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REGULATIONS OF NEURONAL PLASTICITY BY THE NOGO SYSTEM

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REGULATIONS OF NEURONAL PLASTICITY BY THE NOGO SYSTEM

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my understanding

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vår hjärna har kapacitet att skapa nya minnen under hela livet, så vi kan lära oss och anpassa oss till nya miljöer. Denna förmåga brukar kallas *hjärnans plasticitet*. Plasticiteten beror på att hjärnans nervceller, oftast kallade neuron, har en förmåga att anpassa signaleringen via sina synapser (kopplingarna mellan nervceller) beroende på vilken situation som nervsystemet utsätts för. Neuron i det centrala nervsystemet (hjärna och ryggmärg) har denna plasticitet till trots en mycket begränsad förmåga att växa och återhämta sig om de skadas. Bristen på återhämtning beror delvis på att det finns olika system som begränsar neuronens förmåga att växa. Ett av dessa system kallas för Nogo-systemet. Denna avhandling handlar om Nogo-systemet och dess inverkan på plasticitet.

Den receptor som framförallt är associerad med Nogo-systemet är Nogo receptor 1 (NgR1). Avhandlingens första arbete visar att NgR1 försämrar förmågan att lagra minnen. Det visar också att NgR1 har en inverkan på hjärnans struktur, på såväl synapsernas form och antal som på individuella neurons storlek.

I arbete nummer två konstateras att Nogo-systemet har ett varierat uttryck i hjärnans olika regioner. Uttrycket i regionerna skiljer sig dessutom åt mellan olika faser i livet. Resultatet skulle kunna spegla hur vi har olika lätt att lära och läka under livets gång.

I arbete tre undersöktes om Nogo-systemet också kan bidra till sjukdom. Migrän är en vanlig sjukdom som är associerad till en strukturellt annorlunda hjärna men vi vet inte än vad som orsakar sjukdomen. En genetisk undersökning av flera av Nogo-systemets gener hos 749 migränpatienter påvisade dock att det inte föreligger något samband.

Avhandlingens sista delarbete berör den psykedeliska substansen psilocybin, och dess förmåga att påverka hjärnans plasticitet. På universitet världen över undersöks främst huruvida psilocybin skulle kunna ha en roll i behandlingen av flertalet hitintills svårbehandlade sjukdomar, framförallt psykiatriska tillstånd. Dock finns få studier tillgängliga, och än färre bedrivs avseende psilocybinets inverkan på nivån av individuella neuron och deras funktion. Denna studie påvisar en snabb och robust inverkan av psilocybin på synaptiska markörer. Det talar för en dito inverkan på synaptisk plasticitet.

Sammantaget berör denna avhandling hjärnans plasticitet ur olika perspektiv, från det prekliniska till det kliniska. Resultaten understryker Nogo-systemets betydelse avseende både beteende och hjärnans utformning. Den lyfter även fram psilocybin som en intressant substans att studera vidare gällande vad som styr och påverkar hjärnans oumbärliga plasticitet. Att förstå vad som styr hjärnans plasticitet vore nyckeln till behandling av ett flertal hjärnsjukdomar.

ABSTRACT

Our brain has a lifelong capacity to create new memories, to learn and to adapt. This is due to the ability of neurons to modify their signaling depending on the situation to which the nervous system is exposed. The neurons of the CNS have despite this plasticity, a very limited ability to grow and recover if damaged. The lack of recovery is partly due to the presence of growth inhibitory molecules. This thesis approaches the control of nerve growth plasticity provided by Nogo-type signaling.

Nogo receptor 1 (NgR1) is a key receptor for Nogo, MAG and OMgp. In Paper I, transgenic mice with forebrain overexpression of NgR1 were used. These mice did not differ from controls at baseline when investigating behavior and gross anatomy. However, a series of testing showed that NgR1 overexpression impairs memory consolidation and affects underlying neuronal microanatomy affecting both shape and number of synapses, as well as size of individual neurons.

Paper II reveals that genes involved in Nogo-type signaling have individual and region dependent expression patterns in the brain. The expression of mRNA species differs depending on age, from a plastic youth to a rigid adulthood. The results could reflect how our ability to learn and heal changes over the course of life.

Since Nogo-type signaling is important for a healthy brain, Paper III investigated whether a differently expressed Nogo system might contribute to disease. Strong repeated neuronal activity has been shown to cause lasting changes and this is the hallmark of migraine. While structural changes have indeed been found in migraine patients its pathophysiology remains elusive. Although, when a possible link was investigated between 749 migraine patients and SNPs in key genes contributing to Nogo-type signaling, no correlation between migraine and these genes was found.

Paper IV investigates the psychedelic substance psilocybin, and its ability to affect plasticity. Psilocybin is a serotonin receptor agonist, currently increasingly investigated worldwide. The interest concerns whether psilocybin should have a role in the treatment of illnesses hitherto lacking accurate therapy, e.g. addiction and depression. However, few studies are available or being conducted regarding the effects of psilocybin on neurons and their function. In paper IV, a rapid and robust effect of psilocybin on pre- and postsynaptic markers is demonstrated. This suggests a similar effect on synaptic activity.

In conclusion, this thesis addresses structural synaptic plasticity of the brain from different perspectives, and from preclinical to clinical. It emphasizes the relevance of Nogo-type signaling in terms of both behavior and microstructure of the brain. It also highlights psilocybin as an interesting serotonin receptor agonist to study further, in the quest to unravel what controls and affects our indispensable structural synaptic plasticity. To understand what regulates plasticity would unlock possibilities to treat neurological conditions such as stroke and traumatic brain injuries.

LIST OF SCIENTIFIC PAPERS

I. Karlsson, T.E., **Smedfors, G.**, Brodin, A.T.S., Åberg, E., Mattsson, A., Högbeck, I., Wellfelt, K., Josephson, A., Brené, S., Olson, L. (**2016**). NgR1: A Tunable Sensor Regulating Memory Formation, Synaptic, and Dendritic Plasticity. Cereb. Cortex 26, 1804–1817.

II. **Smedfors, G.**, Olson, L., Karlsson, T.E. (**2018**). A Nogo-Like Signaling Perspective from Birth to Adulthood and in Old Age: Brain Expression Patterns of Ligands, Receptors and Modulators. Front Mol Neurosci 11.

III. **Smedfors, G.,** Liesecke, F., Ran, C., Olson, L., Karlsson, T.E., Carmine Belin, A. (2020). Genetic Screening of Plasticity Regulating Nogo-Type Signaling Genes in Migraine. Brain Sciences 10, 5.

IV. **Smedfors, G.**, Papatziamos Hjelle, C., Horntvedt, O,. Wellfelt, K., Brodin, A., von Kieseritzky, F., Olson, L., Karlsson, T.E. Psilocybin induces rapid and robust increase of pre- and postsynaptic markers. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ADAM22	Disintegrin and metalloproteinase domain-containing protein 22
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
CiC	Cingulate cortex
CNS	Central Nervous System
CSD	Cortical spreading expression
CSF	Cerebrospinal fluid
DMT	N,N-dimethyltryptamine
DOI	4-iodo-2,5-dimethoxyphenylisopropylamine
FAC	Frontal association cortex
GPI	Glycophosphatidylinositol
GWAS	Genome wide association study
ISH	In situ hybridization
LA	Lateral amygdala
LgI1	Leucine-rich, glioma inactivated 1
LOTUS	Lateral olfactory tract usher substance
LSD	Lysergic acid diethylamide
LTP	Long term potentiation
MAF	Minor allele frequency
MAG	Myelin associated glycoprotein
MAP2	Microtubule associated protein 2
MD	Mean diffusivity
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NAc	Nucleus accumbens
NgR1	Nogo receptor 1
OMgp	Oligodendrocyte-myelin glycoprotein
OR	Odds ratio
QC	Quality control
RPM	Revolutions per minute
RT	Room temperature
S1pr2	Sphingosine-1-phosphate receptor 2
SCI	Spinal cord injury
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
TROY	Tumor necrosis factor receptor superfamily, member 19

INTRODUCTION

Throughout life our brain is highly competent to undergo structural changes and modifications. It makes us able to learn, remember and adapt until the very end. This capacity is generally referred to as *neuronal plasticity* and takes place mainly between neurons in the form of altered synaptic connections, for every experience we have. At the same time the brain and spinal cord, the constituents of the *central nervous system* (CNS), are highly restricted when it comes to axon regeneration. The Nobel prize winner Ramón y Cajal demonstrated a century ago the inability of axons to regenerate after injury (Ramón y Cajal, 1928), and still today scientists struggle to induce regeneration of injured CNS axons. A regeneration which is needed for treatments of conditions such as spinal cord injury, stroke and traumatic brain injury.

However, it has long been known that injured CNS axons, given the right environment, are able to regenerate such as when pieces of peripheral nerves are grafted to the brain. Originally described by one of Cajal's students Tello in 1911 (see MacLaren, 1998). How CNS axons can regenerate when put in a peripheral nerve graft, was later demonstrated in more detail (Benfey and Aguayo, 1982; Richardson et al., 1980), and has been used to bridge a complete transection of the rat spinal cord (Cheng et al., 1996). These findings paved the way to investigate the properties of central vs peripheral myelin. Potent growth inhibitory proteins were identified in the oligodendrocytes and central (but not peripheral Schwann cell-derived) myelin, as pioneered by Schwab, who identified Nogo-A together with Caroni (Caroni and Schwab, 1988a, 1988b).

This thesis is aimed to increase understanding of the impact and implications of neuron derived inhibitors in general, regarding their effect on neuronal plasticity, and to study Nogo-type signaling in particular.

NOGO-TYPE SIGNALING: A POTENT INHIBITORY FUNCTION IN THE CNS

Neuronal plasticity is the ability of the nervous system to respond and adapt to internal and external stimuli by rearranging itself. These rearrangements may occur at the molecular, synaptic and cellular level, leading to structural modifications, functional changes and behavioral adjustments (Cramer et al., 2011). The neural growth inhibitory capacity associated with Nogo-type signaling was initially discovered in 1988 when Schwab and colleagues found two CNS-specific protein fractions in myelin which had a negative effect on neuronal outgrowth (Caroni and Schwab, 1988b). The protein fractions were demonstrated to share a functional domain, as both had the ability to bind two different antibodies (Caroni and Schwab, 1988a). The protein was later isolated, cloned and named Nogo-A (Chen et al., 2000). Nogo-A, also known as RTN4, is a member of the reticulon family, thus found in and associated with the shape of the endoplasmic reticulum (Teng and Tang, 2008; Zelenay et al., 2016). The growth inhibitory properties of Nogo-A are however associated with the

transmembrane bound Nogo-A (Chen et al., 2000; GrandPré et al., 2000). Nogo-A has two extracellular domains, Nogo-66 and Nogo-A Δ20, each capable of dysregulatory effects on neurite growth and cell migration (Oertle et al., 2003). Nogo-A executes its inhibition along with other *myelin associated inhibitors* (MAIs), namely *myelin associated glycoprotein* (MAG) (McKerracher et al., 1994) and *Oligodendrocyte-myelin glycoprotein* (OMgp) (Wang et al., 2002a). Among these, Nogo-A has been reported to mediate the strongest inhibition (Cafferty et al., 2010). Unlike MAG only found in myelin, both Nogo-A and OMgp are also found in neurons (Josephson et al., 2001; Wang et al., 2002a). While Nogo-A Δ20 has affinity to bind sphingosine-1-phosphate receptor 2 (S1PR2) and Syndecans (Atwal et al., 2008; Kempf et al., 2014, 2017), all three ligands can bind the primary receptor associated with MAI activity: *Nogo receptor 1* (NgR1). They can also bind paired immunoglobulin-like receptor B (PirB) (McGee and Strittmatter, 2003).

The common receptor of Nogo-type signaling: NgR1

NgR1 was first identified as the receptor for the extracellular loop Nogo-66 on Nogo-A (Fournier et al., 2001) and soon after NgR1 was also linked to MAG and OMgp (Domeniconi et al., 2002; Wang et al., 2002a). NgR1 is a neuron specific, *glycosylphosphatidylinositol* (GPI) linked receptor and lacks an intracellular portion (Wang et al., 2002b). Therefore, co-receptors are needed for transmission of signals into the cell. The first established coreceptors were p75 and *Leucine rich repeat and immunoglobin-like domain-containing protein 1* (Lingo-1), both of which were required for NgR1 activation (Mi et al., 2004). Another pair of proteins were later identified as potential coreceptors for NgR1, *Tumor necrosis factor receptor superfamily, member 19* (Troy) and *Amphoterin-induced protein 3* (Amigo3), which respectively could substitute P75 and Lingo-1 (Ahmed et al., 2013; Shao et al., 2005).

When successful activation of NgR1 occurs by either of the ligands, downstream signaling via the RhoA/ROCK pathway is initiated (Niederöst et al., 2002). This signaling destabilizes the cytoskeleton actin in a Cofilin dependent manner, and this in turn result in collapse of the axonal growth cone (Hsieh et al., 2006; Iobbi et al., 2017; Kellner et al., 2016; Montani et al., 2009).

NgR1 is not limited to myelin derived ligands. It can also be activated by a potent B-cell activator named *B lymphocyte stimulator* (Blys) (Zhang et al., 2009) and by *chondroitin sulphate proteoglycans* (CSPGs) (Dickendesher et al., 2012). CSPGs are extracellular matrix proteins involved in various cell processes and can similarly to MAIs mediated growth inhibition. The two homologous receptors to NgR1 are: NgR2 and NgR3 and their ligands are (so far) MAG (Venkatesh et al., 2005) and CSPGs (Dickendesher et al., 2012) with similar results as NgR1.

Nogo-type signaling inhibitors

NgR1 can not only be activated by the previously mentioned ligands, it can also be antagonized in various ways by molecules that act to allow synaptic changes and alterations. *Ligand leucine-rich glioma inactivated 1* (LGI1) was the first identified NgR1 antagonist, and its inhibitory effect was later found to be potentiated when bound to *Disintegrin and metalloproteinase domain-containing protein 22* ADAM22, a co-receptor to NgR1 which acts to suppress NgR1 signaling (Thomas et al., 2010; VanGuilder Starkey et al., 2013). Another way of inhibiting NgR1 activation is through *Lateral Olfactory Tract Usher Substance* (LOTUS) which blocks Nogo-A from binding to NgR1 (Kurihara et al., 2014; Sato et al., 2011). Olfactomedin 1 acts instead directly on NgR1 by decreasing its binding to its co-receptors thus inhibiting NgR1 signaling (Nakaya et al., 2012). The final way NgR1 signaling is known to be antagonized is through *membrane-type matrix metalloproteinase-3* (MT3 MMP or MMP-16) which cleaves NgR1, thereby shedding it from neurons (Ferraro et al., 2011).

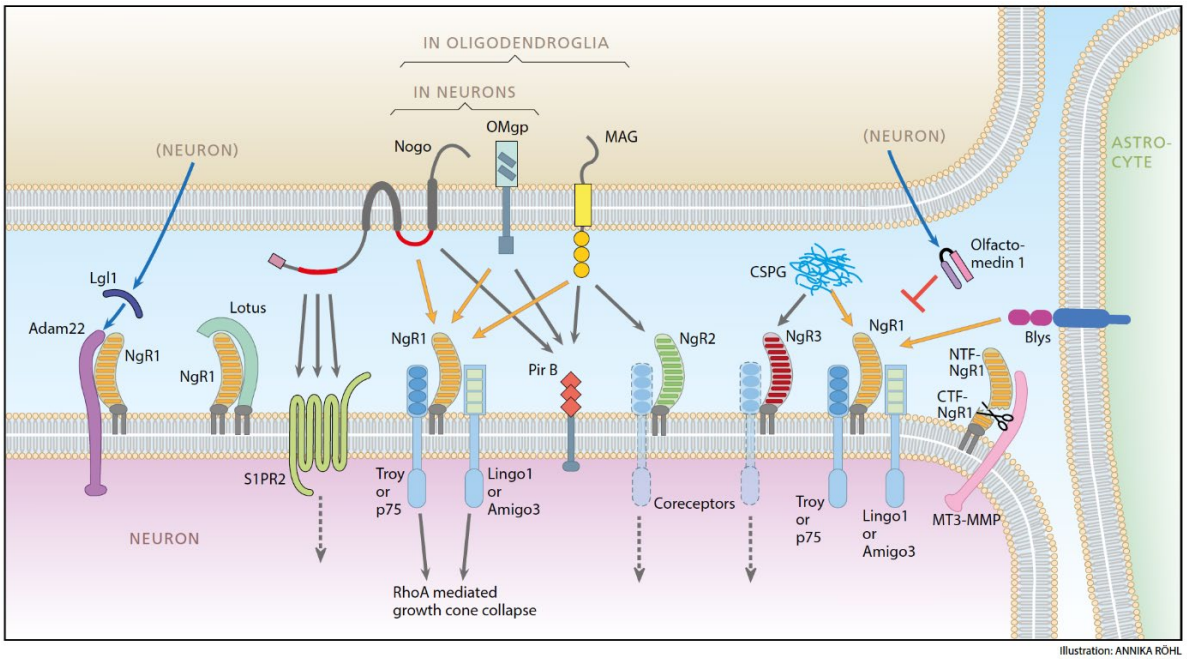

Figure 1: Schematic illustration of Nogo-type signaling. There are three NgR1 ligands, Nogo-A, OMgp and MAG of which Nogo and OMgp are present in both CNS myelin and neurons, while MAG is present only in myelin. NgR1 and PirB both serve as receptors for the three ligands. Ligands for the homologous Nogo receptors NgR2 and NgR3 are MAG and CSPG, respectively. NgR1 has coreceptors, Lingo-1, p75 Troy and Amigo3. ADAM22, LgI1, Lotus, Olfactomedin-1 and MT3-MMP are NgR1 antagonists. Illustrated is also that Nogo-A has a second binding sequence, proposed to bind to Sphingosine 1 phosphate receptor 2 (S1PR2). Illustration by Annica Röhl presented in (Karlsson et al., 2017).

Regional expression as a mean to unravel function

The brain is composed of a large number of functional regions which communicate in an intricate, and so far, incompletely known manner. The geographical distribution of specific proteins may imply regulatory effects on regional functions. Experiments regarding individual as well as clusters of Nogo-type signaling genes have revealed specific expression patterns (Barrette et al., 2007; Habib et al., 1998; Haybaeck et al., 2012; Hunt et al., 2003; Josephson et al., 2001, 2002; Kumari and Thakur, 2014; Tozaki et al., 2002). Available data suggests that the expression patterns do not always correspond to known interactions between Nogo-related molecules, thus the full range of implications for each protein has probably not been found.

The mentioned studies reveal a broad expression for NgR1 mRNA throughout the two brain hemispheres, but limited expression in the brainstem, and interestingly, no detectable NgR1 mRNA in striatum (Yager et al., 2015). NgR2 generally follows the expression of NgR1 except being expressed in striatum. NgR3 remains elusive due to few studies in the matter. Nogo-A is widely expressed in myelin throughout life, with tendencies to decline with age in areas less associated with plastic changes (Huber et al., 2002; Hunt et al., 2003; Josephson et al., 2001; Mingorance et al., 2004). OMgp demonstrates a general increase after myelination to later decrease with age (Vourc'h et al., 2003). MAG is not specific to the CNS and appears earlier in the PNS arguing for an earlier myelination peripherally (Inuzuka et al., 1991). The co-receptor Lingo-1 has a more abundant expression in the CNS than NgR1 and P75 (Lee et al., 2007). Troy and P75 have demonstrated a generally scarce expression throughout the CNS. They were detectable in parts of hippocampus, cerebellum, and midbrain. P75 was also detected in striatum (Barrette et al., 2007). LgI1 has been found, throughout cortex in cerebellum (Irani et al., 2010) and in company with ADAM22, having decreasing levels in hippocampus over time (VanGuilder Starkey et al., 2013). Olfactomedin is highly expressed in cortex, the olfactory bulb and hippocampus (Nakaya et al., 2012). Notably, there has been a broad interest in localizing Nogo-type signaling molecules, while a collected picture of expression patterns over time has been lacking.

