

Involvement of neuronal nitric oxide synthase (NOS-I) PDZ interactions in neuropsychiatric disorders

Der Einfluss von PDZ Interaktionen der neuronalen Stickstoffmonoxidsynthase (NOS-I) auf neuropsychiatrische Störungen



**Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section Neuroscience**

**submitted by
Esin Candemir
from Istanbul, Turkey**

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Summary

Neuronal nitric oxide (NO) synthase (NOS-I) and its adaptor protein (NOS1AP) have been repeatedly and consistently associated with neuropsychiatric disorders in several genetic association and linkage studies, as well as functional studies. NOS-I has an extended PDZ domain which enables it to interact with postsynaptic density protein 95 (PSD-95) bringing NOS-I in close proximity to NMDA receptors. This interaction allows NMDA receptor activity dependent calcium-influx to activate NOS-I, linking NO synthesis to regulation of glutamatergic signaling pathways. NOS1AP is a PDZ-domain ligand of NOS-I and has been proposed to compete with PSD-95 for NOS-I interaction. Studies performed on post-mortem brain tissues have shown increased expression of NOS1AP in patients with schizophrenia and bipolar disorder, suggesting that increased NOS-I/NOS1AP interactions might be involved in neuropsychiatric disorders possibly through disruption of NOS-I PDZ interactions. Therefore, I have investigated the involvement of NOS-I in different endophenotypes of neuropsychiatric disorders by targeting its specific PDZ interactions *in vitro and in vivo*. To this end, I used recombinant adeno-associated virus (rAAV) vectors expressing NOS1AP isoforms/domains (NOS1AP-L: full length NOS1AP; NOS1AP-L_{C20}: the last 20 amino acids of NOS1AP-L, containing the PDZ interaction motif suggested to stabilize interaction with NOS-I; NOS1AP-L_{ΔC20}: NOS1AP-L lacking the last 20 amino acids; NOS1AP-S: the short isoform of NOS1AP), residues 396-503 of NOS1AP-L (NOS1AP₃₉₆₋₅₀₃) encoding the full NOS-I interaction domain, and N-terminal 133 amino acids of NOS-I (NOS-I₁₋₁₃₃) encoding for the extended PDZ-domain.

Neuropsychiatric disorders involve morphological brain changes including altered dendritic development and spine plasticity. Hence, I have examined dendritic morphology in primary cultured hippocampal and cortical neurons upon overexpression of constructed rAAV vectors. Sholl analysis revealed that overexpression of NOS1AP-L and NOS1AP-L_{ΔC20} mildly reduced dendritic length/branching. Moreover, overexpression of all NOS1AP isoforms/domains resulted in highly altered spine plasticity including significant reduction in the number of mature spines and increased growth of filopodia. These findings suggest that NOS1AP affects dendritic growth and development of dendritic spines, which may involve both, increased NOS-I/NOS1AP interaction as well as interaction of NOS1AP with proteins other than NOS-I. Interestingly, the

observed alterations in dendritic morphology were reminiscent of those observed in post-mortem brains of patients with neuropsychiatric disorders.

Given the dendritic alterations *in vitro*, I have examined, whether disruption of NOS-I PDZ interaction would also result in behavioral deficits associated with neuropsychiatric disorders. To this end, rAAV vectors expressing NOS1AP-L, NOS1AP₃₉₆₋₅₀₃, NOS-I₁₋₁₃₃, and mCherry were stereotaxically delivered to the dorsal hippocampus of 6-week-old male C57Bl/6J mice. One week after surgery, mice were randomly separated into two groups. One of those groups underwent three weeks of chronic mild stress (CMS). Afterwards all mice were subjected to a comprehensive behavioral analysis. The findings revealed that overexpression of the constructs did not result in phenotypes related to anxiety or depression, though CMS had an anxiolytic effect independent of the injected construct. Mice overexpressing NOS-I₁₋₁₃₃, previously shown to disrupt NOS-I/PSD-95 interaction, showed impaired spatial memory, sensorimotor gating, social interaction, and increased locomotor activity. NOS1AP overexpressing mice showed mild impairments in sensorimotor gating and spatial working memory and severely impaired social interaction. NOS1AP₃₉₆₋₅₀₃ overexpressing mice also showed impaired social interaction but enhanced sensorimotor gating and reduced locomotor activity. Taken together, these behavioral findings indicate an involvement of NOS-I PDZ interactions in phenotypes associated with positive symptoms and cognitive deficits of psychotic disorders.

In summary, this study revealed an important contribution of NOS-I protein interactions in the development of endophenotypic traits of neuropsychiatric disorders, in particular schizophrenia, at morphological and behavioral levels. These findings might eventually aid to a better understanding of NOS-I-dependent psychopathogenesis, and to develop pharmacologically relevant treatment strategies.

