavior, especially the negative were obtained only after pharmacological induction (Sams-Dodd, Lipska et al. 1997; Javitt and Zukin 1991, Rung, Carlsson et al. 2005). Our rat model is one of the first showing cognitive deficit and negative signs for schizophrenia obtained through a genetic manipulation.

The miRNA induced downregulation of the CNS protein Nogo clearly demonstrated a reduction in Nogo-A brain expression and led to cognition impairment beside a sensorimotor gating impairment and decrease of social behavior.

6.2 NOGO-A KNOCK-DOWN AND STAU2 INDUCIBLE KNOCK-DOWN

The Nogo-A knockdown rat resulted a good model for the study of schizophrenia. With its cognitive and negative schizophrenia-like symptoms it give a valid support on schizophrenia research, defining more clearly the genetic background of this still poorly understood disease. This animal model may also provide a tool for testing compounds created selectively for negative or cognitive symptoms, considering the non-satisfactory patient response to current antipsychotics, This animal model can help to increase our therapeutic power for

example by controlling or better preventing the altered characteristics of the disease in its evolution, rather than focusing only on reverse symptoms.

The time conditional and forebrain–specific knockdown of Stau2 was performed for the first time in a rat and validates the transgenic miRNA technology as an useful tool in neuroscience, in a preferred model, such the rat. These molecular and behavioral data increase our knowledge about the role of mRNA transportation before, during and after a synaptic event and even more underline the importance of this mechanism in memory formation. Further experiment are required to discover Stau2 specific target molecules and the mechanism through which Stau2 protein plays its role in dendritic mRNA localization and local protein synthesis at individual synapses with functional and structural remodeling of synapses during long-term memory.

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