Nogo-type signaling and plasticity

NgR1 has a profound expression in the CNS at both pre- and postsynaptic sites in areas associated with plasticity, such as the cerebral cortex (Barrette et al., 2007; Josephson et al., 2002; Lee et al., 2008), It has been demonstrated how activity causes a temporal downregulation of NgR1 (Josephson et al., 2003; Karlsson et al., 2017; Wills et al., 2012). This downregulation is considered a window for structural plastic changes.

Experience driven plasticity

Vision is one of our primary senses and functional plasticity was initially associated with Nogo-type signaling by experiments investigating the visual cortex. Sensory input robustly impact neuronal connections in the young brain, and it has for long been known that there is a critical period in early life, when closing of one eye results in a shift of ocular dominance

towards the open eye (Wiesel and Hubel, 1963). This is the same plastic window utilized among children who present with strabismus and are administered a patch over one eye for a period of time to correct it (Daw, 1998). Importantly the ocular dominance shift has however been identified as prolonged when mice are lacking the receptor PirB (Syken et al., 2006) or the main ligand NgR1. NgR1 knockout mice even kept their critical window into adulthood, (McGee et al., 2005).

Nogo-type signaling and structural plasticity

The support for Nogo-type signaling as an important contributor to the plastic response in the healthy CNS is multifaceted. It was early demonstrated that blocking of Nogo-A with antibodies resulted in increased axonal sprouting. That sprouting appeared after two days on adult Purkinje cells, it peaked after almost a week and was almost reversed after a month (Buffo et al., 2000). These results were supported when neurons treated with a Nogo-A blocking antibody was shown to induce prominent alteration in the dendrite structure of hippocampal pyramidal neurons. The Nogo-A neutralization also shifted spine types toward more immature phenotypes, without affecting spine density. Axonal complexity and length were also greatly increased (Zagrebelsky et al., 2010). Later studies have demonstrated somewhat inconsistent results. Transfected hippocampal cultures where NgR1 expression was reduced, increased dendritic spine density while overexpressing NgR1 reduced spine density by half (Wills et al., 2012). In vivo experiments however, have also found a shift in spine type distribution, but not identified differences in spine density, neither in NgR1 overexpressing mice nor in NgR1 knockout mice (Akbik et al., 2013; Karlén et al., 2009; Lee et al., 2008; Park et al., 2014). Spine turnover is a measure of plasticity and it occurs at a higher rate in the juvenile brain. Spine turnover in NgR1 knockout mice has both been demonstrated to increase (Akbik et al., 2013; Zemmar et al., 2018), but also to be at the same level as controls (Park et al., 2014). An important aspect regarding the importance of Nogo-type signaling for structural plasticity, was that NgR1 knockout mice had a higher expression of *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid* (AMPA) receptor (Jitsuki et al., 2016). AMPA receptors mediate excitatory synaptic transmission and they are critical for lasting synaptic changes (Malinow, 2003). The higher expression of AMPA receptors in NgR1 knockout mice thus imply an increased plastic potential. Nogo-A was later demonstrated to decrease the number of AMPA receptors (Kellner et al., 2016) in addition to the known plasticity inhibitory signaling (Fricke et al., 2019)

Broad implications of myelin associated inhibitors in the diseased CNS

A healthy CNS relies on a balanced level of plasticity to adequately wire and rewire neuronal connections. As the number of molecules associated with Nogo-type signaling keeps increasing, so does the diseases and conditions linked to their actions. The first aspect to receive careful attention was if blocking Nogo-type signaling would enhance axonal repair after *spinal cord injuries* (SCI). In an initial paper significant growth was identified in SCI mice lacking NgR1 (Kim et al., 2004). Later, antibody blockage of Nogo-A signaling was reported to have positive effects on physical exercise after SCI in rats. This was true both regarding functional recovery measured by gait and structural recovery by measuring corticospinal tract sprouting distal to the injury (Chen et al., 2017). Improved recovery after stroke has also been observed in mice where NgR1 signaling was blocked with an antagonist (Sozmen et al., 2016), and a reduction in dying cells was recently demonstrated in rats suffering cerebral infarction after suppressing the Nogo-A/NgR1/RhoA signaling pathway (Xie et al., 2020).

Pathological Nogo-type signaling has over the years been associated with epilepsy, Parkinson's disease, cancer, amyotrophic lateral sclerosis and schizophrenia (Amy et al., 2015; Budel et al., 2008; Dazzo et al., 2016; Fukata et al., 2010; Schawkat et al., 2015; Seiler et al., 2016; Thomas et al., 2016). Nogo-A has been mentioned as a prognostic marker for gastric cancer (Chi et al., 2015) more specifically, Nogo-B has been linked to the induction of cervical cancer metastasis (Xiao et al., 2013). Nogo-A - NgR1 signaling has on the other hand been mentioned to potentially restrict the migration of tumor cells in human astrocytomas (Xiong et al., 2012). Among these conditions, the connections to schizophrenia is arguably stronger, as microstructural changes of myelin have been identified among these patients and as NgR1 is located in the same genetic region as a known locus highly linked to schizophrenia (Willi and Schwab, 2013).

Multiple components in Nogo-type signaling have been associated with *multiple sclerosis* (MS). Both Nogo-A and NgR1 are expressed in MS lesions (Satoh et al., 2005) and levels of LOTUS in cerebrospinal fluid (CSF) have been associated with disease activity in MS patients (Takahashi et al., 2018). Lower levels of LOTUS were identified in relapse patients and secondary progressive MS, indicating a larger possibility for Nogo-type signaling to occur (Takahashi et al., 2015). LINGO-1 has also been associated with negative regulation of remyelination and LINGO-1 has thus been identified as an interesting target for MS treatment. A phase 2 study investigating the efficiency of LINGO-1 antibodies in relapsing multiple sclerosis did however so far not result in a significant dose-linear improvement (Cadavid et al., 2019).

MEMORY

Our memory is what makes us who we are. It sublimely guides us through life, allowing the ability to ride a bike, follow and dynamically contribute to an ongoing discussion, and allows us to remember what happened twenty years ago. The main categories of memory are our working, declarative, and procedural memories. The working memory has a short endurance and capacity and will not engage processes which manifests long-term memories. Declarative and procedural memories are both long-term memories, where declarative memory is one's collection of facts and experiences while procedural memory is our motor skills and learnt procedures (Squire and Wixted, 2011).

Memory consolidation

The theory of memory consolidation derives from the first proposal of change in synaptic strength in neural circuits suggested by Cajal, in the beginning of the last century (Ramón y Cajal, 1909). It took almost half a century before Donald Hebb presented his theory on how memories are results of synaptic plasticity. He proposed that when a presynaptic cell repeatedly excite a postsynaptic one, the connection stabilizes and becomes stronger, thus, this improved connection becomes a carrier of parts of a memory (Hebb, 1949). These theories were experimentally supported in 1973 when *long term potentiation* (LTP) first was described (Bliss and Lomo, 1973). The long lasting increase in synaptic strength associated with LTP is still considered a key underlying mechanism in memory formation (Lisman, 2017; Morris, 2003). As LTP is synapse specific, any of the 10.000 synapses on an average neuron can be individually modified (Matsuzaki et al., 2004). However LTP does not always lead to lasting memories, and lasting memories can form without preceding LTP (Meiri et al., 1998). Several other molecules have been identified as important for memory consolidation, but in addition to LTP, only Calcium-Calmodulin Protein Kinase type II is known to be critical to memory storage (Lisman, 2017).

The substrate for memory consolidation appears at the level of synapses, but the location and storage of memories in the brain have been hard to pinpoint. An important piece of the puzzle came from a clinical case. Henry Molaison was an epileptic patient who underwent medial temporal lobe resection to reduce the severity of his attacks. Following surgery, the number of attacks drastically decreased while working memory and IQ were intact. However, his declarative memory was drastically diminished, and he suffered from severe anterograde amnesia until his death. Furthermore, his memories for several years before the surgery were also affected while earlier memories remained intact, clearly suggesting that the final storage location for lasting memories is not in hippocampus (Dossani et al., 2015). Rather, hippocampus appears critical for learning and hippocampus has been demonstrated to change in structure when we learn. This was originally suggested when it was shown that London taxi drivers had larger posterior hippocampi than bus drivers (Maguire et al., 2006). In another study Draganski et al tested medical students and could show that as students studied intensely before a test, their hippocampi increased in size compared to a control group of students who were not studying for an exam (Draganski et al., 2006).

Dendritic spines are correlated to memories

Dendritic spines are postsynaptic protrusions, able to change size, shape, and number depending on the level of stimulation (Bhatt et al., 2009; Engert and Bonhoeffer, 1999). In morphological studies in mice, learning has been shown to be strongly correlated to plastic changes in the neuropil (Lesburguères et al., 2011). In a seminal study, Yang et al showed that when mice learnt persistent memories, there was a significant correlation between their performance and the number of new spines that was formed (Yang et al., 2009). Hayashi-Takagi et al (Hayashi-Takagi et al., 2015) followed up on this study with a protocol where transgenic mice learnt a similar procedural memory, but in which light enabled specific destabilization of the newly formed spines. Destabilizing the spines before retrieval of the memory, resulted in the mice losing the memory. LTP has been shown to increase the number of dendritic spines (Engert and Bonhoeffer, 1999; Toni et al., 1999) while long term depression correspondingly causes shrinkage of dendritic spines (Zhou et al., 2004).

Structural plasticity in response to drug sensitization

One of our most robust forms of memory is addiction. Structural plasticity is correlated to its development (Li et al., 2004; Robinson and Becker, 1986; Russo et al., 2010). When animals are subjected to a repeated exposure of a neuro-stimulatory drug, they experience sensitization. Sensitization is an increasingly stronger response to the same administered dose over time, most often measured by increased locomotion (Robinson, 2014). Cocaine is a prime example of a highly potent neuro-excitatory drug which is known to initiate sensitization upon repeated exposure, and apart from behavioral differences, this sensitization is also associated with an increase in the number and density of dendritic spines in drug related areas such as nucleus accumbens (Li et al., 2004).

The memory as an engram

Neural correlates of memory are referred to as engrams (Josselyn and Tonegawa, 2020), a term initially stipulated a century ago by Richard Semon (Semon, 1921). The engram was described as the phenomenon where a group of cells are changed upon stimulation of an external stimulus, and upon reactivation of these cells one retrieves this stimulus, hence remember the experience. Today consensus is that memories are multivariate phenomena involving several regions in the brain, thus the engram is the entire trace of actions and connections which underlie the memory and its potential to be retrieved (Poo et al., 2016). As previously mentioned, memories have been shown to require hippocampus for their formation, but their final location and how they end up there have remained elusive (Marr, 1971). Tonegava's group showed that neurons become activated in the frontal association cortex on the very first day of learning. Although, these neurons require up to two weeks of maturation before they can drive the memory without help from hippocampus (Kitamura et al., 2017). It is thus clear that learning is a process that requires dynamic signaling for structural changes to occur concurrently in several different brain regions. Support for the engram has also been demonstrated by stimulating a subset of hippocampal neurons that were

active during memory consolidation, and through this induce artificial retrieval of a memory (Frankland et al., 2019). Thus, as the brain is activated in response to a memory inducing event, an opportunity for structural changes is required.

Suitably, it has been shown that strong neuronal activity down-regulates mRNA levels of NgR1 (Josephson et al., 2003), presumably to increase the plastic abilities of the nervous system. Based on this hypothesis, Nogo-type signaling functions as a regulator of activity induced plastic changes and may act as a gatekeeper for lasting memories. To test this hypothesis, a mouse that overexpress NgR1 in forebrain neurons was generated. The genetically modified mice have normal LTP and 24 h memory as shown by their ability to learn the Morris water maze as well as controls. However, when retested after 60 days it was shown that their lasting memories were significantly impaired (Karlén et al., 2009). Works from other labs have conversely shown that lack of NgR1 results in increased plasticity as shown by the monocular deprivation test (McGee 2005). Nogo-A has later been shown important for spatial learning as well as capable to regulate spine plasticity (Zagrebelsky et al., 2017).

Future perspectives on the engram

While significant progress in the understanding of memory has been made, key questions remain. How and why are specific neurons chosen for the destination of a memory? How are optimal synapses equipped to consolidate memories? What decides which information to store? As single cells can be activated by an image (Quiroga et al., 2005), how many memories can be associated with one neuron, and how many neurons can be associated with one memory? Are there genes we can isolate which correlate to memory-performance? Is Nogo-type signaling a primary effector of memory consolidation? With answers to these and further questions, we will be able to better fight the detrimental diseases related to memory-deficits.

STRUCTURAL PLASTICITY IN RESPONSE TO PSILOCYBIN

Neuronal activity is known to rearrange and affect the function of neuronal circuits in the brain (Ganguly and Poo, 2013). Such plasticity promoting capacity is associated with upregulation of neurotrophins, e.g. *brain-derived neurotrophic factor* (BDNF) (Humpel et al., 1993; McAllister et al., 1999), and downregulation of plasticity opposing signaling (Josephson et al., 2003; Karlén et al., 2009). and also with modulatory neurotransmitters such as serotonin (Kraus et al., 2017). Classic psychedelic compounds (Figure 2) such as psilocybin, *lysergic acid diethylamide* (LSD), mescaline, *N,N-dimethyltryptamine* (DMT) and *4-iodo-2,5-dimethoxyphenylisopropylamine* (DOI) have individual pharmacological properties, but all involve signaling through serotonergic receptors (Nichols, 2016). LSD has previously been associated with increased activity of immediate early genes such as Arc and c-Fos (Nichols and Sanders-Bush, 2002), The increase of Arc was especially strong in tissue from prefrontal cortex where it increased fivefold. C-Fos increased twofold in prefrontal cortex, hippocampus and midbrain. Stimulation of serotonergic receptors via DOI affects BDNF in a region specific manner, with a robust increase in parietal cortex and decrease in the dentate gyrus (Vaidya et al., 1997). Positive effects on neurite- as well as spine sprouting were also recently shown to be induced in cultured cortical neurons by a number of psychedelic substances, with the most pronounced effects seen with LSD (Ly et al., 2018). Taken together, these observations strongly suggest that psychedelic compounds can have plasticity evoking effects.

One of the substances investigated was psilocin, the biologically active metabolite of psilocybin (Passie et al., 2002). Psilocybin was originally isolated from the Central American mushroom *Psilocybe Mexicana* and its structure determined by independent chemical synthesis by Albert Hoffman in 1958 (Passie et al., 2002). It is an unspecific serotonin agonist (Halberstadt and Geyer, 2011; McKenna et al., 1990), where signaling through the 5-HT2A receptor is believed to be responsible for hallucinogenesis (Nichols, 2004). In line with the other classic psychedelic substances, psilocybin has a favorable safety profile, including non-addictive properties and a large margin of exposure compared to most CNS-active drugs (Amsterdam et al., 2011; Brown et al., 2017; Moreno et al., 2006; Nichols, 2004).

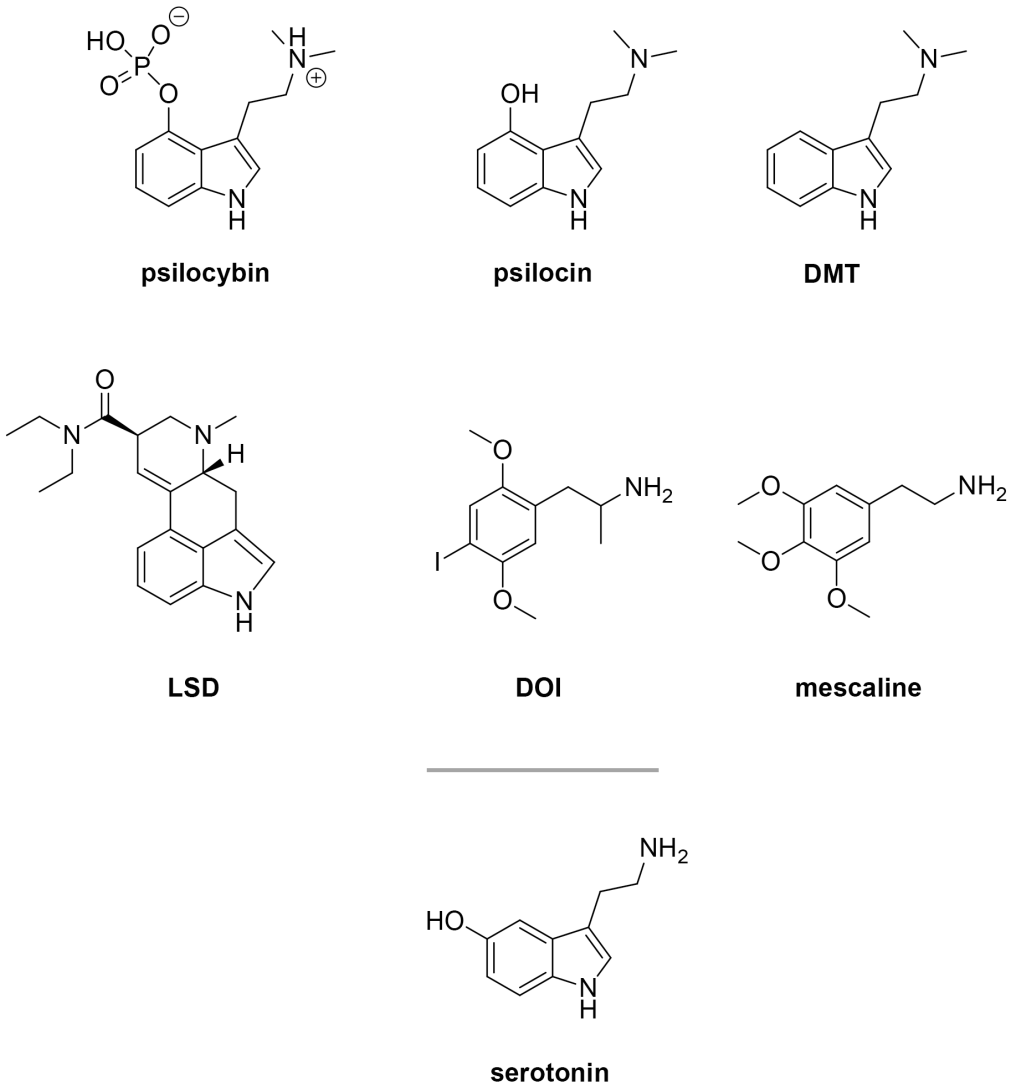

Figure 2: Chemical structures of classic psychedelic compounds (psilocybin, psilocin, DMT, LSD, DOI, mescaline) and the endogenous neurotransmitter serotonin. Illustration with permission from Fredrik von Kieseritzky.

Psilocybin is currently being widely investigated and evidence is accumulating that this substance could play a role in the treatment of several common CNS disorders. For example, several ongoing clinical trials test the effects of Psilocybin in depression and addiction (Nichols et al., 2017). Also the excruciating and utterly resilient pain associated with cluster headache has emerged as a potential target for psilocybin therapy (Schindler et al., 2015; Sewell et al., 2006). Yet, little is known about the mechanistic effects of psilocybin, and its long lasting effects after a few or a single administration (Barrett et al., 2020; Bogenschutz et al., 2015; Carhart-Harris et al., 2016; Griffiths et al., 2016). Psilocybin has previously been shown to have a dose-dependent effect on hippocampal neurogenesis (Catlow et al., 2013), but it is also important to investigate plasticity enhancing properties at the synaptic level related to psilocybin. Such endeavors will both broaden pharmacodynamic understandings and guide clinical research – and, ultimately, be imperative for future drug discovery.

STRUCTURAL PLASTICITY AND DISEASE

Migraine is a very common disease and affects about 15 % of the population world-wide (Vos et al., 2017)**.** Migraine is a chronic disease with an individual frequency of headache episodes, varying from a few episodes per year to several per week. The episodes normally last up to 72 h and can for some individuals be triggered by external stimuli and/or foods (Rizzoli and Mullally, 2017). The International Classification of Headache Disorders has formulated the criteria underlying the diagnosis (Box 1) (IHS, 2018).