Zusammenfassung

Die neuronal Stickstoffmonoxid(NO)synthase (NOS-I) und deren Adapterprotein (NOS1AP) wurden in mehreren Genassoziations- und Genkopplungsstudien, sowie funktionellen Studien, wiederholt und konsistent mit neuropsychiatrischen Störungen assoziiert. NOS-I trägt eine erweiterte PDZ Domäne, die eine Interaktion mit postsynaptic density protein 95 (PSD-95) ermöglicht und es in die Nähe von NMDA Rezeptoren bringt. Diese Interaktion erlaubt es NMDA Rezeptoraktivitätsabhängigen Kalziumeinstrom NOS-I zu aktivieren, was die Synthese von NO an die Regulierung glutamaterger Signalwege koppelt. NOS1AP ist ein Ligand der NOS-I PDZ Domäne und NOS1AP kompetiert mit PSD-95 um die Bindung mit NOS-I. Post mortem Untersuchungen zeigten eine erhöhte Expression von NOS1AP im Gehirn von Patienten mit Schizophrenie und bipolarer Störung, was eine erhöhte NOS-I/NOS1AP Interaktion (was möglicherweise zu gestörter NOS-I PDZ Interaktion führt) mit neuropsychiatrischen Störungen verbindet. Daher habe ich den Einfluss von NOS-I auf Endophänotypen neuropsychiatrischer Störungen untersucht, indem ich spezifische PDZ Interaktionen von NOS-I *in vitro* und *in vivo* gestört habe. Dazu verwendete ich rekombinante Adenoassoziierte virale (rAAV) Vektoren, die NOS1AP Isoformen/Domänen (NOS1AP-L: Volllänge NOS1AP; NOS1AP-L_{C20}: Die letzten 20 Aminosäuren von NOS1AP-L, welche das PDZ Interaktionsmotiv enthalten, das zur Stabilisierung der Interaktion mit NOS-I beiträgt; NOS1AP-L_{ΔC20}: NOS1AP-L dessen letzte 20 Aminosäuren fehlen; NOS1AP-S: die Kurzform von NOS1AP), Aminosäurereste 396-503 von NOS1AP-L (NOS1AP₃₉₆₋₅₀₃), welche die volle NOS-I Interaktionsdomäne kodieren, und die N-terminalen 133 Aminosäuren von NOS-I (NOS-I₁₋₁₃₃), welche die erweiterte PDZ Domäne enthalten.

Bei neuropsychiatrischen Störungen kommt es zu morphologischen Änderungen des Gehirns, einschließlich veränderter dendritischer Entwicklung und Plastizität dendritischer Dornfortsätze („spines“). Daher habe ich die dendritische Morphologie in primär kultivierten Hippokampal- und Kortexneuronen nach Überexpression der konstruierten rAAV Vektoren untersucht. Eine Sholl Analyse ergab dabei, dass die Überexpression von NOS1AP-L und NOS1AP-L_{ΔC20} die Länge und Anzahl dendritischer Verzweigungen leicht reduzierte. Zudem führte die Überexpression aller NOS1AP Isoformen/Domänen zu einer stark veränderten Plastizität dendritischer „spines“, einschließlich einer signifikanten Reduktion der Anzahl ausgereifter „spines“ und einem erhöhten Wachstum von Filopodien. Diese Ergebnisse zeigen, dass NOS1AP einen Einfluss auf das

dendritische Wachstum und die Entwicklung dendritischer ‚spines‘ hat, dem sowohl eine erhöhte NOS-I/NOS1AP Interaktion, sowie Interaktionen von NOS1AP mit anderen Proteinen zugrunde liegen könnten. Interessanterweise, ähnelten die beobachteten Veränderungen solchen, die in post mortem Gehirnen von Patienten mit neuropsychiatrischen Störungen beobachtet wurden.

Aufgrund der Beobachtungen *in vitro*, habe ich untersucht, ob eine Störung der NOS-I PDZ Interaktion auch zu Verhaltensdefiziten, die mit neuropsychiatrischen Störungen assoziiert sind, führt. Zu diesem Zweck, wurden rAAV Vektoren, die NOS1AP-L, NOS1AP₃₉₆₋₅₀₃, NOS-I₁₋₁₃₃, und mCherry exprimieren, stereotaxisch in den dorsalen Hippokampus von sechs Wochen alten männlichen C57Bl/6J Mäusen injiziert. Eine Woche nach der Operation wurden die Mäuse zufällig in zwei Gruppen aufgeteilt. Eine dieser Gruppen wurde für drei Wochen dem ‚chronic mild stress‘ (CMS) Paradigma unterzogen. Im Anschluss daran wurden alle Mäuse einer umfassenden Verhaltensanalyse unterzogen. Die Ergebnisse zeigten, dass die Überexpression der Konstrukte nicht zu Angst- oder Depressionsassoziierten Phänotypen führten. Jedoch hatte das CMS Paradigma einen anxiolytischen Effekt, der unabhängig vom injizierten Konstrukt war. Eine Überexpression des NOS-I₁₋₁₃₃ Konstruktes, von welchem zuvor eine Störung der NOS-I/PSD-95 Interaktion nachgewiesen wurde, führte zu Störungen des räumlichen Kurzzeitgedächtnisses, der Reaktionsunterdrückung (‚sensorimotor gating‘) und der sozialen Interaktion, sowie zu erhöhter lokomotorischer Aktivität. NOS1AP überexprimierende Mäuse zeigten leichte Störungen in der Reaktionsunterdrückung und des räumlichen Kurzzeitgedächtnisses, sowie erheblich gestörte soziale Interaktionen. NOS1AP₃₉₆₋₅₀₃ überexprimierende Mäuse zeigten ebenfalls gestörte soziale Interaktion, jedoch eine erhöhte Reaktionsunterdrückung und verminderte lokomotorische Aktivität. Zusammengefasst, deuten diese Verhaltensuntersuchungen auf einen Beitrag der NOS-I PDZ Interaktionen zu Phänotypen, die mit positiven Symptomen und kognitiven Defiziten bei Psychosen assoziiert sind, hin.

Zusammengefasst konnte diese Studie einen wichtigen Beitrag der NOS-I Proteininteraktionen bei der Entstehung endophenotypischer Züge (morphologisch sowie im Verhalten) neuropsychiatrischer Störungen, insbesondere der Schizophrenie, aufzeigen. Diese Erkenntnisse könnten zu einem besseren Verständnis NOS-I abhängiger Psychopathogenese, sowie zur Entwicklung relevanter pharmakologischer Behandlungsstrategien führen.