The migraine attack has different phases of which the initial phase known as the "aura" is found among a third of migraine patients (Goadsby, 2012). The migraine aura precedes each or some migraine attacks (Rossi et al., 2005). The aura most frequently involves blurred vision in the form of scintillating scotomas. These normally subside after 30 minutes to be replaced by an increasingly severe headache. The aura can also involve other sensory, language or brainstem disturbances (Dodick, 2018). The migraine aura is since the 1940's associated with *cortical spreading depression* (CSD) (Charles and Baca, 2013). CSD is a slowly spread depolarization which results in suppression of brain activity. It is thought that CSD might contribute to the pain alongside trigeminal nerve fiber activation which together results in vasodilatation and inflammation throughout the brain (Akerman et al., 2017). The full pathophysiology of migraine is however not known.

Box 1: Migraine diagnostic criteria according to ICHD-3

A: At least 5 attacks fulfilling criteria B - D

B: Headache attacks lasting 4-72 h (untreated or unsuccessfully treated)

C: Headache has at least 2 of the following four characteristics:

1: unilateral location

2: pulsating quality

3: moderate or severe pain intensity

4: aggravation by or causing avoidance of routine physical activity (eg, walking or climbing stairs)

D: During headache at least one of the following:

1: nausea and/or vomiting

2: photophobia and phonophobia

E: Not better accounted for by another ICHD-3 diagnosis.

The migraine brain has been associated with structural differences in comparison to the healthy brain, both regarding white and gray matter. It is consistent in the literature that the migraine brain appears structurally different, but the exact regions and to what extent they are affected is not (Coppola et al., 2017; Lovati et al., 2016a; Maleki et al., 2013; Messina et al.,

2018; Neeb et al., 2017; Soheili-Nezhad et al., 2019; Zhang et al., 2017). Arguably could the many faces of migraine, with different intensity, frequency etc., be part of the explanation why the migraine brain can be structured in different ways. This is supported as attack frequency has been correlated to the level of structural deviance (Lai et al., 2015a; Lovati et al., 2016b). Structural plasticity has also been recognized to fluctuate in relation to migraine attacks (Coppola, 2015).

Genetic studies identified migraine as both mono- and polygenic due to its many subtypes (Sutherland and Griffiths, 2017). The most recent *genome wide association study* (GWAS) identified a series of genes with some potential contributory effects (Gormley et al., 2016), but none which so far substantially increased the understanding of the disease. This does not however indicate genetic studies as redundant; rather, GWAS studies include multiple testing and can identify larger effects. For a complex disease like migraine, many smaller effects are probably contributing to the disease. These small effects can be lost in the GWAS setting.

Thus, instead of blindly fishing for candidate genes genome wide, identified abnormalities could instead be approached. The structural deviations in the migraine brain could either be a result of the disease or a predisposing phenomenon. In either case, potent regulation of neuronal circuitry does occur. Whether Nogo-type signaling contributes to the structural changes in the migraine brain is probable. If Nogo-type signaling is differently expressed in migraine patients remains unknown.

AIMS

The general aim of this thesis was to increase our understanding of the impact and implications of Nogo-type signaling on neuronal plasticity.

The specific aims were:

Paper I: To determine if an innate overexpression of NgR1 impairs the formation of lasting memories and affects the underlying structural plasticity.

Paper II: To investigate the regional expression of ligands, receptors, co-receptors and modulators involved in Nogo-type signaling, from the early postnatal to the mature brain.

Paper III: To investigate a potential role of Nogo-type signaling in the migraine brain.

Paper IV: To investigate the response of psilocybin on pre- and postsynaptic markers.

MATERIAL AND METHODS

To carry out the aims of this thesis, i.e., to expand the knowledge about Nogo-type signaling, several methods were used depending on the different samples that were investigated. In each subsection information about methods used in the chosen experiments building this thesis is provided, in the order of appearance in papers I - IV.

MATERIAL

Animal models

All animal experiments were performed with approval from the local ethics board, Stockholms Norra Djurförsöksetiska nämnd. Mice were housed in animal facilities at Karolinska Institutet. For publication 1 and 2, mice were housed at the Department of Neuroscience. Animals for experiments in later publications were housed in the Comparative Medicine Biomedicum facility. Mice lived under standardized conditions in cages with a maximum of five mice, with water and food *ad libitum*. A paper house and paper tissues were available for nesting. Lights were on a 12h hour alternating on/off cycle, representing day and night. Temperature was kept at 22-23 °C and the relative humidity was set to 60%.

In paper 1 we used three genetically different mice, NgR1 −/−, NgR1 overexpressing mice and naïve controls. In paper II and IV we only used naïve C57BL6 mice.

NgR1 –/–

The NgR1 knockout mice used in paper 1 were a kind gift from Dr. Marc Tessier-Lavigne (Genentech,USA) and was previously described (Zheng et al., 2005).

Innate overexpression of NgR1 in forebrain neurons, NgR1 +/+

In paper I, a bitransgenic mouse with an innate overexpression of NgR1 in forebrain neurons was used. It was previously created by mating a mouse with a CamKII promoter and a tetracycline transactivator (Jackson laboratories) with a mouse carrying a miniCMV promoter followed by the tetracycline transactivator responsive element and a NgR1 gene (Karlén et al., 2009). This resulted in mice carrying two transgenes: the transactivator and the NgR transgene construct (Figure 3 lower left box). The tetracycline transactivator leaves the possibility to turn off the NgR-overexpression by adding Doxycyclin to the drinking water (Figure 3 red symbol in lower right box). As turning off the overexpression has been shown to impact memory formation in a reversible manner these mice were named MemoFlex (Karlén et al., 2009). As the mice in paper I were not put under a doxycycline regimen, they will further on be referred to as NgR1 overexpressing mice.

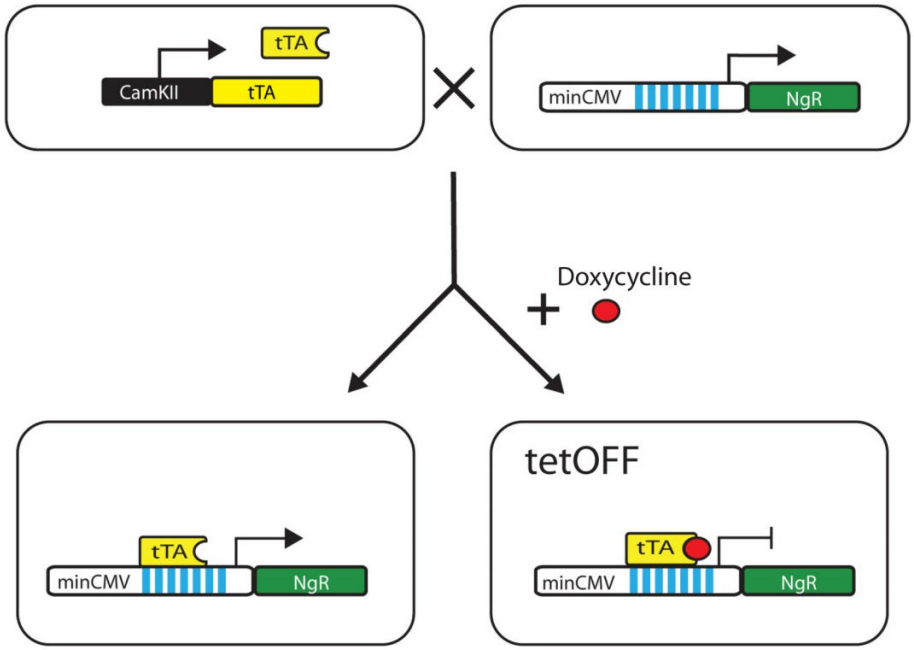

Figure 3: Schematic illustration of strategy used to obtain mice with inducible (tet-off) expression of a NgR1 transgene in forebrain neurons. Illustration from Karlén et al 2009.

Swedish twin registry for genetic association study

For the migraine association study in paper number 3, we used a cohort from the unique Swedish twin registry (https://ki.se/forskning/svenska-tvillingregistret). The whole registry contains information and data from 85,000 pairs of mono and dizygotic twins (Lichtenstein et al., 2006). Our cohort consisted of 4781 individuals, 4032 controls and 749 cases of migraine based on the ICHD II (IHS, 2004). The individuals had responded to a lifestyle and health questionnaire and had donated blood from which DNA was obtained with the Puregene extraction kit. Genotyping was done on the Illumina HumanOmniExpress 12 v1.1 chip at the SNP&SEQ Technology Platform, Uppsala University. *Quality control* (QC) of the material did not motivate removal of any subjects. QC included missing genotype rate per person <0.1, missing genotype rate per *single nucleotide polymorphism* (SNP) <0.1, *minor allele frequency* (MAF) <0.01 and Hardy–Weinberg equilibrium (1×10^{-6} for controls and 1×10^{-10} for cases) (Marees et al., 2018).

METHODS

Memory tests

Novel object recognition

Novelty recognition is considered a potent test of memory and when performed in the classical way as described below, parietal and frontal cerebral cortex as well as hippocampal functions are tested (Antunes and Biala, 2012).

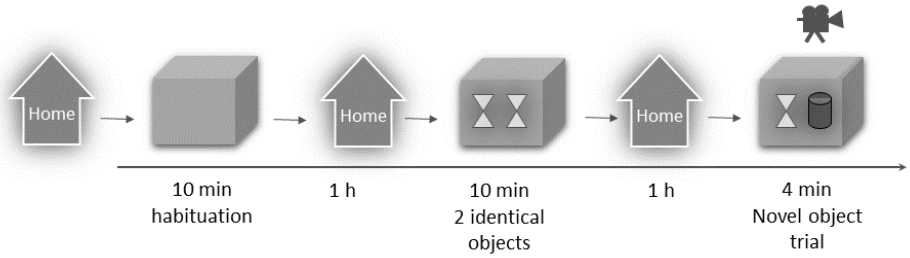

Figure 4: Timeline of Novel object recognition task

The experiment (Figure 4) was initiated with 10 min of habituation in 42 x 42 cm boxes after which mice were returned to their home cages. One hour later, mice were put back in the box for 10 min for familiarization with 2 identical objects. Following this, they were again returned to their home cage for 1 h and then returned for the novel object phase. During the novel object phase one of the previous two identical objects had been replaced by an unfamiliar one. To evaluate mouse behavior, digital recordings were made. These films were analyzed by an individual blinded to genotype and novelty of the objects. The time mice spent investigating each object was measured and the preference for the objects was scored during 4 min.

Barnes maze

Barnes maze (Barnes, 1979) is a dry version of Morris maze described below. The maze is a test of visuospatial capacity and it is considered to depend on hippocampal function. The experiment was housed in a brightly lit room where a circular table measuring 1.25 m in diameter was placed. The board has 36 holes along the outer perimeter and the task for the mouse was to find the one hole that allowed the mouse to escape the light into a small dark compartment. Mice were trained 4 times per day for 5 days (180 s per day). For the probe trial on day six, the escape compartment had been removed. The memory of the escape hole was estimated as the proportion of pokes into the previous escape hole and the 2 closest neighboring holes. Mice that did not move significantly were excluded.

Morris water maze

This classic test was used to assess spatial memory (Morris, 1984). It consists of a 1.8m circular pool with a submerged hidden escape platform. Mice were put into the pool at 4 different starting positions (that varied semi-randomly) and the distance mice swam before

reaching the target was digitally analyzed. Mice were trained with 4 trials per day for 7 days and on the 8th day a probe trial without a platform was performed. In searching for the removed platform, the average distance to its previous position is considered a measurement of their memory. The test for presence and strength of a lasting memory was performed after 45 days and consisted of a second probe trial. Mice that did not engage in swimming "floaters" were removed from the experiment.

Locomotor and drug sensitization tests

Rotarod

Motor learning is effectively evaluated in the Rotarod test (Jones and Roberts, 1968) and it reflects function in the basal ganglia, premotor and motor cortex as well as cerebellum (Hikosaka et al., 2002). During five consecutive days, mice were trained 4 times per day on a Rotarod with an allowance of 40 min inter-trial rest. The mouse ran on the accelerating rod starting at the speed of 4 and reaching 80 *revolutions per minute* (RPM) in 7.5 minutes. The RPM which made the mouse fall off was recorded. In the situation when a mouse was hanging on the rod without running, it was lightly touched to recommence running. If running was not continued the RPM which was noted at the cessation of running was recorded.

Cocaine sensitization paradigm

Naïve mice that are repeatedly given the same dose of a psychostimulant will typically respond with stronger locomotor activity over time. This phenomenon is referred to as sensitization, and it is strongly associated with increases in spine density in nucleus accumbens and several other brain regions (Li et al., 2004; Robinson and Kolb, 2004).

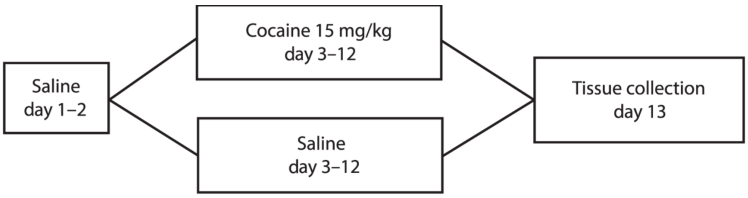

Figure 5: Timeline of sensitization paradigm

After three days of handling mice to make them familiar with the investigator, the baseline locomotor activity in response to intraperitoneal injections of saline was registered for two days (Figure 5). The locomotor registration was done in individual plastic chambers measuring 42 x 42 cm (AccuScan Instruments, OH, USA) that automatically measured activity parameters in X, Y and Z axis during 1.5 h. The movements registered were locomotion and stereotype-like behavior (measured as repeated crossings of the same light beam as occurring during grooming, head bobbing, etc.). Mice were randomized into equally big groups receiving either injections of saline or cocaine (15 mg/kg) followed by registrations of behavior for ten days. The amount of locomotion was used as a measure of the strength of sensitization and development of stereotypies. The experimenter was blinded regarding genotype until the final data were analyzed.

Analysis of structural plasticity in response to cocaine

To study dendrites and dendritic spines in paper I, mice were sacrificed 24 h after the last injection of saline or cocaine. Tissue was cryo-sectioned at −25°C (Microm HM500M; Microm HM560; Thermo Scientific) and coronal 160 µm sections were mounted on coated slides. Tissue was then processed using the rapid Golgi stain technique according to the manufacturer's manual (FD rapid GolgiStain Kit, FD NeuroTechnologies, Ellicott City, MD, USA). Spine counts and dendritic branching were obtained using a dedicated analysis system (NeuroLucida MBF Bioscience, VT, USA). Spines were analyzed in nucleus accumbens due to its involvement in reward behavior (Robinson and Kolb, 2004), in frontal association cortex due to its role in decision making (Kennerley and Walton, 2011) and to take the limbic system into account, the anterior cingulate cortex was included.

Neurons suitable for analysis were randomly selected in the areas of interest with soma and dendritic trees contained in the section, and without disturbance from other close Golgi-stained cells. Dendritic spines were analyzed on distal branches, (≥fourth order dendrites) by light microscopy (Zeiss Axio Imager M2 light microscope, Carl Zeiss Microscopy, Germany) and dedicated software (Neurolucida). Spines were quantified and morphologically categorized as filopodia (clearly more than 2 times as long as wide), thin (approximately twice as long as wide), or mushroom (a head at least 2 times wider than the neck) types. The filopodia-type spines were too few to allow sufficient power for further analysis and were therefore excluded. Approximately 8 neurons were counted per brain and area. The analysis was blinded to genotype and drug treatment. A total of 77,452 spines from 1115 neurons were counted and classified (frontal association cortex: 368 neurons, cingulate cortex: 363 neurons, nucleus accumbens: 384 neurons, used to analyze 4 groups with 12 animals in each group). All counting and classification of spines was carried out by one trained individual.

The Sholl analysis principle (Sholl, 1953) was applied to the same neurons as for dendritic spine analysis using the same software (Neurolucida). The Sholl analysis was used to plot the dendritic complexity at different distances from the soma of the neurons. The total dendrite length, the number of dendrite endings, and the distribution of different dendrite lengths.

Ex Vivo Magnetic Resonance Imaging (MRI)

As the overexpression of NgR1 previously has been associated with difference in the capacity to store lasting memories (Karlén et al., 2009; Karlsson et al., 2013), the possibility of a structural difference in the underlying brain anatomy was investigated in these mice. To do this, brains from mice overexpressing NgR1 and controls were imaged in a horizontal 9.4 Tesla magnetic resonance scanner (Agilent, Yarnton, UK) equipped with a birdcage coil (16 mm inner diameter, Rapid Biomed, Rimpar, Germany). This was to investigate cortical thickness in five regions, the volume of four major brain regions and the volume of three white matter tracts. Fractional anisotropy (FA) which reveals the uniformity of axonal tracts and mean diffusivity (MD) was also used to reveal diffusion patterns in the tissue which can reveal information about the tissue architecture.

Data were collected from formalin fixed brains. Brains were positioned in syringes filled with Fomblin (Solvay Solexis, Italy) to avoid image artifacts which easily arise when tissue is removed from the skull and the CSF (Shatil et al., 2016). A diffusion-weighted spin echo with diffusion-weighted gradients applied in 30 different directions, as well as a reference image with the diffusion encoding gradients set to zero, was used (TR = 2.1 s, TE = 20.65 ms, NEX = 4, matrix = 192 × 128, field of view = 19.2 × 19.2 mm2, 50 contiguous 0.3-mm-thick slices). The data were zero-filled to 256 × 256 points before Fourier transformation. Images were analyzed (ImageJ, NIH) by a person blinded to the genotype.

In situ hybridization

Paper II was designed to better understand how a system that potently impacts plasticity behaves during phases in life associated with various levels of CNS stability. Using *in situ hybridization* (ISH) mRNA expression of 11 genes related to Nogo-type signaling was quantified in 18 relevant anatomical regions at 6 different ages (P0, 1 w, 2 w, 4 w, 14 w and 2 years (104 w)).

The analyses were made in tissue from naïve C57BL6 mice sacrificed by decapitation. Fresh frozen tissue was sectioned using a cryostat (Microm HM500M; Microm HM560; Thermo Scientific). Serial coronal sections of 14 µm were mounted on coated slides (VWR Superfrost Plus Micro Slide). Every 10^{th} sectioned was chosen for the analysis of each gene. Sections were labeled with oligonucleotide probes (table X) using a high stringency radioactive ISH, protocol derived from Dagerlind et al (Dagerlind et al., 1992)**.** Each probe was measured for radioactive strength after ^{33}P labeling and before hybridization. After hybridization, slides were placed over night on phosphoimaging plates (Fujix BAS-3000; Fuji Photo Film Co, Tokyo, Japan) to evaluate strength and quality of isotope labeling. The phosphoimaging results decided the needed exposure time for further autoradiography. Hybridized and air-dried sections were then exposed to film for autoradiograhy (Carestream Kodak BioMax MR-Film, Sigma-Aldrich) for 8-57 days depending on probe strength. Developed films were scanned for further analysis (Epson Perfection V750 Pro, Dual lens system, High pass optics; Digital ICE Technologies, Long Beach, CA, USA).

Oligonucleotide probes

Quantitative measurements of mRNA were made using ISH with oligonucleotide probes of ~50 base pairs. The majority of the probes had been previously used and documented in the laboratory. New probes where quality controlled towards USCS genome browser (https://genome.ucsc.edu/) where they were aligned to all publicly known sequences. Folding energy was estimated by Mfold version 3.6 (Zuker, 2003). The final probes to be used are found in Table 1.

Table 1: Oligonucleotide DNA probes used for in situ hybridization

Gene	^{33}P -labeled oligonucleotide DNA probes
Nogo-A	“TCT GGA GCC GTC CTT CAC AAG TTC TGG GGT CCT GGG GAA AGA AGC ATTC”
OMgp	“AGG TTC TCC TTC GTT TCA GAA ACT GCA GAC CCT CAG CCT GCC AGC ATG AC”
MAG	“TCG TCT GGG TGAT GTA GCA CAC AAT GGC AAT CAG GAT GGC AAA GGC GACC”
NgR1	“GTG CAG CCA CAG GAT AGT GAG ATT TCG GCA TGA CTG GAA GCT CGC AGC”
NgR2	“AAG GAC AGC GGC ACT GAG AAG TTG GCC TGG CAG CTC ACG GT”
NgR3	“AGG GCG CTC AGT CCA CAC TTA TAG AGG TAG AGG GCG TGA AG CTT C”
LINGO-1	“AGC TGA GCT GAT GCC TGC GTC CGA TTT CCG GGG CAC ATA CTC AAT TTC G”
ADAM22	“AGT CTT TGC ATC ATT GTG AGG AAA GTG GGT GCC GCA GTC AGC CCC TG”
Troy	"TTT CCC CAG CTG TGT CTG TCT TTG AAG TCC ATC AGG GCT CGG CAT GTG G"
Olfactomedin	“GGG TTG TTC TTG GGT GAG AGG GGC CAA CAG TCA TCG CCT TGC TTC TTT G"
LgI1	“TTG TGG ATG GCT TGC TCT TTA ATG GCT GCC CCT TAG GTG GGA AGG TC"

Quantification of mRNA

mRNA expression was quantified in 18 anatomical regions (Table 2). The regions were chosen to cover a broad number of functions associated with limbic properties, motor and sensory functions. Identification of regions was made with neuroanatomical atlases (Lein et al., 2007; Paxinos and Franklin, 2007). Manual mappings (ImageJ, NIH) of the areas of interest revealed levels of optical density. The acquired measurement was finally converted to expression levels after corrections for radioactive decay before and during film exposure, for background detection levels and isotope labeling. For each age and gene, an average of five sections (2-15) was measured per region and animal.

Table 2: The 18 investigated anatomical regions

Hippocampal areas	CA1 CA3 lateral CA3 medial Dentate gyrus
Cortical areas	Anterior cingulate area Primary motor area Somatosensory area Retrosplenial Visual area Auditory area Entorhinal cortex Frontal association area
Subcortical areas	Caudoputamen Globus pallidus Lateral Amygdala Basolateral Amygdala Central Amygdala Mammilary bodies

Genetic association study

To investigate the potential association of selected plasticity regulating genes involved in Nogo-type signaling, and the structural deviations noted to correlate to aggravation of symptoms in the migraine brain (Lai et al., 2015b; Messina et al., 2018; Schmitz et al., 2008), a logistic regression analysis in a cohort from the Swedish twin registry (described above) was performed. The cohort consisted of 4781 individuals, 4032 controls and 749 cases of migraine. Fifteen SNPs associated with five key genes of Nogo-type signaling (ligands Nogo-A, OMgp, MAG, receptor NgR1 and co-receptor LINGO-1) were investigated (Figure 6). Due to an overrepresentation of migraine among women, the analysis was performed with sex as covariate. Genetic analyses were made with PLINK version 1.07 and 1.9 (Chang et al., 2015; Purcell et al., 2007). Haplotypes were analyzed with Haploview (Barrett et al., 2005). A power analysis was performed with help of the Genetic Association Study Power Calculator (available online) (Jennifer Li Johnson, 2017)

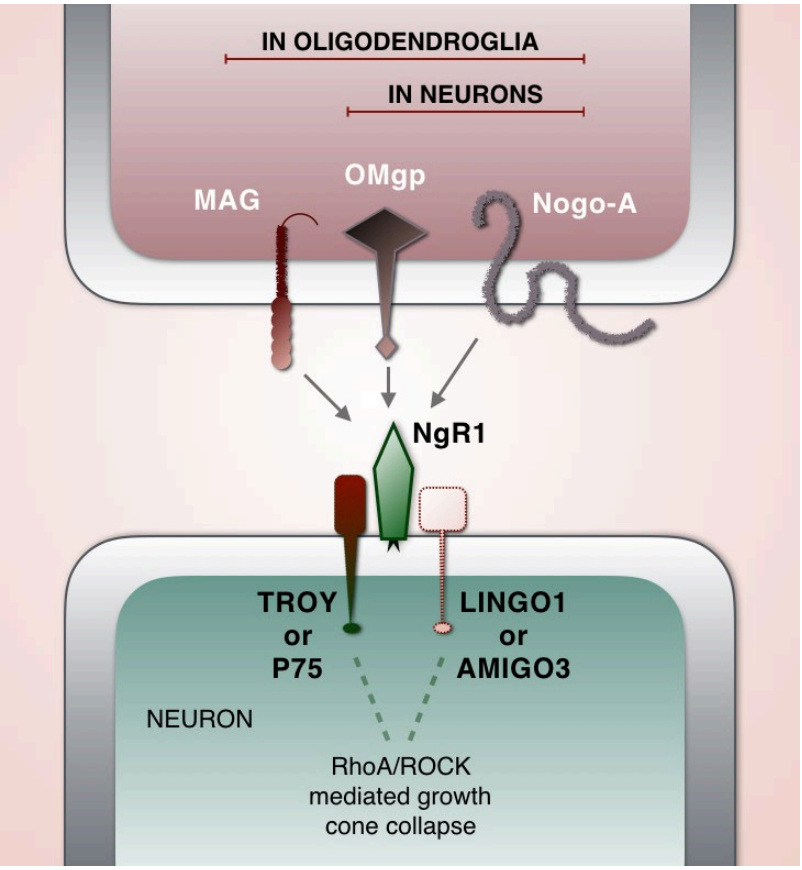

Figure 6: Illustration of key genes associated with Nogo-type signaling. Genes investigated for association with migraine were: ligands Nogo-A, OMgp, MAG, receptor NgR1 and co-receptor LINGO-1. (Smedfors et al., 2019).

The impact of psilocybin on synaptic markers

Psilocybin vs psilocin

When psilocybin is metabolized in vivo, it undergoes rapid dephosphorylation into its CNS-active form psilocin (Anastos et al., 2006). Psilocybin should thus be considered a prodrug for psilocin. Previous in vitro and in vivo work investigating effects of psilocybin on behavior and neuronal plasticity has often been carried out directly with psilocin (Ly et al., 2018; Rambousek et al., 2014; Sakashita et al., 2015). However, the hydroxy-indole psilocin is notoriously unstable making it difficult to store and handle adequately avoiding auto-degradation (Truscott and Manthey, 1989). Given the intrinsic instability of psilocin, the fact that dephosphorylation is an abundant biochemical transformation in prokaryotes - and eukaryotes, and the fact that relevant phosphatases, such as tissue-nonspecific alkaline phosphatases, are found in neuronal cultures (Brun-Heath et al., 2011), we chose to use psilocybin (Merck & Co, USA). There are good reasons to assume that under our reaction conditions, psilocybin is effectively converted to psilocin, and that observed effects should be attributed to psilocin. It should be noted that also psilocybin is somewhat sensitive to air and light (Anastos et al., 2006), which is why our experiments were designed to minimize reagent exposure to ambient conditions.

Primary cell cultures

For paper IV, cell cultures were made with hippocampal tissue harvested from E18 C57BL6 mice (Janvier labs). Cells were dissociated by incubating the fetal brain tissue in trypsin in a 37°C water bath for 15 min. Tubes were turned every 3rd min to evenly distribute the trypsin. The trypsinized tissue was cleaned three times in HBSS and subsequently separated with Pasteur pipettes, normal and fire polished. Cells were stained with Trypan blue and counted in a Bürker chamber. Cells were plated on polyornithine coated coverslips in 24-well plates at concentrations of 37.500 cells per well (75.000 cells per ml). Attachment media consisted of Neurobasal media, 200 mM glutamine and B27 supplement serum (all ingredients from Thermo Fisher Scientific). Plates were put in an incubator (37°C, 5% CO^2). After three hours the medium was changed to maintenance medium, a medium similar to the attachment medium with the addition of penicillin and streptomycin. Cultures were then returned to the incubator. Cultures were analyzed at different time-points depending on experiment. For analysis of neuritogenesis at day 6 and 9 in vitro (DIV6 and DIV9). For analysis of synapse markers at DIV17.

Neuritogenesis

To investigate neuritogenesis in response to psilocybin treatment psilocybin solution (Merck & Co, USA) was diluted in Neurobasal media into a final concentration of $1x10^{-7}$ M when added to the treatment wells. Due to the manufacturer's dilution of psilocybin in 50 % acetonitrile : 50 % distilled water, both a plain Neurobasal media control and a vehicle control with the corresponding dose of Acetonitrile (Sigma-Aldrich, Germany) were used.

The treatment was administered at DIV3 and cells were harvested after three days (72 h) or six days by fixation in 4 % formalin at room temperature (RT) for 10 min.

Immunohistochemistry

Subsequent immunohistochemistry was initiated after 3 PBS washings. Cells were permeabilized during 10 min in 0.1 % Triton. Blocking was done during 1 h with donkey serum in PBS before primary antibodies were administered. Staining with DAPI was done to visualize nuclei of cells, MAP2 is a neuron specific marker (Soltani et al., 2005) chosen for staining of microtubules to reveal the dendritic tree (Caceres et al., 1984). Cells were then incubated at 4°C overnight. Secondary antibodies were administered the next day after 3 washings with PBS and left on shaker for 1 h at RT. Coverslips were mounted on glass slides using a drop (~5 µl) of Prolong Gold (Thermo Fisher Scientific) after which the slides were left at RT to harden overnight before imaging.

Synapse marker Time curve

For insight into the impact of psilocybin on synaptic markers in hippocampal cells, they were harvested at 4 time-points following treatment (5 min, 15 min, 30 min, 1h). Treatment was performed with the same concentration of psilocybin as for the neuritogenesis experiment. The vehicle control had the corresponding concentration of acetonitrile. Cells were simultaneously harvested at DIV17 by the same protocol as above. Cells were stained with DAPI for nucleic navigation. To optimize visualization of dendrites and their spines by staining of actin, Alexa Fluor™ 488 Phalloidin was used. A Synapsin I antibody was used as a presynaptic marker. Equivalent cell cultures were investigated at four other timepoints after either psilocybin or vehicle treatment (1, 3, 6, and 24 h), and were stained with the presynaptic (Piccolo) and postsynaptic (Homer1) antibodies to verify and complement the initial time curve.

Imaging and analysis

For the dendritic analysis an inverted fluorescence microscope (Zeiss Axio Observer 40x) was used. Fifteen to twenty neurons were imaged per well for dendrite analysis, 15 were later chosen based on tracing suitability. Number and distribution of dendrites were traced with dedicated software (Imaris Bitplane, Andor Technology Ltd, Great Britain). For synaptic markers, Airyscan imaging was applied (Zeiss LSM800-Airy 63x oil). It was performed in the Biomedicum Imaging Core (BIC) with support from the Karolinska Institutet. Second order dendrites from ten individual cells were imaged per well and analyzed. Pre- and postsynaptic markers were quantified after thresholding to eliminate background signal using ImageJ (ImageJ, NIH). The number of puncta were normalized based on width of the individual dendrite. The investigators performing imaging and analyses were blinded to treatment groups until the data were analyzed.

Statistical analyses

In paper I, t-tests were applied to analyze data when two groups were compared. All tests consisting of multiple groups were analyzed with a general or a generalized linear model. Generalized estimated equations were used for longitudinal data. Post hoc tests were performed with Bonferroni correction for multiple testing when suitable. All analyses were performed using the same analysis program (SPSS 22, IBM, USA).

In paper II, data were split per gene and group of regions (cortical regions, hippocampal regions and subcortical regions) and analyzed with a mixed model analysis. A post-hoc analysis was performed using the estimated marginal means if a significant effect was found. Each area was compared to the other areas in the region at each timepoint. Age groups were compared to their neighboring groups during postnatal development and maturation (P0 to 4 weeks) and all age groups were compared to the 14-week (adult) group. P-values were adjusted with Bonferroni correction for the number of tests per group. All analyses were performed using the same software (SPSS 23, IBM, USA).

In paper III a logistic regression analysis was performed with sex as covariate and Bonferroni corrections were applied for the number of statistical tests. This was done in PLINK versions 1.07 and 1.9 (Chang et al., 2015; Purcell et al., 2007).

In paper IV data were analyzed with a generalized linear model using a Gaussian distribution. Dunnett's post hoc analysis was applied, comparing all entries to the control group without adjustment of p-value.

For paper III and IV, statistical calculations and graphical illustrations were made in R and RStudio version 1.1456 (R Core Team, 2019; RStudio Team, 2015).

ETHICAL CONSIDERATIONS

The mammalian brain is probably the most complex structure known; studying it is demanding. To obtain information, including behaviors, with more direct relevance for humans, studies of mammals is advantageous. Hence in vitro and in vivo studies were used with mouse cells and mice, respectively. In vitro experiments are important and can often point out directions for further studies. In vivo experiments in animals are necessary to access the dynamics of the living brain and resulting behaviors.

All our animal experiments are performed in accordance with "Stockholms djurförsöksetiska nämnd". We are reducing the number of animals, replacing them when possible, using the best possible methods and refining protocols to minimize stress.

Our methods to embrace these three aspects are power calculations to ensure that the experimental group sizes neither are too big nor too small, for detection of an effect. For the genetic study, we used already available data to replace the need of new substantial experiments. Refinement is never-ending, with a continuous improvement in skills and continuity in the animal facility, accompanied by a dialogue about why and how we perform animal experiments.

Paper III involved research on volunteer human participants. All study participants gave their informed consent and the study is approved by the local ethical committee; Regionala etikprövningsnämnden in Stockholm (registration numbers: 2007/644-31). All experiments on human subjects were conducted in accordance with the Declaration of Helsinki.

The research project involved physical interventions on the study participants in the form of taking venous blood samples which is an invasive, but not harmful technique. The project involved collection of biological samples and all samples are stored in a biobank “KI Biobank” at Karolinska Institutet, Stockholm, Sweden in accordance with ”Lagen om biobanker i hälso- och sjukvården” (2002:297). The sampling done in this study is according to clinical routine. We do not believe that this study poses any risks or complications to study participants, except for any discomfort in collecting blood samples.

The project involved personal data collection and/or processing and partly the collection and/or processing of sensitive personal data (e.g.: health, lifestyle, and ethnicity). It also involves processing of genetic information. The data are pseudonymized into a database in accordance with the EU's General Data Protection Regulation (GDPR). This data will allow studies to which various genetic markers combine in individual patients, and how such haplotypes may correlate to different forms of the disorders, treatment response, age of onset, known heredity etc. Genetic information is and will be recorded in such a way that subjects cannot be identified. As the significance of the analyzed genetic markers is unclear today, participants will not be informed about individual results. All results are presented in the form of statistics, there is no way to identify individuals.

RESULTS AND REFLECTIONS

This thesis focuses on our lifelong neuronal plasticity through the perspective of Nogo-type signaling. The impact of NgR1 on synaptic and structural plasticity in relation to memory consolidation has been investigated. The regional brain expression of many Nogo-type signaling genes has been mapped throughout life. A potential association of Nogo-type signaling with migraine has been analyzed at the genetic level. Finally, the impact of psilocybin on synaptic markers has been studied. The results are as follows:

PAPER I

NGR1 IMPAIRS MEMORY CONSOLIDATION AND STRUCTURAL PLASTICITY

To investigate the importance of NgR1 signaling in memory consolidation, a series of behavioral experiments were performed. Three groups of mice were used: mice overexpressing NgR1, mice lacking NgR1 (NgR1 −/−) and controls.

Overexpression of NgR1 does not affect rotarod performance while NgR1 knockout impairs motor learning

In the first experiment motor-skill performance was investigated in the accelerating rotarod. We found both NgR1 overexpressing mice and controls to improve their motor skills by training, but no significant difference was noted between the groups (genotype $P= 0.3$, day $P < 0.001$ and genotype × day $P = 0.5$). Neither did maximal speed differ between the groups ($p=0.22$). Knockouts however, did initially perform worse than controls (Figure 7C, genotype $P = 0.34$, day $P < 0.001$ and genotype × day $P = 0.003$) but they caught up, both regarding average and maximal speed, and did not differ significantly from controls by the end of the experiment (Figure 7D, $P = 0.66$).

NgR1 overexpressing does not alter recognition of a novel object while this is impaired in NgR1 knockout mice

With the setup described above, the impact of NgR1 on 1h short term memory was investigated. All groups but the NgR1 knockouts demonstrated a significant preference for the novel object (controls $P = 0.034$, NgR1 overexpressing mice $P < 0.001$, NgR1 +/− mice 0.003 and NgR1 −/− $P = 0.467$). As this difference could not be explained by an altered behavior during the habituation phase, this suggests that complete lack of NgR1 impairs 1 h recognition memory.

NgR1 overexpressing mice, but not NgR1 knockouts, demonstrate impaired sequential spatial learning

The two spatial tasks, Barnes maze and Morris water maze were performed sequentially. While no difference could be detected in any group in Barnes maze during the training (Overexpressing mice vs controls Figure 7G, genotype $P = 0.94$, day $P < 0.001$ and genotype × day $P = 0.22$ and NgR1 −/− vs NgR1 +/− Figure 7I**,** genotype $P = 0.34$, day $P < 0.001$ and genotype × day $P = 0.3$), Following training a probe trial was performed (day 5) that did not reveal any difference between the groups ($P = 0.46$). Both NgR1 −/− and NgR1 +/− mice showed a robust memory for the former escape location although NgR1 +/− mice demonstrated an even stronger preference than NgR −/− ($P = 0.03$). The behavior in both NgR1 overexpressing and mutant mice suggests an intact day-to-day learning. Following this, the same cohort of NgR1 overexpressing mice performed significantly worse in Morris water maze than their controls. This could not be explained by any initial difference as mice performed equally during the two training days. The ability to swim was not impaired in NgR1 overexpressing mice as their swim speed (Figure 8) did not differ. Subsequent to the habituation phase, mice were trained in the maze for seven days to find a hidden platform. NgR1 overexpressing mice were significantly impaired in this task (Figure 7K**,** genotype $P < 0.001$, day $P < 0.001$, genotype × day $P = 0.72$) and also in the probe trial (Figure 7L, $P = 0.013$) one day after the seven days of training. No difference was detected between NgR1 −/− and NgR1 +/− mice neither during the seven days of training (Figure 7M, genotype $P = 0.47$, day $P < 0.001$, genotype × day $P = 0.29$) nor in the probe trial ($P=0.56$).

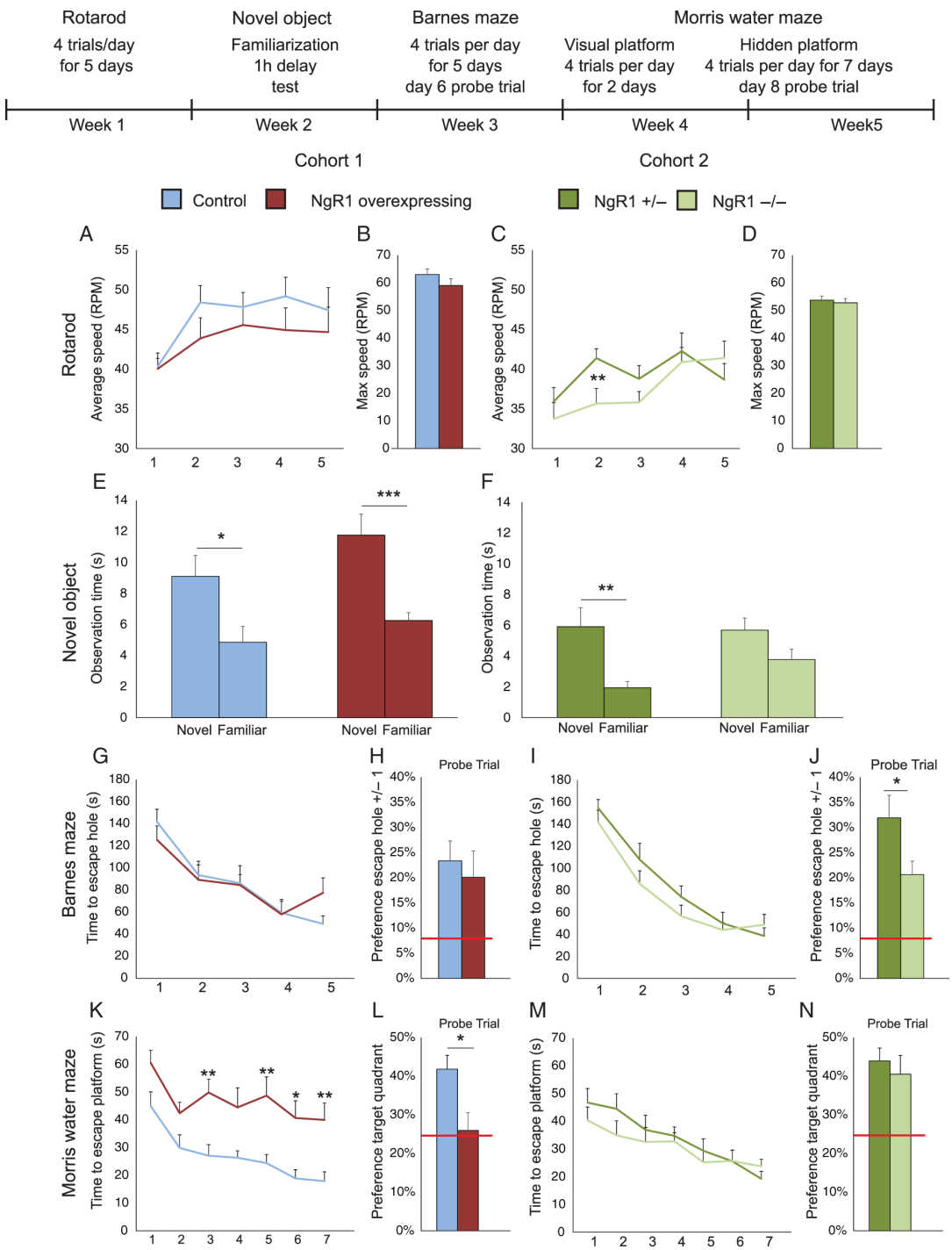

Figure 7: Overexpression of NgR1 impairs sequential spatial learning. Lack of NgR1 impairs locomotion and novel object recognition. Impact of NgR1 overexpression or the lack thereof in four consecutive behavioral tests. This figure illustrates two parallel studies, one where NgR1 overexpressing mice were compared with litter mate controls, one in which NgR1−/− mice were compared with littermate NgR1+/− mice. Statistical comparisons are made within each cohort, not between NgR1 overexpressing and knockout mice. The timeline of the tests is shown at the top: Performance during 5 days (4 trials per day) of Rotarod training (A,C). Average of maximal speeds (B,D). Novel object recognition, illustrated as time spent interacting with the novel, compared to the familiar object (E,F). Time to enter the escape hole during 5 days of training in Barnes maze with 4 trials per day (G,I). Probe trial performed 1 day after the 5 days of training (H,J). Time to reach escape platform during 7 days of training in Morris water maze (K,M). Probe trials of the same groups in Morris water maze 1 day after the last training session (L,N). Red bars in (H,J,L,N) indicate chance levels of performance. *P < 0.05, **P < 0.01, ***P ≤ 0.001.