1. Introduction

1.1. Neuropsychiatric disorders

Neuropsychiatric disorders compromise emotional, cognitive, executive and social functioning in different ways. The most prevalent neuropsychiatric disorders are anxiety disorders, mood disorders and psychotic disorders such as schizophrenia (Edwards *et al*, 2016). These disorders disrupt not only behavior and social relationships but also professional-occupational functioning (Edwards *et al*, 2016) causing severe disability to those affected and their families, and create a tremendous social and economic burden on societies. A recent study has shown that mental and behavioral disorders caused 7.4 percent of global disability-adjusted life years in 2010 (Murray *et al*, 2012). Latest reports estimated the number of affected individuals by any mental disorder at 10-30% (Wittchen *et al*, 2011; World Health Organisation, 2016). Due to their complex nature, neuropsychiatric disorders might occur comorbidly with other forms of disorders increasing their burden (Edwards *et al*, 2016). In a study based on the analysis of German patients' symptoms in a period of 12 months, researchers showed 39.5% of patients showed comorbid disorders and for example 40% of patients with psychotic disorders had comorbidity with at least 3 or more psychiatric disorders (Wittchen *et al*, 2011). Another factor escalating the burden is the age of onset. Early onset causes disrupted education and sociability during transition from adolescence to adulthood. Kessler *et al*. (2007) suggested the age of onset for these disorders at >4-7 years for impulsivity disorders, <20 years for anxiety disorders, >25 years for mood disorders, >18 years for substance use disorders and >15 years for schizophrenia spectrum diagnoses. Even though patients who receive an early diagnosis with good life-time social and medical support can achieve a relatively high quality of life, unfortunately that is not the case for many affected individuals (Edwards *et al*, 2016).

Despite their high heritability (Plomin *et al*, 1994), it has been long known that most of these disorders do not fit to Mendelian genetics and they occur due to a complex interaction of genetic susceptibility factors, biochemical imbalance influencing neurotransmission, and environmental factors (Arslan, 2015). There is a significant amount of research being performed per year to uncover the underlying neurobiological mechanisms and their interactions with influential environmental factors, however our understanding of disease pathogenesis and precise risk factors

remains still inadequate. I will discuss research approaches and integration of these approaches in following sections in more detail (see section 1.3).

1.1.1. Gene-environment interactions in neuropsychiatric disorders

Evidence from studies focusing on genotype-phenotype associations remained insufficient to provide scientists a direct pathway to understand the development of neuropsychiatric disorders (Gottesman and Gould, 2003; Hyman, 2008). Many of these disorders have already been known to have environmental causes (e.g. maternal substance abuse, childhood maltreatment, stressful life events...) and individuals might show differences in their susceptibility or coping mechanisms. Inter-individual differences against similar environmental risk factors and insufficient evidence from gene-disorder studies caused research to be directed towards investigating the interplay of genetics and environmental influences (Caspi and Moffitt, 2006; Hunter, 2005).

The brain undergoes critical developmental periods starting from early prenatal stages till late adolescence (Stiles and Jernigan, 2010). Human and animal studies have shown that acute or chronic exposure to stress, especially at the early stages of life, are decisive in terms of programming and plasticity of the brain that will have life-long enduring behavioral outcomes. Genetic liability might cause a stronger vulnerability/resilience under the influence of specific environmental factors or an environmental influence might be stronger/weaker on an individual depending on their genetic background (Homberg, 2012).

Interaction of life stressors and genetic disposition have been very well established for psychiatric conditions. In humans, prenatal stress (maternal stress or depression) has been linked to higher glucocorticoid response (essential mediators of stress responses) in offspring (O'Connor *et al*, 2005). In addition, prenatal stress in combination with low postnatal care might be associated with reduced hippocampal volume in adulthood (Buss *et al*, 2007). In adulthood, chronic exposure to stressors in combination with genetic liability increases the risk of developing psychiatric disorders. These stressors might include employment (i.e. work load, low appreciation), neighborhood socio-economic status, urbanization, belonging to a minority, violence, and substance abuse (Edwards *et al*, 2016). For illustration, a recent study has revealed that single nucleotide polymorphisms (SNPs) in neuronal nitric oxide synthase (*NOS1*) gene mediates the risk of depression when it is combined with exposure to economic and psychosocial stressors (Sarginson *et al*, 2014).

1.1.2. Synapse and dendrite maintenance

Emerging evidence has linked gray matter loss and disrupted dendritic development to a variety of psychiatric and neurodegenerative disorders. Dendritic branching is a crucial process for proper signal integration in neuronal circuits and mediated by microtubule and actin organization. As a post-synaptic neuron matures, microtubule polarity leads to formation of dendritic protrusions called filopodia which will be stabilized to dendritic branches (Jan and Jan, 2010; Penzes *et al*, 2011; Sweet *et al*, 2011). On the other hand, dendritic filopodia are also suggested to be an important intermediate structure during spinogenesis, initiating synaptic communication with surrounding axons. Upon sufficient transmission, filopodia are replaced by shaft synapses and dendritic spines (Fiala *et al*, 1998; Yuste and Bonhoeffer, 2004). Previous studies have shown decreased number and reduced motility of those filopodia via increased activity of post-synaptic membrane associated guanylate cyclases (MAGUKs) in developing neurons indicating a potential role for filopodia as dendritic spine precursors (Sweet *et al*, 2011; Yuste and Bonhoeffer, 2004). Time-lapse imaging studies have shown that spine plasticity is a reversible mechanism which is highly dependent on synaptic activity, in particular N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type glutamate receptors (Hering and Sheng, 2001).