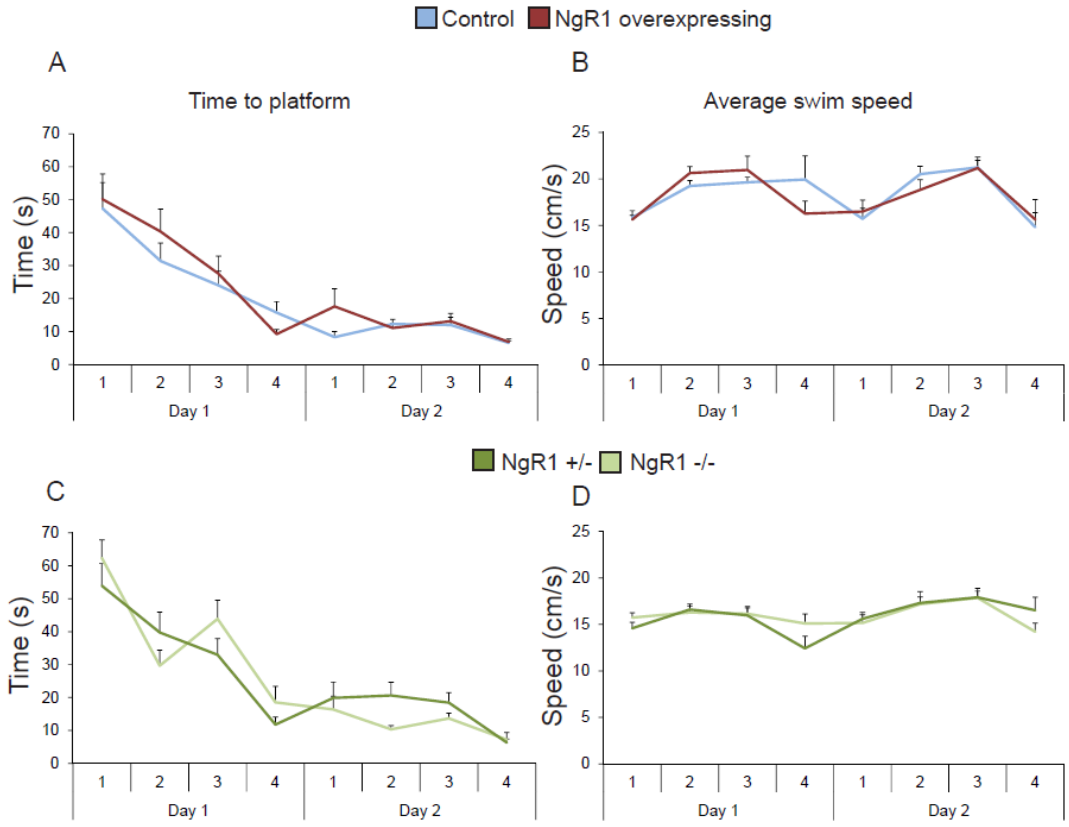

Figure 8: Time to visual platform or swim speed did not differ between NgR1 overexpressing mice and controls or between NgR1 knockout mice and controls during the two initial days of training in the Morris water maze.

Ex vivo MRI in NgR1 overexpressing mice propose intact gross anatomy including white matter tracts

To examine if NgR1 overexpression from birth causes changes of brain structure that could explain the behavioral differences noted in NgR1 overexpressing mice, brains were imaged using T2-weighted ex vivo 9.4 T MRI scans. Cortical thickness was measured at several different locations with no significant difference detected between the groups. Neither was there a significant difference in the volume of the cerebrum, hippocampal formation, cerebellum or brainstem (Figure 9).

As Nogo-type signaling is associated with myelination in both health and disease (Chong et al., 2012; Sozmen et al., 2016), possible effects of an innate overexpression of NgR1 on major white matter tracts in the CNS were also studied. Volume, fractional anisotropy to reveal potential dissimilarity in the axonal tracts and *mean diffusivity* (MD) reflecting the quantity of inhibited water that were assessed in corpus callosum, the anterior commissure and the internal capsule. MD was slightly but significantly higher in NgR1 overexpressing mice (Figure 9H, genotype $P = 0.02$, region $P = 0.546$ genotype × region $P = 0.675$) but altogether the results indicate similar structures in both white and gray matter in mice overexpressing NgR1.

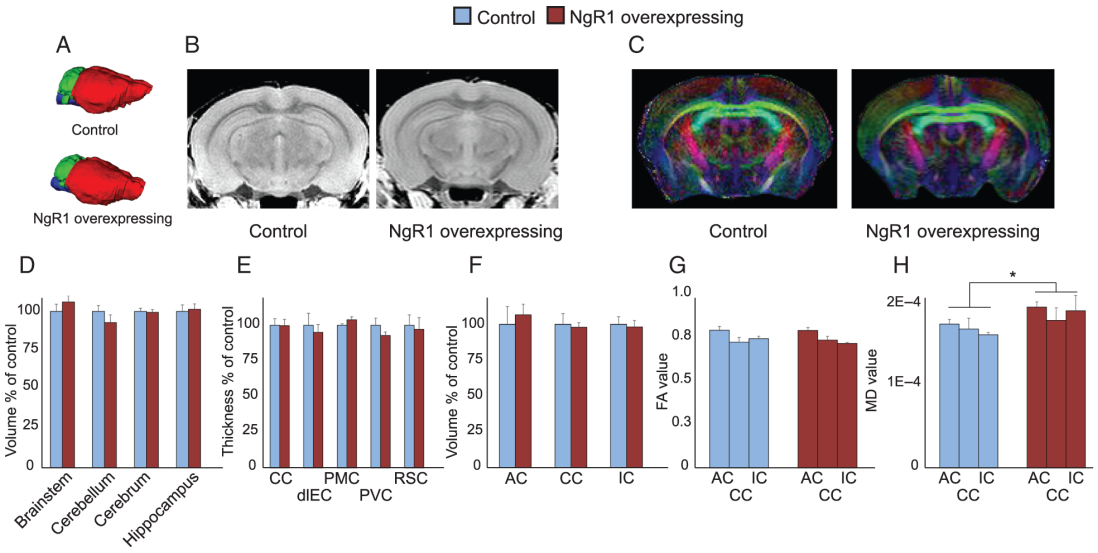

Figure 9: NgR1 overexpression does not affect gross brain anatomy as shown by ex vivo MRI analysis in NgR1 overexpressing mice and controls. (A) 3D rendering of a representative control and NgR1 overexpressing brain structures, in red forebrain, in green cerebellum and in blue brianstem. (B) T2 images used for cortical thickness. (C) DTI images used for white matter analysis. (D) Volumes of four major brain regions. (E) Thickness of the cortical areas: CC, cingulate cortex; dlEC, dorsolateral entorhinal cortex; PMC, premotor cortex; PVC, primary visual cortex; RSC, retrosplenial cortex. (F) Volumes of white matter tracts: AC, anterior commissure; CC, corpus callosum; IC, internal capsule. (G) Fractional anisotropy and (H) mean diffusivity of the same white matter areas as shown in (F). *P < 0.05.

NgR1 overexpression enhances locomotor sensitization while inhibiting the development of stereotypic behavior

A new cohort of mice overexpressing NgR1 and litter mate controls were subjected to a sensitization paradigm in response to cocaine or saline (in total four groups) to investigate the impact of increased NgR1 signaling on locomotor sensitization and subsequently on structural plasticity.

The locomotor response to intraperitoneal injections in a new environment did not differ between the groups during the two first days, but NgR1 overexpressing mice did habituate slower than controls (Figure 10A, genotype $P = 0.035$, day $P < 0.001$ and genotype × day $P = 0.039$). However, over time the saline groups behaved similarly. In cocaine treated mice there was no difference during the initial days of saline injections. Notably, both groups responded thereafter with the strong and expected sensitization to cocaine injections when comparing locomotion day 12 with locomotion on day 3 (Figure 10C controls $P < 0.001$ and NgR1 overexpressing mice $P < 0.001$), with time NgR1 overexpressing mice developed a stronger sensitization than controls.

Sensitization paradigms are known to cause not only locomotor sensitization but also the development of stereotypies (repetitive movements measured as crossings of the same light beam several times) (Reith et al., 1986; Robinson and Berridge, 2008). In our study control mice had a stronger development of stereotypies than NgR1 overexpressing mice when

comparing the time spent on stereotypies day 3 and day 12 (Figure 10E controls P = 0.0043; NgR1 overexpressing mice P = 0.85). This was due to control mice significantly increasing time for stereotypies (Figure 10, genotype P = 0.007, day P < 0.001 and genotype × day P = 0.051) which NgR1 overexpressing mice did not. Thus, the inability to downregulate NgR1 appears to impair the known effect to develop stereotypy-like behavior.

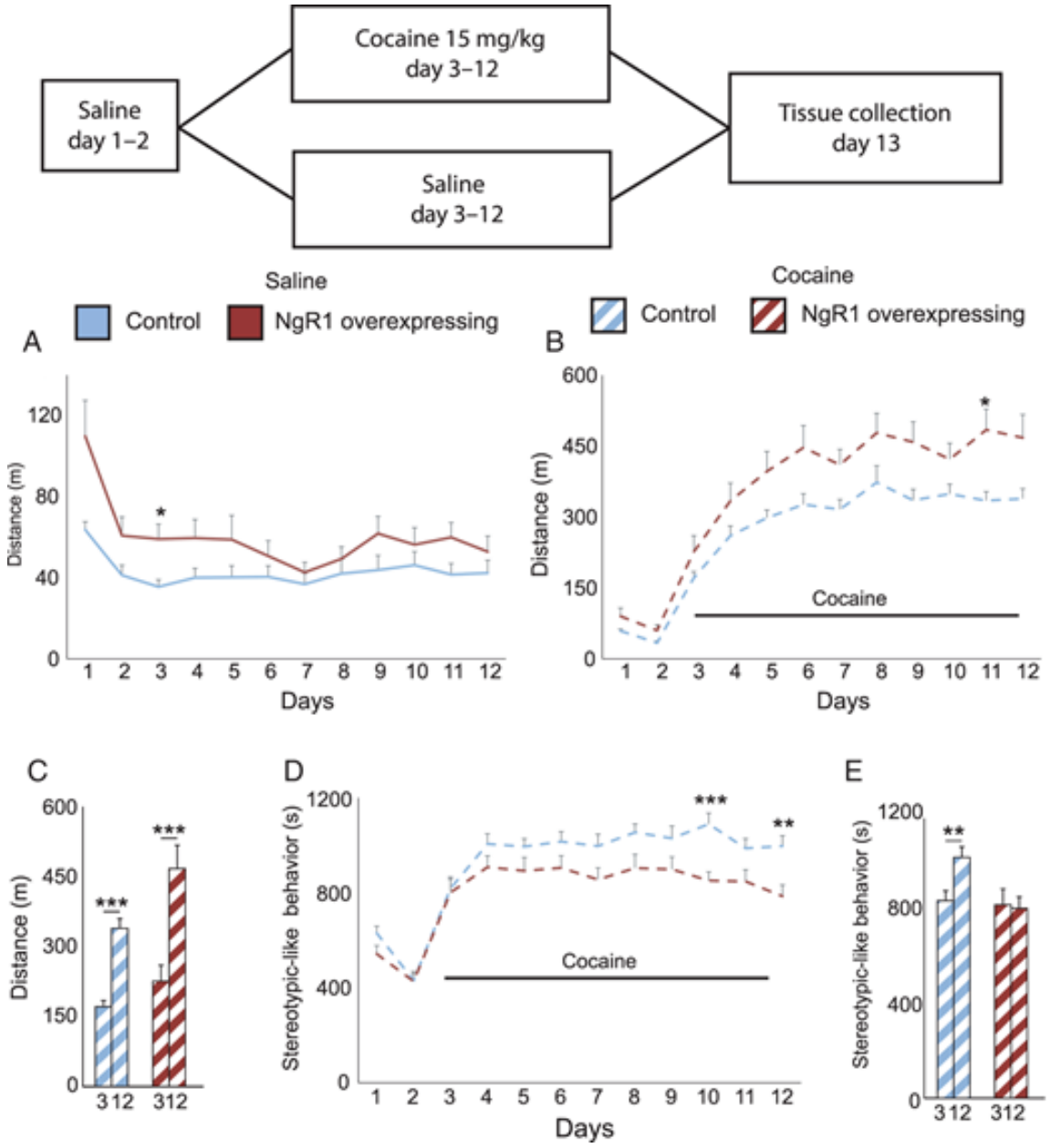

Figure 10: Increased locomotor sensitization but restricted development of stereotypy-like behaviour in NgR1 overexpressing mice. At the top of the figure is a time-line of the experimental setup. (A) Locomotor activity during the two days of saline and ten days of saline or cocaine injections with mice recieveing only saline injections. (B) Mice receiving saline followed by cocaine injections. (C) Locomotor responses of controls and NgR1 overexpressing mice to the first (day 3) and the last day (day 12) of cocaine. (D) The daily time spent performing stereotypy-like behavior measured in seconds. (E) The amount of stereotypy-like behavior after the first and the last cocaine dose measured in seconds. *P < 0.05, **P < 0.01, ***P < 0.001.

Forebrain NgR1 levels regulate dendritic spine densities and spine responses to cocaine

To further investigate if the different response to the cocaine sensitization paradigm correlated with changes in underlying neuronal architecture, the mice from the sensitization paradigm were sacrificed 24 h after the last injection. Spine and dendrite analysis were made in the *frontal association cortex* (FAC), the *cingulate cortex* (CiC), and in *nucleus accumbens* (NAc), in Golgi stained brain sections from all four groups (Figure 11).

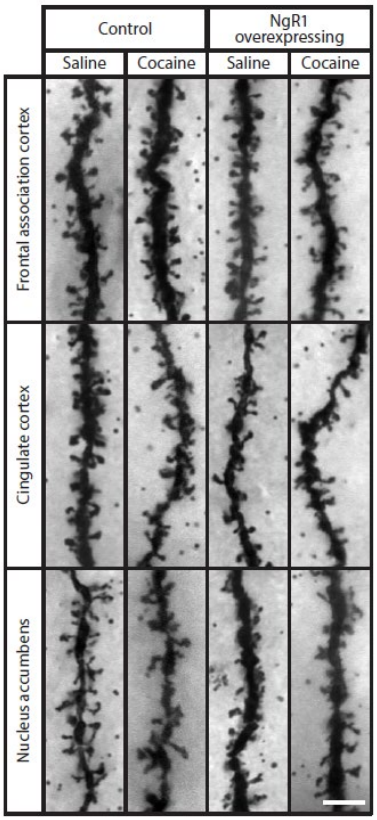

Figure 11: Representative Golgi stained dendrites chosen for spine analysis. Three anatomical areas (FAC, CiC and NAc) for each treatment group.

Dendritic spines are postsynaptic protrusions receiving input and their individual morphology is associated with the stability of a memory (Yang et al., 2009). The spines with a rounded head over a robust neck, consequently called mushroom spines, are associated with more stable synapses than the thinner and longer spines (Golden and Russo, 2012).

The three anatomical regions where we analyzed spines and dendrite were chosen based on their involvement in different aspects of behavior. In the FAC, which is important for decision making (Kennerley and Walton, 2011), apical dendrites of pyramidal neurons had significantly lower spine densities in NgR1 overexpressing mice compared with controls (Figure 12A, genotype $P = 0.003$). While thin spine density did not differ between the groups, mushroom spine density was significantly lower in NgR1 overexpressing mice (Figure 12B, genotype $P < 0.001$) and there was a tendency for cocaine to increase the density of mushroom spines in both control and NgR1 overexpressing mice (treatment $P = 0.088$).

In the cingulate cortex, observed for its emotional regulation and its involvement in memory retrieval (Rajasethupathy et al., 2015), a lower spine density was observed on the apical dendrites of pyramidal neurons in NgR1 overexpressing mice than in controls (Figure 12F). Interestingly, while cocaine decreased spine densities in control mice, it increased spine densities in NgR1 overexpressing mice (Figure 12F, genotype × treatment $P = 0.016$). A similar result was seen for thin spines only when investigating the effect of genotype on treatment (Figure 12G, genotype × treatment $P = 0.025$). The density of mushroom spines in NgR1 overexpressing mice was however significantly lower (Figure 12I; genotype $P = 0.046$) but no interaction between genotype and treatment was found for mushroom spines.

In the reward and addiction related region NAc (Russo et al., 2010) there was a reduction in the total density of spines on medium spiny neuron dendrites in mice overexpressing NgR1

(Figure 12K, genotype P = 0.045), but there was no significant effect of cocaine treatment. As the number of thin spines was similar in all groups, this spine type could not explain the reduction. The difference was instead due to a significant decrease in the density of mushroom spines in NgR1 overexpressing mice (Figure 12N, genotype P < 0.001). In line with previous research (Robinson and Kolb, 2004), controls showed a significantly higher density of mushroom spines in response to cocaine sensitization than NgR1 overexpressing mice. The mushroom spine density distribution mirrored that in CiC and FAC (Figure 12O). This resulted in an overall lower spine density in overexpressing mice when compared with controls. We conclude that NgR1 is a robust limiter of the number of mature dendritic spines in vivo with clear functional consequences.

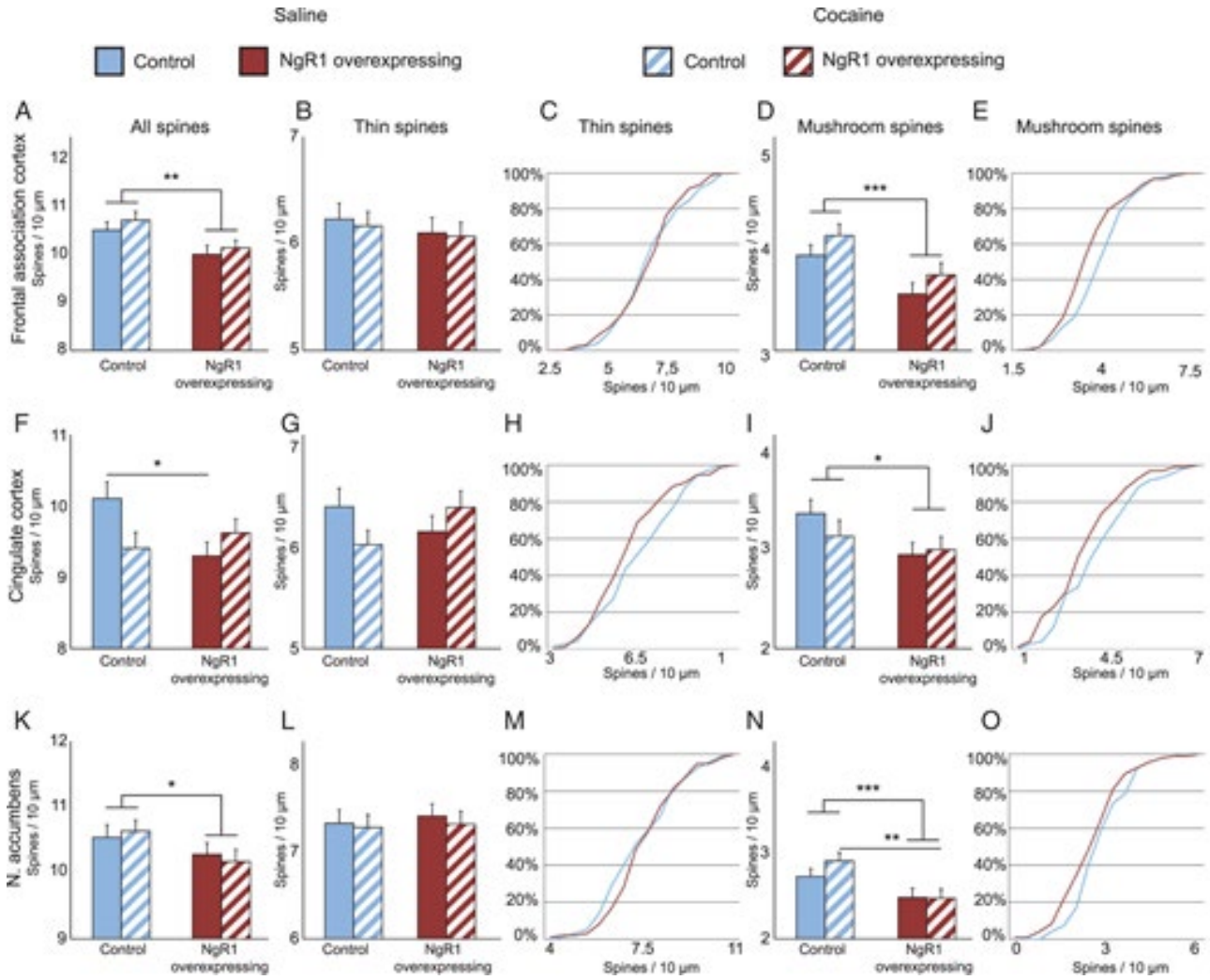

Figure 12: NgR1 limits the number of mature mushroom spines in vivo; cocaine affects control and NgR1 overexpressing mice differently. Density of all spines (A,F,K), thin spines (B,G,L), mushroom spines (D,I,N) in 3 brain areas from control and NgR1 overexpressing mice 24 h after last treatment with saline or cocaine. Frequency distribution charts for thin (C,H,M) and mushroom spines (E,J,O) (n > 60 neurons per group from 12 mice per group). Significances between groups with Bonferroni correction: *P < 0.05, **P < 0.01, ***P < 0.001.

NgR1 affects dendritic structure in the cerebral cortex but not in accumbens

The structure and complexity of the dendritic arbor mirrors the afferent information to a neuron. As the blocking of the NgR1 ligand Nogo-A has previously been associated with limit dendritic plasticity and complexity (Papadopoulos et al., 2006; Zagrebelsky et al., 2010) we therefore examined the total length of the dendritic tree, the distribution of lengths of the individual branches, the number of endings and as well the complexity using Sholl analysis (Figure 13).

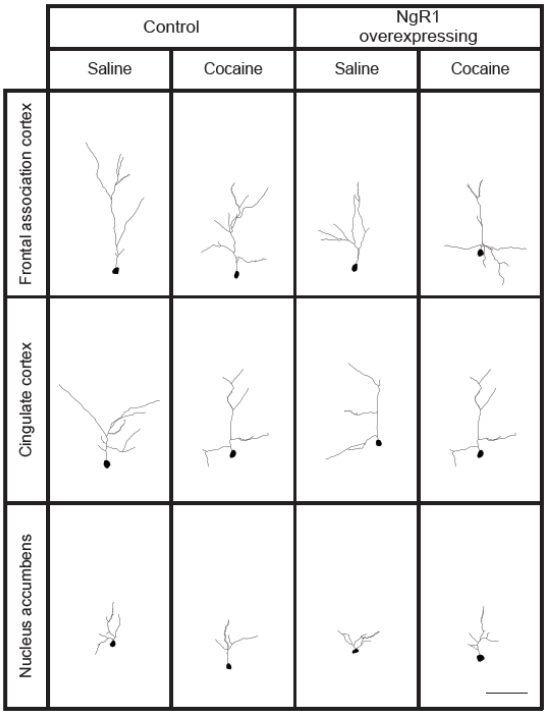

Figure 13 Representative examples of traced neurons from the three anatomical areas (FAC, CiC and NAc) for each treatment

In the frontal association cortex, the dendritic tree tended to be shorter in NgR1 overexpressing mice than in controls (Figure 14A, genotype P = 0.1), but after 10 days of cocaine exposure the length of the dendritic trees increased significantly in the cocaine treated groups compared to saline treated groups (P = 0.02). The number of endings per dendrite did not change significantly in frontal association cortex but the Sholl analysis did in line with the dendritic length reveal a significant difference in NgR1 overexpressing mice. A frequency distribution of the length of individual branches did not reveal any difference between any groups.

In the cingulate cortex did NgR1 overexpressing mice have significantly smaller dendritic trees than controls after saline treatment (P = 0.008). The exposure to cocaine on the other hand, resulted in a significant decrease in the length of the dendritic arbor in control mice (P = 0.037) while there was a nonsignificant increase in dendritic length in NgR1 overexpressing mice (Figure 14E). When combining the effect of genotype and cocaine, ergo the cocaine-induced decrease of dendritic length in control mice and the nonsignificant increase in NgR1 overexpressing mice, a significant interaction was noted (genotype × treatment P = 0.001). The same significant interaction was noted when looking at the number of endings (genotype × treatment P = 0.003).

Saline treated NgR1 overexpressing mice had a significantly reduced number of dendritic endings, hence a reduced branching of the dendritic tree (P = 0.02). Reduced branching was confirmed by the Sholl analysis (Fig. 5G, genotype P < 0.001). However, the dendritic branch length distribution analysis (Fig. 5H) showed no difference, indicating that NgR1 mostly affects formation of new branches and not the growth of existing ones.

In the nucleus accumbens the dendrites were stable and neither affected by overexpression of NgR1 nor by exposure to cocaine. Dendritic length, number of dendrite endings, Sholl analysis, and dendrite branch length frequency analysis were all similar between the groups (Figure 14I–L)

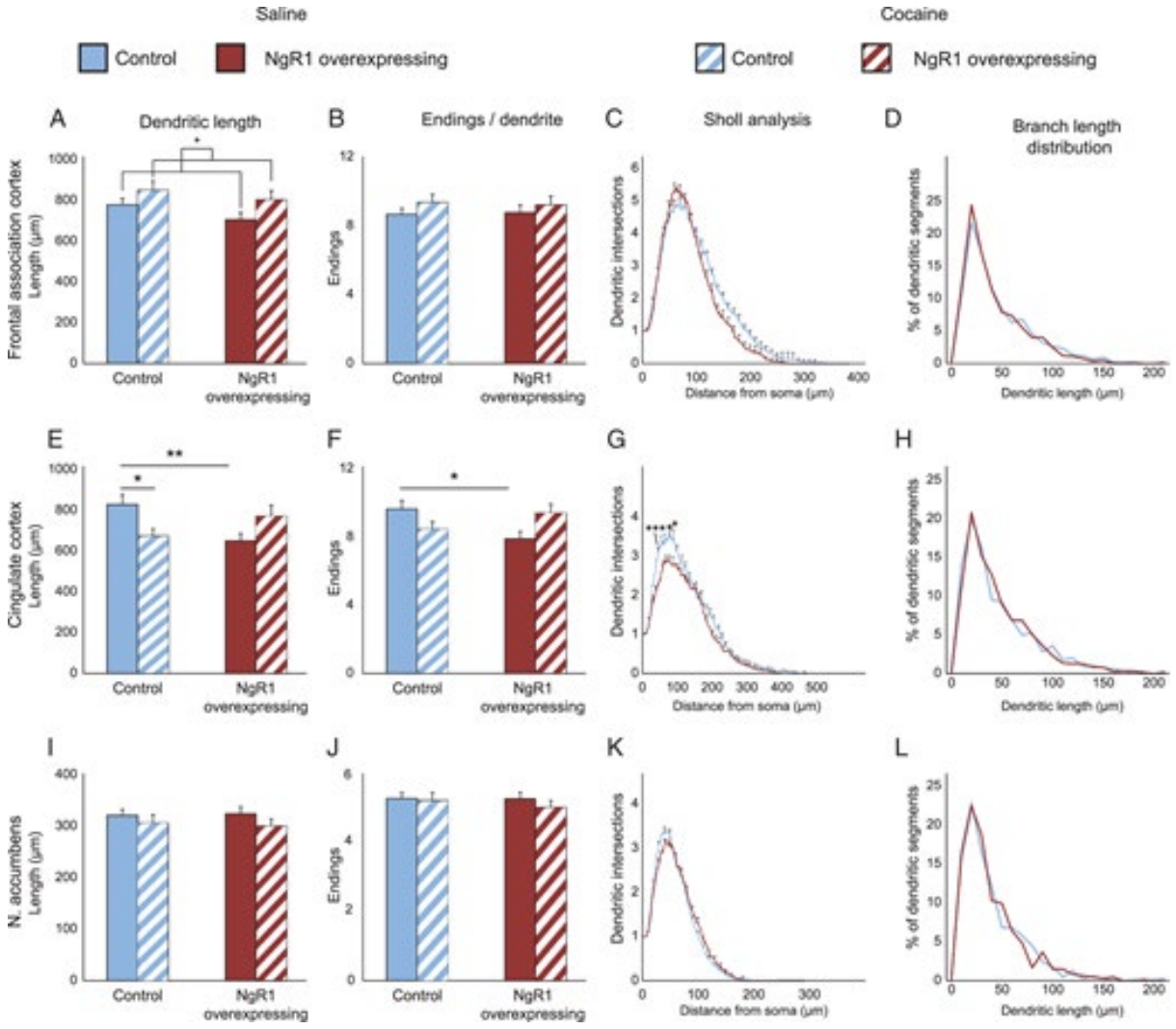

Figure 14: **Dendritic length, number of branch points, and complexity are limited by NgR1.** Same groups as in Figure **X**. (A,E,I) Total length of dendrites of neurons used to analyze dendritic spines in 3 brain areas of control and NgR1 overexpressing mice treated with saline or saline followed by cocaine. (B,F,J) Number of dendritic endings per analyzed dendrite in the same 4 groups. (C,G,K) Sholl analysis of dendrite complexity with respect to distance from cell body. (D,H,L) Frequency distribution of different dendritic lengths. Significance between groups with Bonferroni correction: *P < 0.05, **P < 0.01.

The impact of cocaine and saline on mice overexpressing NgR1 and controls in three important anatomical regions

Regional and genotype specific alterations show how cocaine potently affects neuronal architecture in a NgR1 dependent manner. The results point out cingulate cortex as an area where NgR1 type signaling plays a profound role for cocaine-induced plasticity of dendrite architecture.

PAPER II
DYNAMIC REGULATION OF NOGO GENES OVER TIME

Nogo-type signaling has a potent effect on structural plasticity, but knowledge about expression during different stages of life associated with various levels of growth and stability have been limited. Studies in Paper II therefore investigated the mRNA expression of 11 relevant genes in 18 different brain areas (Table 2) at six different ages (P0, 1 w, 2 w, 4 w, 14 w and 2 years (104 w)), using quantitative in situ hybridization (Figure 15).

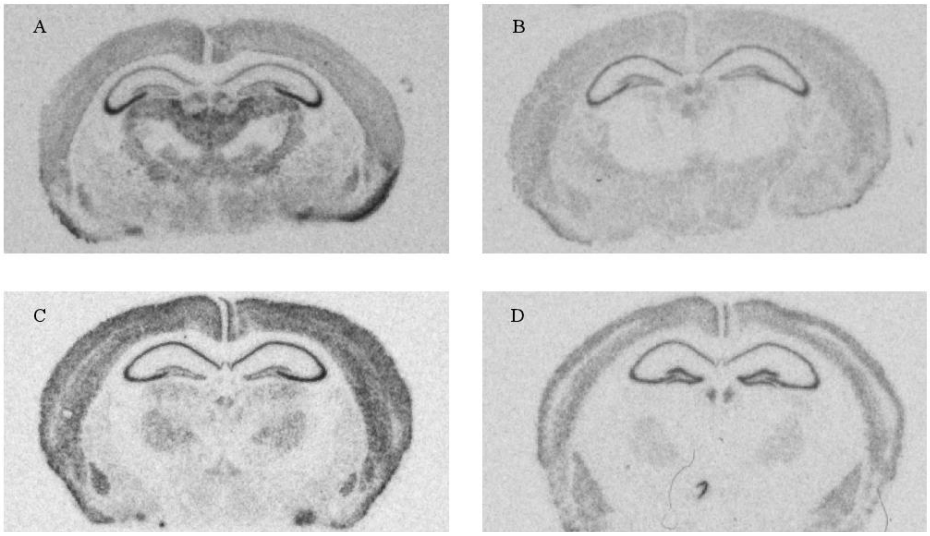

Figure 15: Representative autoradiograms used for quantification of mRNA expression in 18 regions. Picture illustrate in situ hybridization performed with 33P-labeled oligonucleotides for four different genes (A: Olfactomedin 1 mRNA 1 week; B: Nogo-A mRNA 2 weeks; C: Lingo-1 mRNA 4 weeks and D: NgR1 mRNA 104 weeks).

Individual levels of expression for each gene and age

The expression levels of the 18 different areas were pooled into three major regions: cortex, hippocampus and subcortical areas. Each gene demonstrated an individual profile of expression over time, and the level of expression ranged from very low, as for NgR3, to very high as for Olfactomedin (Figure 16).

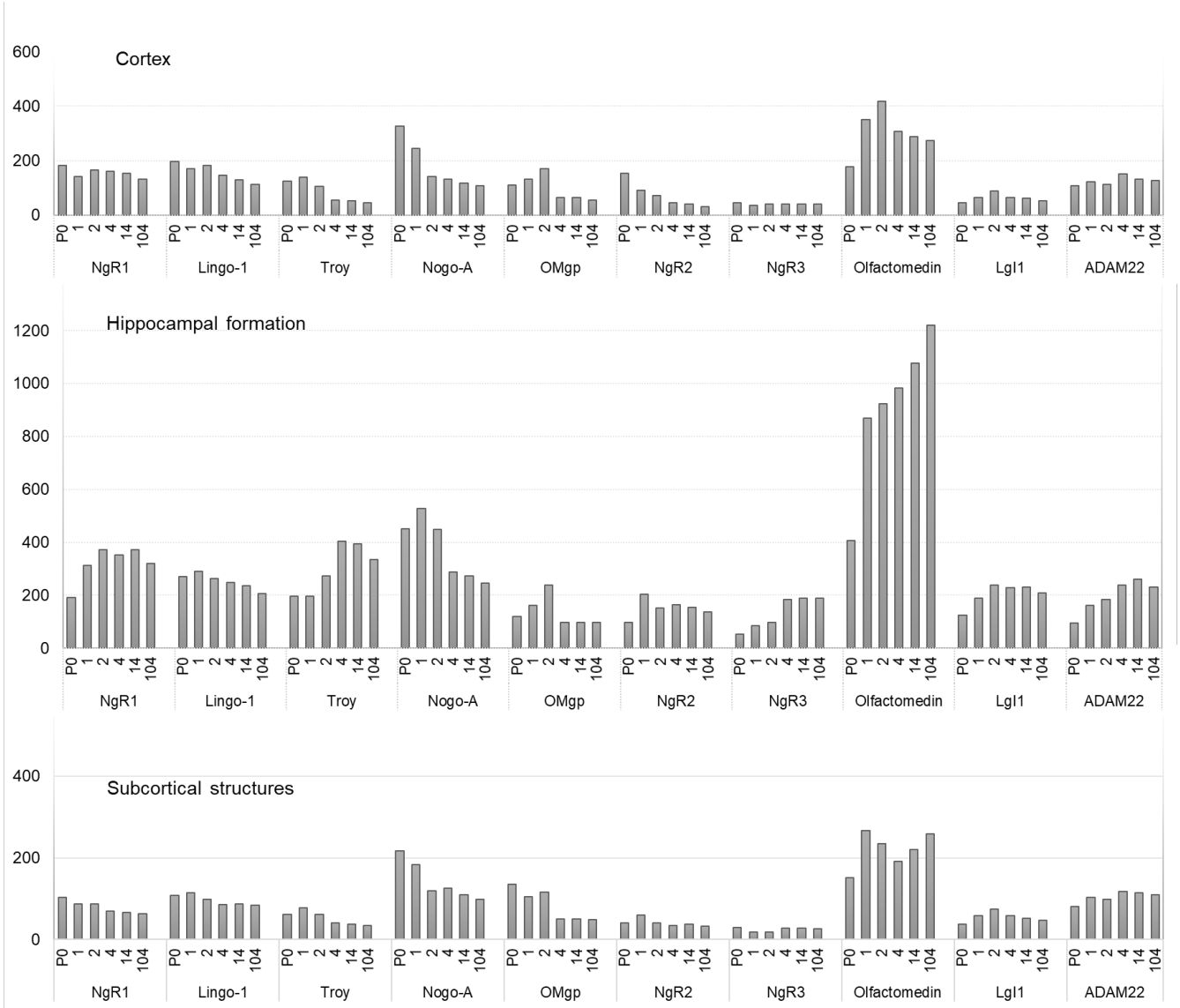

Figure 16: Overview of pooled average expression of all subregions in the three main regions over time for each gene. From the top: The expression of each gene (apart from MAG) in Cortex, hippocampal formation and subcortical structures. X-axis: age in weeks and gene name. Y-axis: expression in nCi/g.

Following this, it was noted that the three main regions underwent different amounts of change for each individual gene over time. While most cortical areas demonstrated similar expression levels, most of the genes in the hippocampal formation and in the heterogenous subcortical regions had a large amount of inter-regional differences in mRNA expression levels over time.

The changes in mRNA expression over time could either represent an increase or a decrease (Table 3). In cortical areas only MAG increased over time. Troy, Nogo-A, OMgp and NgR2 decreased. Nogo-A and OMgp were the only genes to decrease in the hippocampal formation, and they also decreased in the subcortical areas together with NgR1, Troy and MAG. Increases were primarily seen in the hippocampal formation by a majority of the genes. In subcortical areas did only Troy and MAG increase over time.

Table 3: Distribution of 175 changes ($p < 0.05$) of mRNA expression for the 11 studied Nogo-related genes in cortex, the hippocampal formation and subcortical areas over time

Genes involved in Nogo-type signaling	Number of significant changes in mRNA-expression over time					
	Cortex		Hippocampal formation		Subcortical areas	
	(8 areas)		(4 areas)		(6 areas)	
	-	+	-	+	-	+
NgR1	0	0	0	7	2	1
Lingo-1	0	0	0	0	0	0
Troy	26	0	0	1	9	3
Nogo-A	13	0	11	2	5	0
OMgp	15	0	8	3	15	0
MAG	0	4	-	-	3	3
NgR2	4	0	0	0	1	0
NgR3	0	0	0	12	0	0
Olfactomedin	0	0	2	9	0	0
LgI1	0	0	0	5	1	1
ADAM22	0	0	0	9	0	0

Significant change of mRNA expression between either neighboring timepoints (1 week – P0, 2 weeks – 1 week or 4 weeks – 2 weeks) or when comparing the mRNA expression in the adult 14 weeks old mice to that in P0, 1 or 2 weeks old mice. Significant increases are coded in a green scale while significant decreases are coded in a red scale.