1.2. Nitric oxide (NO) system

Nitric oxide (NO) has been shown to involve in pathogenesis of many neuropsychiatric disorders from psychotic disorders to mood disorders (Chung and Park, 2003). NO is a gaseous neurotransmitter, first identified as endothelial derived relaxing factor in blood vessels in the 1980's (Ignarro *et al*, 1987). Garthwaite *et al*. (1988) suggested that following the activation of NMDA receptors in cerebellum, release of NO was triggered and led to activation of soluble guanylate cyclase (sGC), an enzyme that synthesizes cyclic GMP (cGMP). Later studies have shown that production of NO was highly selective to neurons and blood vessel endothelia (Bredt *et al*, 1990). Its selectivity to neurons indicated a role for NO to be an important neural messenger. NO is enzymatically produced by NO synthase (NOS) isoforms which are distinct in their regulatory mechanisms (Blum-Degen *et al*, 1999): endothelial NOS (eNOS or NOS-III) is mainly responsible for vascular tone, neuronal (nNOS or NOS-I) is mostly required for the production of NO in neurons. Both NOS-I and -III are calcium-calmodulin controlled. The, third isoform,

inducible NOS (iNOS or NOS-II), produces NO upon stimulation by cytotoxins in the immune system (Blum-Degen *et al*, 1999; Bredt and Snyder, 1990).

1.2.1. *NOS1* gene

The *NOS1* gene has been consistently associated with psychiatric disorders (Freudenberg *et al*, 2015; Luciano *et al*, 2012; O'Donovan *et al*, 2008). The *NOS1* gene, localized to chromosome 12q24.2-31 in humans (Xu *et al*, 1993) and chromosome 5 in *Mus musculus* (Lee *et al*, 1995), is one of the most complex genes consisting of 29 exons and 28 introns spanning over 240 kb of human genomic DNA (Bros *et al*, 2006; Hall *et al*, 1994; Wang *et al*, 1999). Complexity of the gene in transcription, pre-mRNA splicing and translation levels results in high mRNA and protein diversity. So far, a total of 12 exon 1 variants (1a-1l) of the human *NOS1* gene scattered over approximately 50 kb of genomic region has been demonstrated due to presence of exon-specific alternative promoters which might enhance cell-type specific usage or developmental regulation (Boissel *et al*, 1998; Bros *et al*, 2006; Wang *et al*, 1999). Studies have also shown that there are three alternative exon 1 variants in mouse and six alternative exon 1 variants in rats. In addition, exons 1f and 1g are conserved between humans and rodents (Bros *et al*, 2006). Alternative mRNA splicing is another strong factor effecting *NOS1* gene products (e.g. NOS-I α , NOS-I β , NOS-I γ ...), however regulation and significance of these splicing events remains to be understood. NOS-I α is the full-length transcript also encoding for the PDZ-domain (postsynaptic density protein 95 (PSD95) /discs large 1/zona occludens 1) coding exon which is absent in other splice variants as NOS-I β and NOS-I γ .

1.2.2. Neuronal nitric oxide synthase (NOS-I) protein

Neuronal nitric oxide synthase (NOS-I), in humans is a 1434-amino-acid protein (Molecular weight: 160970 Da) encoded by the *NOS1* (NOS-I α) gene, is the primary source of NO in the central nervous system (Boissel *et al*, 1998). NOS-I ([Figure 1](#)) consists of an N-terminal extended PDZ domain (Tochio *et al*, 1999; Wang *et al*, 2000), an oxygenase domain carrying binding sites for L-arginine, BH₄ ((6R)-5,6,7,8-tetrahydrobiopterin) and haem (iron protoporphyrin IX), a calmodulin-binding site linking the oxygenase domain to the reductase domain, which carries binding sites for FAD (flavin adenine dinucleotide), FMN (flavin adenine mononucleotide) and NADPH (Alderton *et al*, 2001). The NOS-I enzyme is dimeric in its active form, which requires the binding of haem and stabilization of the dimer by binding of L-arginine and BH₄. NOS-I

reduces NADPH to NADP⁺ at the reductase domain and donates the electrons by FAD and FMN redox carriers to the oxygenase domain of the dimer partner where L-arginine and oxygen are converted to L-citrulline and NO.

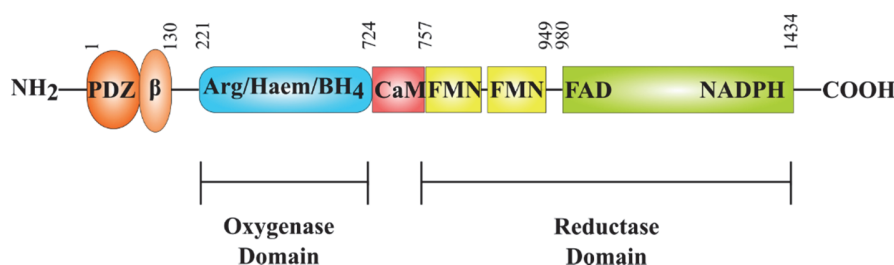


Figure 1. Structure of neuronal nitric oxide synthase (NOS-I). NOS-I carries an N-terminal PDZ domain followed by a β -finger which carries an internal PDZ motif. The oxygenase domain carries binding sites for L-arginine, BH₄ and haem which are required for NOS-I activity. CaM links the oxygenase domain to the reductase domain. Reductase domain consists of binding sites for cofactors FMN, FAD NADPH. Abbreviated binding sites: BH₄: (6R)-5,6,7,8-tetrahydrobiopterin, Arg: L-arginine, Haem: iron protoporphyrin IX, CaM: calmodulin, FMN: flavin adenine mononucleotide, FAD: flavin adenine dinucleotide, NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen. (adapted from Doucet *et al*, 2012)

Even though NOS-I is widely expressed throughout the brain with highest expression in limbic system, cerebellar cortex, striatum, it is present in only 1% of neurons (Blum-Degen *et al*, 1999; Snyder and Ferris, 2000). As a diffusible neurotransmitter, NO cannot be stored in the brain and has to be synthesized when required. Therefore, subcellular localization of NOS-I is decisive for its function. Studies have shown NOS-I localization in neurons either in cytoplasm or membrane-bound (Hecker *et al*, 1994; Rothe *et al*, 1998). It has been also shown to be localized in the nucleus of rat astrocytes (Yuan *et al*, 2004). NOS-I requires specific interactions (Figure 2) with other proteins (i.e. postsynaptic density proteins) which anchors it to postsynaptic membrane (Brenman *et al*, 1996). The core PDZ domain of NOS-I is followed by a 30-residue β -finger, containing an internal PDZ motif, which connects NOS-I to the PDZ2 domain of post-synaptic density protein 95 or 93 (PSD-95 or PSD-95) and anchors it to the post-synaptic density (Doucet *et al*, 2012; Hecker *et al*, 1994; Tochio *et al*, 2000; Wang *et al*, 2000). PSD-95 binds to GluN2 subunits (NR2A or NR2B) of NMDA receptors through its PDZ1 or PDZ2 domain (or even rarely PDZ3 to NR2B)(Vallejo *et al*, 2016). This NOS-I/PSD-95/NMDA receptor complex NMDA receptor activity-dependent Ca²⁺-influx induced activation of NOS-I (Alderton *et al*, 2001). PSD-95 and PSD-93 are not the only proteins that influence the localization or function of NOS-I. A recent study suggested that synapse-associated protein 97 (SAP97) can also act as a scaffolding protein providing a physical bridge between NOS-I and AMPA receptor subunit GluA1. Therefore, Ca²⁺-

permeable AMPA receptors may stimulate NO production through formation of a GluA1/SAP97/NOS-I complex (von Ossowski *et al*, 2017). The NOS-I PDZ domain also interacts with the carboxyterminus NOS1AP. NOS1AP binds to NOS-I PDZ domain via its internal ExF motif (Li *et al*, 2015) and PDZ ligand motif (Jaffrey *et al*, 1998). Jaffrey *et al* (1998) suggested that NOS-I/NOS1AP interaction directly competes with interaction between NOS-I and PSD-95, however a recent study argued that NOS-I/NOS1AP interaction might be induced by NMDA receptor activation. In both cases, NOS-I/NOS1AP interaction alters subcellular localization of NOS-I and mediates interaction with downstream signaling proteins (see section 1.2.3) (Jaffrey *et al*, 1998, 2002; Li *et al*, 2013).

Postsynaptically produced NO can diffuse to the presynaptic terminal of interacting axons and stimulate presynaptic soluble guanylyl cyclase (sGC) activity leading to an increased amount of cGMP (Burette *et al*, 2002; Garthwaite *et al*, 1989) which then triggers neurotransmitter release (Hoque *et al*, 2010; Jayakumar *et al*, 1999; Kiss, 2001; Kraus and Prast, 2001; Lonart *et al*, 1992). Furthermore, NO acts as a modulator of spine development (Kamiya *et al*, 2006; Nikonenko *et al*, 2008), synapse formation (Nikonenko *et al*, 2008; Sancesario *et al*, 2000), regulation of neurogenesis and cell proliferation (Gibbs, 2003). One key mechanisms of NO to regulate protein activity, protein-protein interactions and related cellular signaling pathways is S-nitrosylation. The term refers to a reversible posttranslational mechanism, which leads to the addition of an NO group to a cysteine thiol/sulfhydryl group of the target protein(s) (Shahani and Sawa, 2011). So far studies have shown that S-nitrosylation might protect cells from apoptotic cell death, prevent over-activation of NMDA receptors to reduce excitotoxicity, activate transcription factors such as estrogen receptor (ER) and hypoxia-inducible factor (HIF) which would have important roles in synaptic plasticity and neuronal cell viability respectively. Excessive S-nitrosylation of proteins such as HSP90, parkin and matrix metalloproteinase-9 has been linked to neurodegenerative disorders including Alzheimer's, Parkinson's diseases, and stroke. S-nitrosylation has also been shown to regulate the activity of PSD-95, NMDA receptors, and AMPA receptors (reviewed in Shahani and Sawa, 2011). NMDA receptor activity dependent production of NO promotes S-nitrosylation of AMPA receptor regulatory proteins (e.g. N-ethylmaleimide sensitive factor, NSF) increasing the AMPA receptor surface expression (Huang *et al*, 2005). Enhanced production of NO, can also lead to S-nitrosylation of NMDA receptor subunits (Lipton *et al*, 2000) or the GluA1 subunit of AMPA receptors (von Ossowski *et al*, 2017), which inactivates them providing a

negative feedback, which might regulate glutamate dependent neurotoxicity. Higher activity of glutamate receptors also leads to increased post-translational modification (e.g. palmitoylation) of PSD-95 which enables synaptic clustering of this protein which in turn may increase AMPA receptor surface clustering and/or bring NOS-I and NMDA receptors together to promote NO production. Increased NO then competes with palmitate to stimulate depalmitoylation of PSD-95 and reduced clustering of PSD-95 to the postsynaptic density (Ho *et al*, 2011).