Plastic maturation and stable ageing

Different ages were found to be associated with different frequencies of change. Notably, maturation was correlated with more frequent changes in mRNA expression, explicitly between 2 and 4 week old mice, an age correlated to early adolescence (Ciampoli et al., 2017). This finding was clear when the mRNA expression levels in 14 weeks old mice were compared to the different young ages. This comparison demonstrated that the Nogo system gradually reached its adult expression level between 2 and 4 weeks and remained strikingly stable into aging (Table 4).

Table 4: Distribution of changes ($p < 0.05$) of mRNA levels per region during maturation and aging

Brain area		Significant changes during maturation			Significant changes between 14 w adults and all other ages				Number of changes / region
		P0 vs 1 w	1 w vs 2 w	2 w vs 4 w	14 w vs P0	14 w vs 1 w	14 w vs 2 w	14 w vs 4 w	14 w vs 104 w	
ACA	Anterior cingulate area	0	1	2	3	2	1	0	0	9
Mop	Primary motor area	0	1	0	3	1	0	0	0	5
SS	Somatosensory area	0	1	0	3	1	1	0	0	6
RSP	Retrosplenial area	0	1	0	3	2	0	0	0	6
VIS	Visual area	0	1	1	2	2	1	0	0	7
AUD	Auditory area	0	1	1	2	1	1	0	0	6
ENT	Entorhinal cortex	0	0	0	2	2	0	0	0	4
FAC	Frontal association area	0	0	1	1	1	1	0	0	4
CA1	CA1	0	1	2	1	1	2	0	0	7
CA3 lat	CA3 lateral	4	0	4	5	4	3	0	0	20
CA3 med	CA3 medial	3	2	3	3	4	4	0	0	19
DG	Dentate gyrus	0	2	2	3	4	2	0	0	13
CP	Caudate putamen	0	1	3	2	2	3	0	0	11
GP	Globus pallidus	1	0	1	1	1	2	0	0	6
LA	Lateral Amygdala	1	2	1	1	2	0	0	0	7
BLA	Basolateral Amygdala	1	3	1	4	3	1	0	0	13
CEA	Central Amygdala	0	1	0	1	1	0	0	0	3
MBO	Mammilary bodies	0	0	0	2	1	1	0	0	4
	Number of changes / age	10	18	22	42	35	23	0	0	

Significant change of mRNA expression between either neighboring timepoints (1 week – P0, 2 weeks – 1 week or 4 weeks – 2 weeks) or when comparing the mRNA expression in the adult 14 weeks old mice to that in P0, 1 or 2 weeks old mice.

NgR1 and its coreceptors Lingo-1 and Troy

NgR1 had an even mRNA expression in cortical areas with no significant change over time while the structures in hippocampus and the subcortical regions had more significant regulations of NgR1 mRNA levels (Figure 17).

Like for NgR1 Lingo-1 mRNA did not change significantly over time in cortical areas, neither did Lingo-1 change over time in the hippocampal formation. The hippocampal subregions each had their individual expression profile. The subcortical areas basolateral amygdala (BLA) and lateral amygdala (LA) had a prominently stronger expression of mRNA than the other subcortical regions

Troy mRNA levels behaved differently during the first weeks of life compared to NgR1 and Lingo-1 mRNA. While Troy mRNA levels in several cortical structures decreased significantly with age, the hippocampal expression of Troy mRNA appeared to increase between 2 and 4 weeks although the only statistically significant increase was in CA3. While the majority of the subcortical regions were stable and demonstrated a low expression over time, some areas peaked at 1 week.

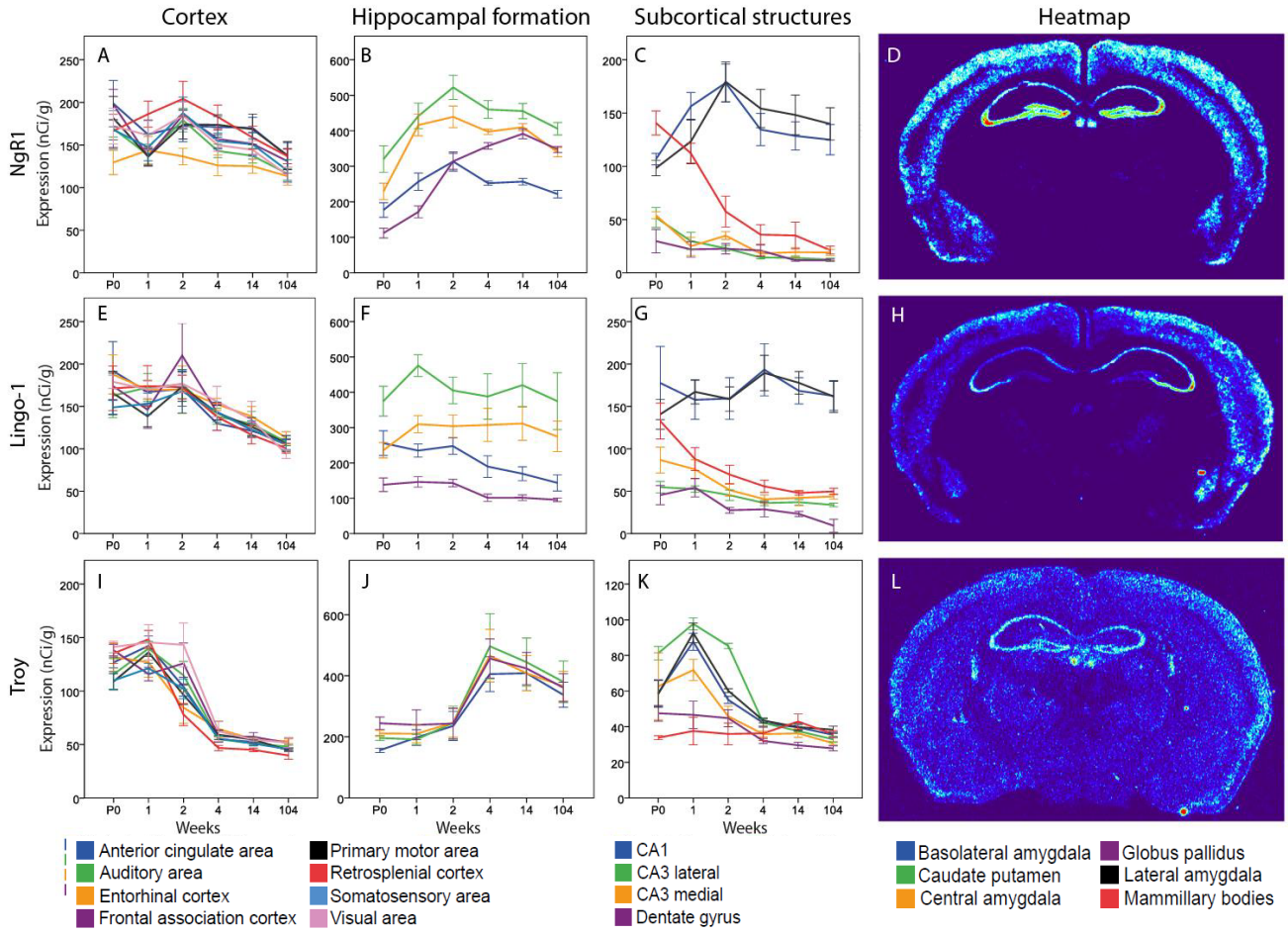

Figure 17: NgR1 (A–C), Lingo-1 (E–G) and Troy (I–K) mRNA levels in 18 brain regions over time. The x-axis indicates age in weeks, the y-axis indicates levels of mRNA expression in nCi/g and is individual for each gene and region. Significant changes defined as $p < 0,05$ are mentioned in the text for each gene. Heatmaps of the three genes (D,H,L) visualizing expression in 14 weeks old mice brains.

Neurite-growth inhibitory Ligands Nogo-A, OMgp and MAG

Nogo-A mRNA was strongly expressed at P0 to later decrease significantly over time (except for frontal association cortex). A similar pattern of decrease over time was noticed in the majority of the regions in the hippocampal formation and subcortical areas (Figure 18, 19).

OMgp mRNA was noted with increasing levels in both cortical and hippocampal regions until the age of 2 weeks from where it dropped profoundly for all regions (Figure 18). All genes had a lower expression in adulthood than in early life in subcortical areas.

As the expression of MAG mRNA follows the myelination of CNS (Inuzuka et al., 1991) no expression was detected before 1 week. The levels in cortical areas were very low throughout life.

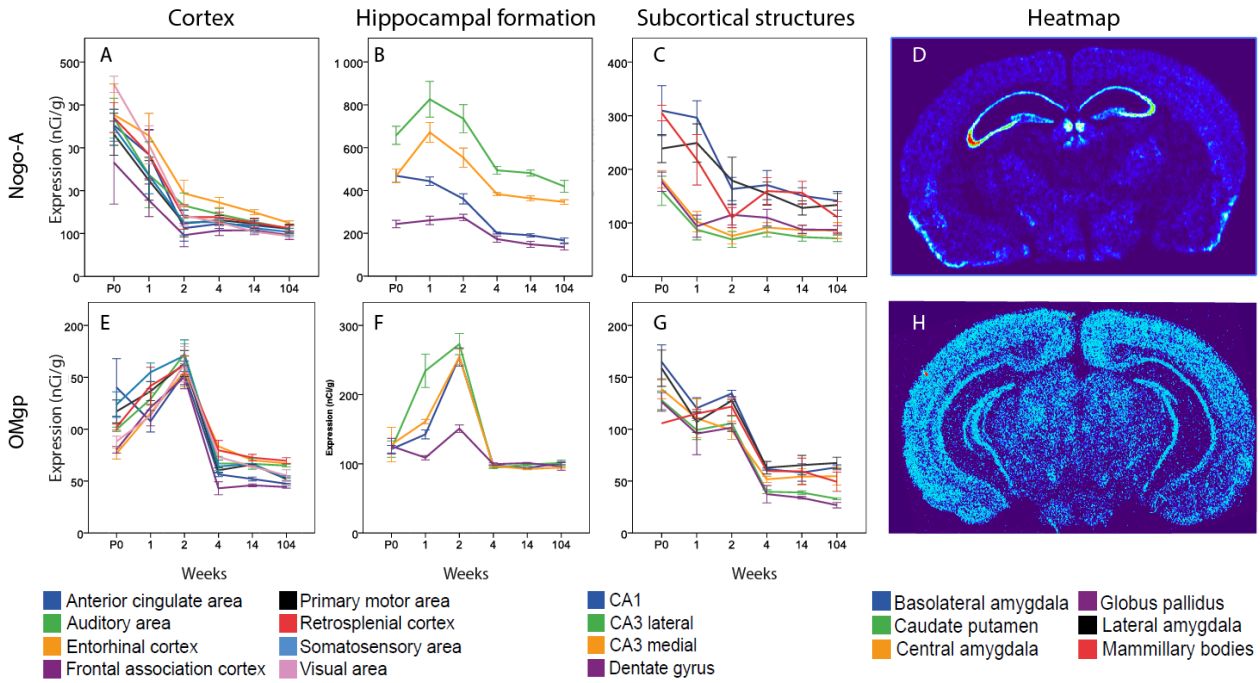

Figure 18 Expression levels of mRNA encoding Nogo-A (A–C) and OMgp (E–G) in 18 cerebral regions over time. For full legend see Figure 17.

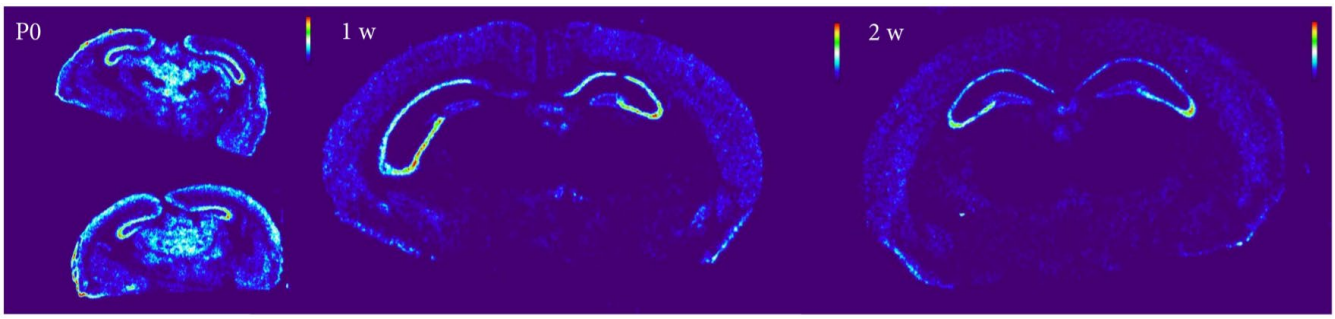

Figure 19: Heatmap of Nogo-A sections at P0, 1 and 2 weeks, illustrating the global decline of mRNA expression during postnatal development.

Receptors NgR2 and NgR3

The global expression of NgR2 mRNA tended to decline over time. In the hippocampal formation there was a non-significant tendency for NgR2 mRNA levels to be increased during the first week of life, to later level off or decrease modestly. NgR2 mRNA levels were robust in BLA and LA and low or non-detectable in other areas.

NgR3 mRNA had generally a very low expression in the developing and adult brain. There was however a tendency for NgR3 to increase between the second and fourth week of life in the hippocampal formation and in some subcortical areas. In the hippocampal formation, BLA, LA and MBO the increase had a tendency to level out on a slightly higher baseline from 4 weeks onwards (Figure 20).

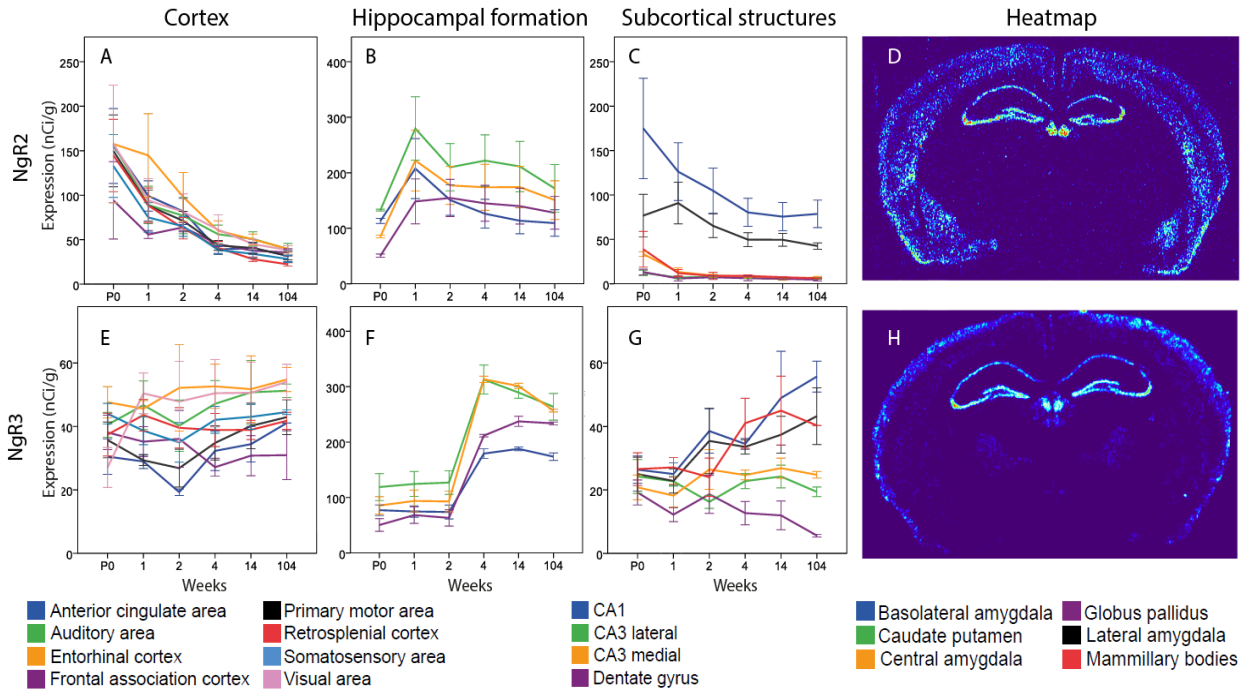

Figure 20: The mRNA expression for NgR2 (A–C) and NgR3 (E–G) in the 18 cerebral regions over time. For full legend see Figure 17.

Nogo-type signaling inhibitors: olfactomedin, LgI1 and ADAM22

Olfactomedin mRNA had the highest expression of all investigated genes and it was present in all cortical areas throughout life without significant change over time. In the hippocampal areas Olfactomedin demonstrated a tendency to initially decrease after birth and later increase in all regions except for CA1. Lateral and basolateral amygdala demonstrated higher levels of olfactomedin mRNA than the other areas.

Measurements LgI1 mRNA showed a tendency for cortical expression to increase from low levels at birth to 3–4-fold higher levels at 2 weeks of age, after which levels declined the next 2 weeks with modest changes thereafter (Figure 21). Hippocampal and subcortical areas did

partly increase initially to later stabilize although BLA was the only region with a significantly higher expression in adulthood than in early life.

The expression of ADAM22 mRNA was stable in all cortical areas from birth to old age while all regions of the hippocampal formation increased with age, with a tendency to decrease at old age.

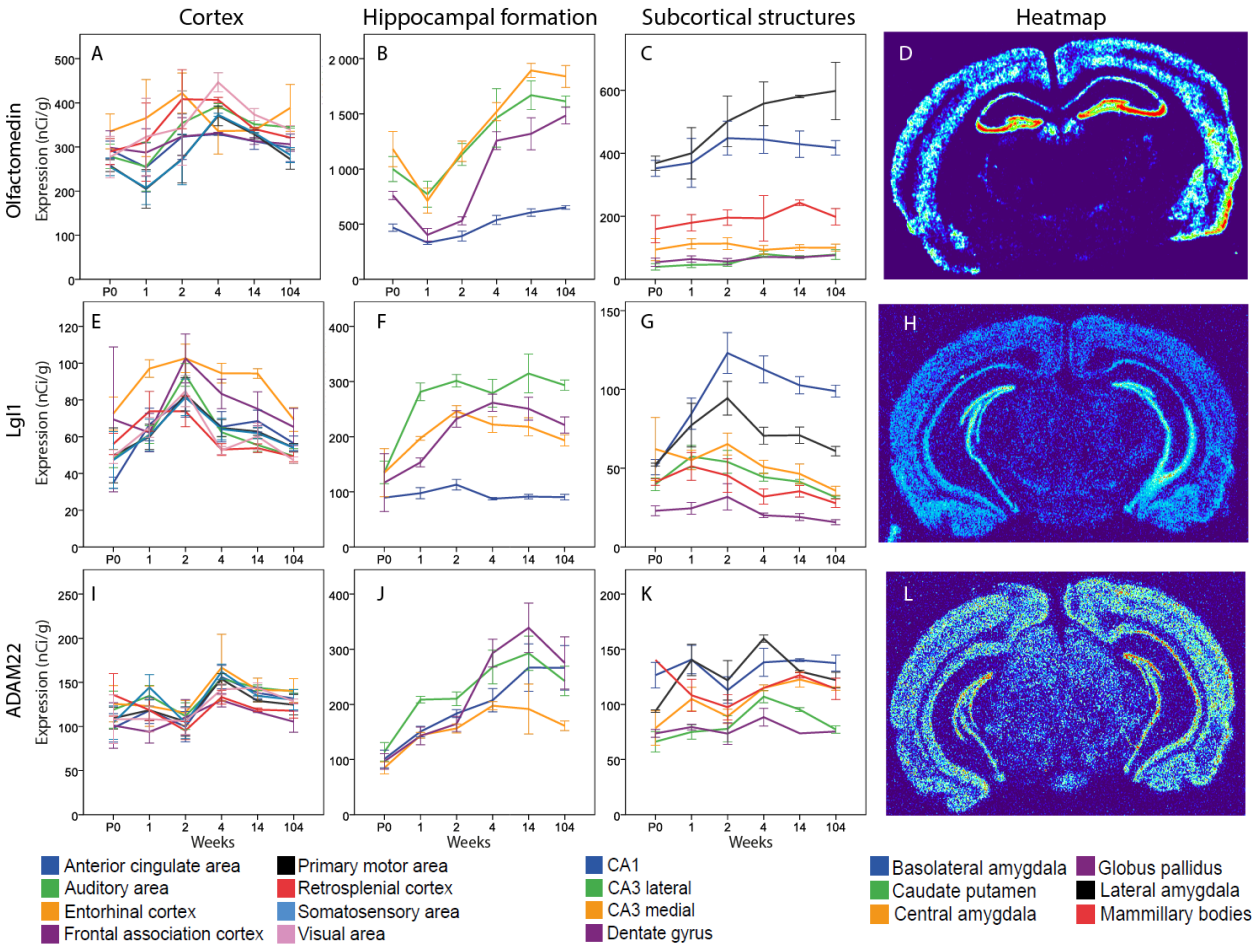

Figure 21: The mRNA expression for Olfactomedin (A–C), Lgl1 (E–G) and ADAM22 (I–K) in 18 cerebral regions over time. For full legend see Figure 17.