1.2.3. NOS-I adaptor protein (NOS1AP)

NOS-I adaptor protein (NOS1AP), previously named carboxy-terminal PDZ ligand of NOS-I (CAPON), was first identified in the rat brain as a highly specific binding partner of the NOS-I PDZ domain, which was detected only in neurons and showed a high co-localization with NOS-I throughout the brain (Jaffrey *et al*, 1998).

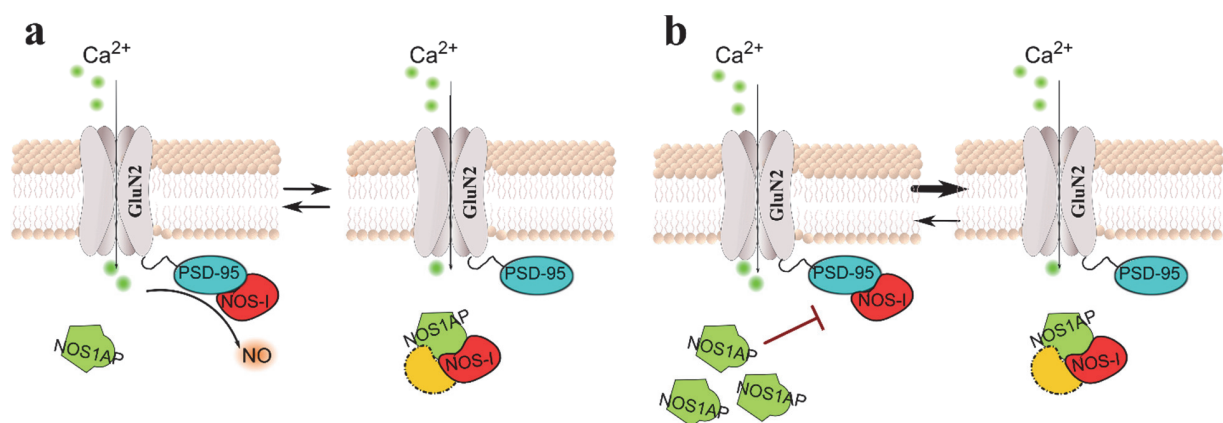


Figure 2. NOS-I PDZ interactions. a) NOS1AP competes with PSD-95 to interact with NOS-I PDZ domain and disrupts the NOS-I/PSD-95/NMDA receptor complex. b) NOS1AP was suggested to interrupt NOS-I interaction with post-synaptic density, possibly requiring interactions with downstream signaling proteins. (adapted from Candemir *et al*, 2016)

NOS1AP contains an N-terminal phosphotyrosine binding domain (PTB), followed by a carboxypeptidase E binding region (CPE) and a C-terminal PDZ-binding region. In humans, there are two known isoforms of NOS1AP; long isoform is the full length protein from ten exons and the other, a short isoform only bearing C-terminal PDZ-binding region and a unique 18-amino acid N terminus from last 2 exons but not PTB and CPE regions (Jaffrey *et al*, 1998; Seki *et al*, 1997; Xu *et al*, 2005). The PTB domain associates NOS1AP to a variety of physiological processes by binding to molecules as DexRas1, synapsin and Scribble (Figure 3). NOS-I/NOS1AP/Dexas1 complex, as mentioned before, activates DexRas1 via S-nitrosylation facilitating iron-dependent

neurodegeneration and NMDA receptor dependent neurotoxicity (Cheah *et al*, 2006; Fang *et al*, 2000). Additionally, NOS1AP enables formation of a ternary complex composed of NOS-I, NOS1AP and synapsin, which in turn determines localization of NOS-I to presynaptic vesicles (Jaffrey *et al*, 2002). Tumor suppressor protein Scribble directly interacts with the NOS1AP PTB domain in the synapse leading to an increase in dendritic protrusions (Richier *et al*, 2010). Binding of CPE to the central domain of NOS1AP has also been shown to regulate dendrite morphology in cultured rat hippocampal neurons (Carrel *et al*, 2009a). Originally, it has been suggested that the C-terminal 20 amino acids of NOS1AP were necessary and sufficient for NOS-I binding. Jaffrey *et al* (1998) were able to show interaction of NOS-I PDZ domain with as little as the C-terminal 13 amino acids of NOS1AP but not with a construct lacking the C-terminal 20 amino acids. They also showed that NOS1AP directly competes with PSD-95 for NOS-I interaction (Jaffrey *et al*, 1998). (Tochio *et al*, 1999) showed that the C-terminal 12 residues of NOS1AP (NOS1AP_{C12}) were sufficient for binding to NOS-I. A recent study showed in vitro that the C-terminus of NOS1AP, encoding the IAV PDZ-motif (residues 504-506 in human; 501-503 in murine NOS1AP), stabilizes and even increases the interaction lifetime of the NOS-I/NOS1AP interaction but not required. NOS1AP residues 400-431 (396-427 in murine NOS1AP), containing an internal ExF interaction motif at residues 429-431 (425-427 in murine NOS1AP), are proposed to be the actual site required for the interaction between NOS-I and NOS1AP (Li *et al*, 2015). The same researchers suggested recently that this regions is not sufficient to block NOS-I PDZ domain binding to other ligands and a third structural element residing between ExF motif and PDZ motif required to enable stable interaction between NOS-I and NOS1AP (Li *et al*, 2017).

Post-mortem studies performed on brain tissues have shown increased expression of NOS1AP short isoform in dorsolateral prefrontal cortex (DLPFC) in patients with either schizophrenia or bipolar disorder (Xu *et al*, 2005) and reduced levels of long isoform in the cerebellum of patients with schizophrenia (Hadzimichalis *et al*, 2010). Researchers have also linked a DNA region containing NOS1AP on chromosome 1q22 to schizophrenia in multiple linkage and association studies and provided SNPs in NOS1AP also associating higher functioning of DLPFC and risk for schizophrenia (Brzustowicz *et al*, 2004; Husted *et al*, 2012; Kremeyer *et al*, 2008; Wratten *et al*, 2009). Moreover, linkage and association studies performed on data collected by Consortium on the Genetics of Schizophrenia have also shown the involvement of NOS1AP to neurocognitive

endophenotypes (e.g. deficits in sensorimotor gating, attention and spatial memory) observed in schizophrenia patients (Greenwood *et al*, 2011, 2016).

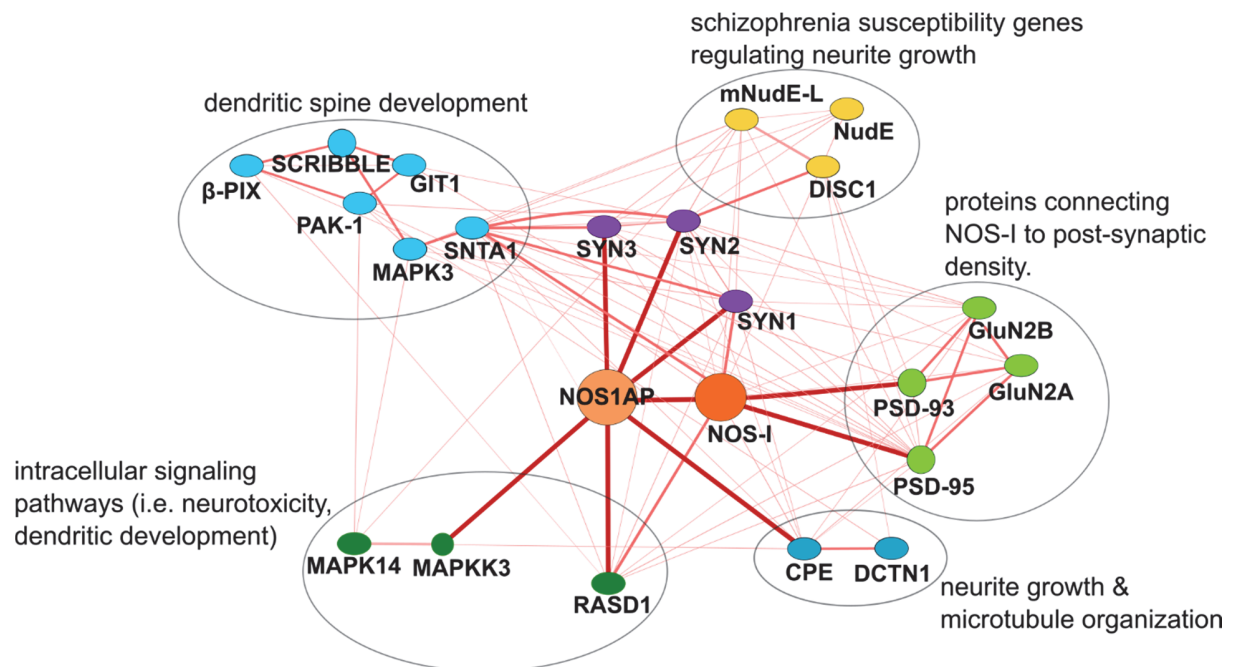


Figure 3. Theoretical model for NOS1AP interactions. Model represents NOS1AP interactions predicted by previous studies (Carrel *et al*, 2009b; Courtney *et al*, 2014; Fang *et al*, 2000; Guan *et al*, 2008; Jaffrey *et al*, 2002; Kamiya *et al*, 2006; Li *et al*, 2013, 2017; Nagasaka *et al*, 2010; Richier *et al*, 2010). (adapted from Candemir *et al*, 2016)

1.3. Research approaches

The human genome project (Venter *et al*, 2001) paved the way for a comprehensive analysis of the relation between genetic architecture and psychiatric phenotype. Twin and adoption studies have proven the high heritability (i.e. 80% or higher for schizophrenia, bipolar disorder and autism) for neuropsychiatric disorders (Dick *et al*, 2010; Plomin *et al*, 1994). Linkage and association studies provided great amount of data on candidate genes and disease-associated risk loci (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Hindorff *et al*, 2009; Maher *et al*, 2008; McGrath *et al*, 2013; Sklar, 2002). Cytogenetics associated a variety of chromosomal abnormalities to different disorders (Corvin *et al*, 2011). However, due to overlaps in genetic liability and phenotypic spectra between different disorders, a vast majority of all this information remained ineffective to provide biologically defined diagnostic criteria (Bondy, 2011; Hyman,

2007). As one possible solution, scientists considered investigating the association of genes with internal phenotypes (so-called endophenotypes), which can be measured by biochemical, cognitive, neurophysiological, neuroanatomical or neuropsychological paradigms (Gottesman and Gould, 2003).

In the last 30 years, an increasing number of studies have been underpinning genotype–phenotype associations through “integrative” experimental models at the molecular (e.g. genomics, epigenomics, proteomics...), cellular (e.g. cell-culture models), anatomical (e.g. neuroimaging), neural-circuit (e.g. system neuroscience), and behavioral levels (e.g. human and animal studies) (Bearden and Freimer, 2006; Quadrato *et al*, 2016). Notable examples include studies investigating the role of candidate genes and protein–protein interactions in regulation of pathological synaptic remodeling and trafficking by making use of postmortem patient tissues (Kristiansen *et al*, 2006; Weickert *et al*, 2013; Xu *et al*, 2005), cell culture samples (Candemir *et al*, 2016; Carrel *et al*, 2009a; Richier *et al*, 2010), and animal models (Freudenberg *et al*, 2013a; Woronowicz *et al*, 2010).