Taken togther, these results including six different ages from newborn (P0) to aged (104 weeks) mice, demonstrate an age and region specific expression of each individual Nogo-related gene. The results also suggest higher levels of expression in regions associated with high levels of structural plasticity.

PAPER III
KEY NOGO GENES NOT CORRELATED TO MIGRAINE

The causes of migraine are incompletely understood, although part of the explanation is due to genetic components. Features such as auras caused by spreading depression in visual cortex, and other brain activities may elicit kindling-like structural plasticity to permanent or aggravate the condition. Paper III thus investigates the potential involvement of five key Nogo-type signaling genes (Nogo-A, NgR1, LINGO1, OMgp, and MAG), by screening 15 SNPs and their potential association to migraine in a Swedish cohort consisting of 749 migraine cases and 4,032 controls. A logistic regression analysis was performed with sex as covariate to take the 3.6 x higher frequency of migraine among women (in our cohort) into account. After Bonferroni corrections the P-values were 1 for each SNP (Table 4). Hence, we did not find a significant association between Nogo-type SNPs and the frequency of migraine.

Table 4: No significant relations between 15 SNPs related to Nogo-type signaling and migraine.

Gene	SNP	Function	Minor allele	MAF cases	MAF controls	OR (95% CI)	P-value
Nogo-A	rs2580765	Intron	C	0.46	0.43	1.09 (0.97-1.22)	0.14
	rs6715980	Intron	A	0.07	0.07	1.04 (0.83-1.29)	0.76
	rs17046589	Intron	G	0.18	0.18	1.003 (0.87-1.16)	0.96
	rs10496037	Intron	T	0.12	0.11	1.08 (0.91-1.29)	0.36
Omgp	rs11080149	Coding	T	0.17	0.15	1.08 (0.92-1.25)	0.35
MAG	rs6510476	Intron	G	0.18	0.18	1.01 (0.87-1.17)	0.92
	rs2301600	Coding	T	0.25	0.23	1.07 (0.94-1.22)	0.33
	rs9304870	Intron	G	0.38	0.38	1.03 (0.91-1.15)	0.66
NogR1	rs701427	Intron	A	0.32	0.34	0.93 (0.83-1.05)	0.26
	rs1567871	Intron	T	0.25	0.25	1.0 (0.88-1.14)	1.00
	rs855050	Intron	G	0.51	0.50	1.04 (0.93-1.17)	0.47
LINGO-1	rs3144	3’ UTR region	C	0.37	0.37	0.97 (0.86-1.09)	0.56
	rs907396	Intron	G	0.40	0.38	1.1 (0.98-1.24)	0.11
	rs8023571	Intron	T	0.12	0.12	1.02 (0.86-1.22)	0.79
	rs8028788	Intron	C	0.05	0.04	1.17 (0.91-1.52)	0.23

Chr =Chromosome, SNP = Single Nucleotide Polymorphism, 3’ UTR = three prime untranslated region, MAF = Minor Allele Frequency, OR = Odds Ratio, CI = confidence interval, P-values = α 0.05, Corrected P-value = Bonferroni correction based on α / 15 (nr of SNPs).

As haplotypes, groups of SNPs inherited together, are considered to correlate stronger to disease than single SNPs alone (Zhang et al., 2002) we also searched for these. We found three haplotype blocks (Table 5), however, none of them with a significant relation to our migraine cohort.

Table 5: Haplotype blocks in genes associated with Nogo-type signaling were not associated with migraine.

	Block	Haplotype	Frequency	Case : Control Frequencies	P-value
LINGO1	rs907396 rs8023571	CC	0.41	0.43 : 0.41	0.17
		AC	0.34	0.32 : 0.34	0.21
		CT	0.25	0.25 : 0.25	0.83
MAG	rs6510476 rs2301600	AC	0.59	0.58 : 0.59	0.23
		AT	0.23	0.25 : 0.23	0.18
		GC	0.18	0.18 : 0.18	0.96
RTN4R	rs701427 rs1567871	TC	0.50	0.49 : 0.50	0.26
		GC	0.38	0.39 : 0.38	0.26
		TT	0.12	0.12 : 0.12	0.89

Power of the results

The lack of identified correlations between 15 SNPs associated with five genes of Nogo-type signaling and migraine should be evaluated by power analysis. The sample size and publicly available MAF values underlying the power analysis indicated that we would need ORs far exciding those later identified in our report (0.97-1.17). Our calculation showed that for 80 % power, our SNPs would need ORs between 1.27 and 1.74. and for 95 % power ORs 1.34-1.97. A power of 80 % is the commonly accepted level when repeated experiments are performed, such as cell cultures. However, since the nature of this cohort is rather distinctive, replication studies where each replicate decrease the risk of missing an actual effect, would be hard to obtain, and as a power of 80 % indicates that we could miss a positive correlation among 3 of our 15 SNPs, we found it reasonable to also look at the ORs needed for a power of 95 %. Thus, rather than considering the results as purely negative, they define a theoretical upper level of how strongly these SNPs would be likely to influence the prevalence of migraine.

PAPER IV
ROBUST IMPACT ON SYNAPTIC PLASTICITY BY PSILOCYBIN

In the last paper the impact of psilocybin on synaptic markers over time was investigated. Two different time curves illustrate how psilocybin has a robust impact on synaptic plasticity. Individual markers are associated with either a decreased (Synapsin I) or increased (Piccolo and Homer1) number of puncta in response to activity.

Psilocybin increases density of synaptic markers, suggesting increased plasticity and connectivity

When neurons are activated, Synapsin I becomes phosphorylated and disperse from the pre-synaptic terminal into the axon leading to a transient reduction in the number of Synapsin I immunoreactive puncta (Chi et al., 2001). Paper IV investigated Synapsin I expression at 4 timepoints after administration of psilocybin to cultured hippocampal neurons. Interestingly, there was a gradual decrease in the number of Synapsin I puncta during the first 30 min after psilocybin treatment. The effect of treatment was ($p = 4.62x10^{-7}$) with significant reductions starting at 15 min ($p = 0.008$) continuing to 30 min ($p = 0.0002$) (Figure 22). This indicates potent activation of pre-synaptic activity. One hour after treatment the number of puncta in psilocybin treated cultures no longer differed from numbers in control cultures ($p = 0.343$).

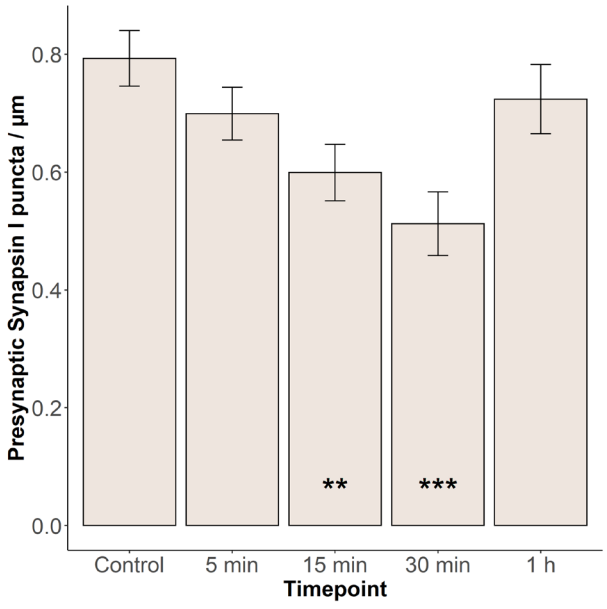

Figure 22: Increase of presynaptic activity during the first 30 min after psilocybin treatment as indicated by the reduction of Synapsin I puncta/µm. From left to right: 1-hour vehicle control followed by 5 min, 15 min, 30 min and 1 h after treatment with psilocybin. N = 20 per timepoint. Stars indicate difference to control treatment. Error bars = SEM.

The reduction in Synapsin I expression could either be due to strongly activated terminals or to a reduction in the number of pre-synaptic sites. To address this issue, an additional time curve was performed where we fixed hippocampal cell cultures after treatment with either

psilocybin or vehicle treatment at four timepoints (1 h, 3 h, 6 h and 24 h). As no significant effect was detected in the vehicle-treated groups they were pooled with the control group. The number of pre-synaptic sites was assessed using an antibody to Piccolo, important for synaptic vesicle retrieval (Ackermann et al., 2019), and to post-synaptic sites using an antibody to the immediate early gene Homer1 (Jaubert et al., 2007).

The second time curve also found hippocampal neurons to respond robustly to psilocybin treatment. Specifically, psilocybin increased the number of pre-synaptic puncta/µm at 1 h and 3 h (Figure 23A). This was mirrored in an increase at the same timepoints for the postsynaptic marker Homer1 (Figure 23B). In this experiment, the group was twice as big as in the Synapsin I time curve and thereby the results were more robust. Also, there was a higher density of synaptic markers than in the previous time curve. The effect of treatment was ($p = 2x10^{-16}$). The post hoc analysis showed significant differences between the 1 h ($p = 6.87x10^{-13}$) and 3 h ($p = 1.56x10^{-9}$) psilocybin treated groups in comparison to controls. Both pre- and postsynaptic markers demonstrated expression levels corresponding to controls at 6 h and 24 h after treatment.

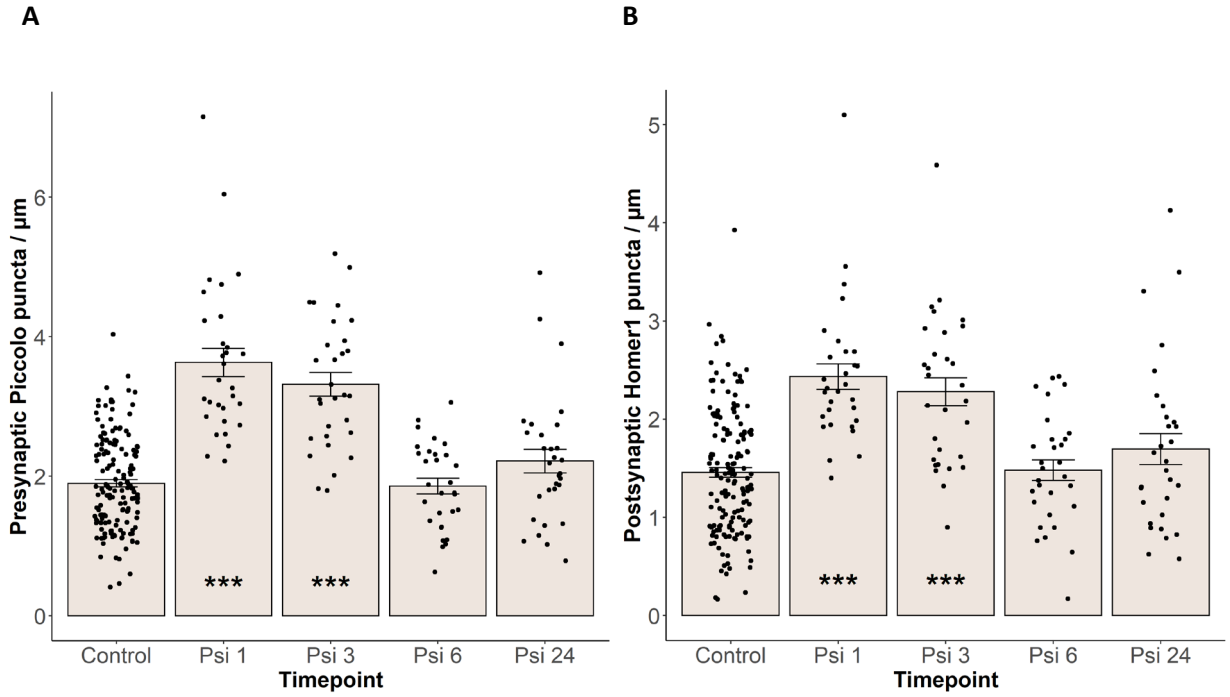

Figure 23: Number of presynaptic Piccolo puncta (A) and postsynaptic Homer1 puncta (B) on second order dendrites, 1, 3, 6 and 24 h after psilocybin or control treatment. Each black dot represents an individual neuron. N = 30 per timepoint after treatment with psilocybin. N = 159 for pooled controls. Stars indicate difference to control treatment. Error bars = SEM.

Our results with three different immunohistological markers show that psilocybin potently impact pre- and postsynaptic protein puncta, in a protein and time-dependent manner. This has to the best of our knowledge not been illustrated before. Previously known is that different serotonergic receptors respond to different concentrations of psilocybin (Halberstadt and Geyer, 2011). As the concentration of psilocybin reasonably differ over time due to

metabolism in our cell cultures, activation of various serotonergic receptors, inhibitory as well as excitatory (Berumen et al., 2012), are probably occurring over the time-scale used in the present study. To find out which level of synaptic activity that permits the lasting effects of the substance require further studies.

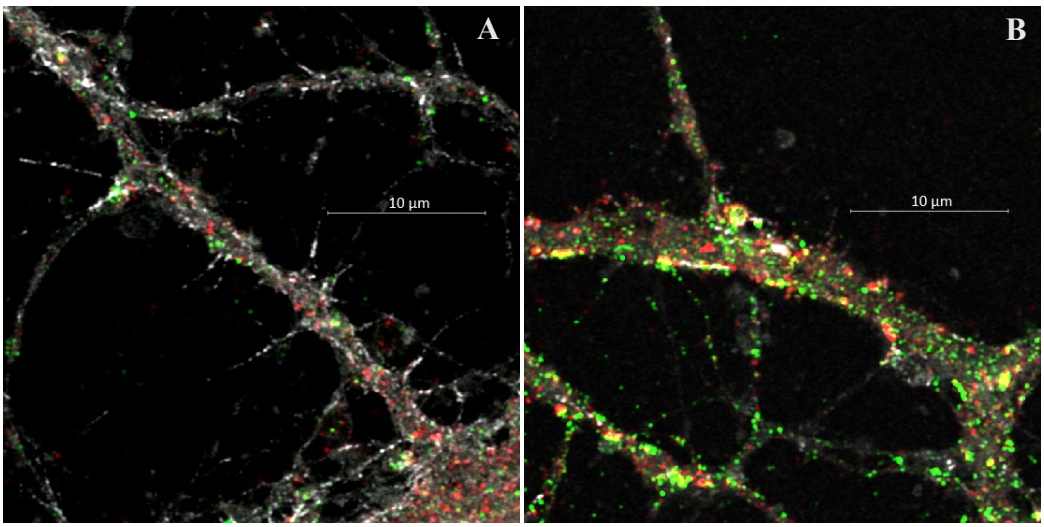

Figure 24: Airyscan images visualizing the distribution of synaptic markers on second order dendrites in cultured hippocampal neurons. (A) Control cell treated with neurobasal media. (B) Cell 1h after treatment with psilocybin. Green: presynaptic Piccolo, red: postsynaptic Homer1, white: actin stained with phalloidin.

Neuritogenesis in response to psilocybin

We did not find effects of psilocybin on neuritogenesis in vitro (data mentioned but not shown in results section). Measurements of dendritic length could only be reliably performed on neurons that were not in close contact with other neurons. Most of our cultured neurons tend to grow rapidly and form extensive dendrites that intertwine with those of other neurons. As neurons that have established connections with other neurons reasonably live in a more representative environment, probably with a higher concentration of growth factors secreted by nearby cells, the issue arises that neurons accessible for tracing can be the least healthy ones. Thus, those data were not presented above. To rightfully analyze healthy cultured neurons regarding neuritogenesis, a method for sparse labelling, e.g. via a GFP-vector, should be applied to enable investigation of individual neurons that are integrated in a network of neurons.

CONCLUSIONS

This thesis focuses on neuronal plasticity from a preclinical to a clinical perspective.

Paper I demonstrates how a constitutive overexpression of ***NgR1 impairs memory consolidation and affects structural plasticity*** in frontal association cortex, cingulate cortex, and nucleus accumbens. Paper I also shows this overexpression to reduce the emergence of stereotypic-like behavior in response to repeated exposure to the stimulatory drug cocaine, and to alter the structural responses to this drug.

Paper II reveals the ***dynamic regulation of Nogo genes over time.*** By investigating mRNA levels, the intricate Nogo-system of ligands, receptors, co-receptors and modulators was mapped. The different mRNA species are expressed throughout the brain and demonstrate individual age-related patterns. Some fluctuation of the analyzed mRNA species was often observed in the young and maturing brain. This was followed by a more static, although not infrequently declining, expression during ageing.

Paper III describes Nogo-type signaling genes in migraine. The migraine brain is known for structural differences but SNPS in ***key Nogo genes were not correlated to migraine*** in Paper III. To rule out a potential role of Nogo-type signaling in migraine further studies are however needed, as the system may be involved in the synaptic regulation even though it is not a predisposing factor for the disease. Future studies will complement the picture of the potential link between Nogo-type signaling and migraine.

Paper IV addresses the possible roles of psilocybin on neuronal structure. Psilocybin is a psychedelic substance with long-lasting effects after a single or few administrations. The substance has previously been shown to induce structural plasticity in cortical neurons, but little is known about the synaptic mechanisms. Paper IV investigated the impact of psilocybin on synaptic markers over time in cultured hippocampal neurons and ***a strong impact on both pre- and postsynaptic markers by psilocybin was found*** with a peak effect at 1 hour after treatment. These findings indicate potent effect on synaptic plasticity.

FUTURE DIRECTIONS THROUGH A CLINICAL PERSPECTIVE

This thesis supports Nogo-type signaling as a contributing actor in the riddle of neuronal plasticity. It also suggests psilocybin (and hence serotonin) as an interesting agent for further studies on this topic. Future work should include, but not be limited to, investigating the impact of psilocybin on Nogo-type signaling. An initial approach would be to study the regulation of NgR1 in response to psilocybin. This experiment would illustrate how a growth inhibitory system behaves in response to a substance that allegedly induce neuronal plasticity. Since synaptic activation by other compounds can downregulate NgR1, it could be speculated that psilocybin causes the same effect.

Another approach which could reveal central information about the complexity before us, would be to apply a corresponding time curve to *in vivo* experiments and collect tissue at the same timepoints after administration of psilocybin as used *in vitro*. Brain tissue from different regions to detect regional differences and to cover different neuronal functions (preferably cortex, hippocampus and cerebellum) could also be used for RNA sequencing (RNA-seq). This would reveal the plethora of genetic regulations supposed to occur over time and mirror the genetics of rapid yet long lasting structural and behavioral modifications.

Having described the temporal patterns of regulation of NgR1, and several other genes known to affect plasticity, using the RNA-seq, it becomes possible to also acquire information about when plastic windows are optimal in relation to a treatment. A window of increased plasticity could be enhanced by psilocybin for treatment of conditions craving this, such as stroke or traumatic brain injuries. Whether such application could have potential for the acute phase, during rehabilitation, or both, is yet another parameter to investigate.

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Due to current COVID-19 circumstances this thesis is not accompanied by a dinner party invitation. The feast is however only postponed, and I am very much looking forward to celebrate my academic freedom with all of you beautiful souls whom I am sincerely grateful to know:

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