Scientists have been using a variety of genetic tools, including transgenic animals, to understand the neurobiological mechanisms underlying psychiatric disorders by reproducing specific behavioral phenotypes in animals. These methods can be time consuming, expensive and lack brain region specificity. Therefore, viral gene delivery has become a strong alternative. Stereotaxic delivery of virally encoded transgenes provide fast and efficient expression of the gene of interest and enables spatio-temporal control of expression (Cetin *et al*, 2006; McClure *et al*, 2011). Among several available viral systems, adeno-associated viruses (AAV) are the most promising tool due to fact that they are non-pathogenic and highly efficient to provide long-term gene expression (Lentz *et al*, 2012; Xiao *et al*, 1997). AAVs are small (~20 nm) parvoviruses, which have only linear and single-stranded DNA of ~4.7 kb flanked by an inverted terminal repeats (ITR). In recombinant AAV (rAAV), coding sequences for the Rep gene for replication and Cap gene for encapsidation are removed and replaced by the DNA of interest. Therefore, rAAVs require helper plasmids to encode Rep and Cap genes for the production of rAAV particles (Wu *et al*, 2006).

1.3.1. Behavioral testing in rodents

Even though several phenotypes of psychiatric disorders require higher cognitive and emotional functioning (e.g. hallucinations and delusions cannot be modeled in rodents), animal studies are

still able to provide extensive information on different behavioral phenotypes including learning, memory, anxiety, hyperactivity, sensorimotor gating, aggression, and depressive-like behaviors.

Chronic mild stress (CMS) is was originally developed to induce depressive-like behavior in rodents (Willner *et al*, 1992) and later it has been a fundamental experimental tool to test antidepressant activity as all CMS-induced phenotypes can be reversed by antidepressant treatment (Willner, 2016). Nowadays, it is a validated research approach of replicating effects of stressors in people's everyday life by repeatedly exposing rodents to several days or weeks of stressful or aversive stimuli (e.g. social defeat, sleep deprivation, food deprivation, restraint...) which induces behavioral alterations related to psychiatric disorders (D'Aquila *et al*, 1994; Willner, 2016). CMS has been also shown to up-regulate NOS-I expression in hippocampus. Increased expression and enzymatic activity of NOS-I upon stress was also associated with impaired neurogenesis in this area and behavioral despair in mice. NOS-I inhibition reversed these CMS-induced neuronal and behavioral effects (Zhou *et al*, 2007). These findings support the involvement of gene and environment interactions and their validity in animal models.

Rodents tend to show increased locomotor activity when they are exposed to a novel environment and this activity declines by time as they get habituated to the environment (Bailey and Crawley, 2009). **Locomotor activity** is mainly evaluated in the open field arena. Studies targeting glutamatergic system components (i.e. NMDA or AMPA receptor activity) revealed that rodents with reduced glutamatergic neurotransmission showed increased locomotor activity resembling stereotypic behavior observed in patients with psychiatric disorders (Iasevoli *et al*, 2014). Studies with *NOS1* knock-out (KO) mice also revealed similar results pointing to increased locomotion (Tanda *et al*, 2009; Weitzdoerfer *et al*, 2004). Lesions in hippocampus have been also shown to increase locomotor activity (Bannerman *et al*, 1999, 2002; Hock and Bunsey, 1998; Teitelbaum and Milner, 1963).

Anxiety-related behaviors can be assessed in several experimental designs. In the open field test, rodents naturally prefer to spend more time investigating the periphery of an open arena. Therefore, increased investigation of the center (unprotected, open part) of the arena has been associated with anxiolytic behavior (Prut and Belzung, 2003; Walsh and Cummins, 1976). The elevated plus maze (EPM) is a widely-used test to detect anxiogenic/anxiolytic behavior by presenting rodents a choice between open, unprotected and enclosed, darker maze arms. It is used to assess rodents'

natural avoidance from unprotected, high and open areas. Therefore, animals exploring the open arms have been accepted to show anxiolytic behaviors (Bailey and Crawley, 2009; Shepherd *et al*, 1994). The elevated zero maze (EZM) is a modification of the elevated plus maze, bearing the advantage of circular apparatus. On the EPM, animals tend to spent more time in the central area rather than making a choice of open or close arm. EZM eliminates this outcome (Shepherd *et al*, 1994). Light-dark box (LDB) is another widely used test to determine anxiety-related behaviors by exposing rodents to a novel environment which has a dark (protected) compartment and a brightly lit (unprotected) compartment. Rodents have innate aversion to bright and unprotected areas, hence increased exploration of the light compartment has been associated with anxiolytic behaviors (Bailey and Crawley, 2009; Crawley and Goodwin, 1980). In a recent study, researchers increased NOS1AP expression by using lentiviral vectors in mouse hippocampus to induce anxiogenic-like behaviors. Mice with overexpression of NOS1AP spent less time in the open arms of EZM and light compartment of LDB which was reversed by disruption of this interaction (Zhu *et al*, 2014).

Depression-like behaviors are modeled in rodents by investigating symptoms such as self-neglect, anhedonia, and behavioral despair. Building a nest is an important indicator of survival and wellness in rodents, because in nature nests function as shelters for protection from predators and harsh environmental conditions (Deacon, 2006). Therefore, nest building can be assessed as a measurement of well-being by simply providing animals pressed cotton materials. Anhedonia, the loss of ability to experience pleasure, is a core feature of depression (Katz, 1981; Strekalova *et al*, 2004) and reflects the negative symptoms of psychotic disorders such as schizophrenia (Wilson and Koenig, 2014). It can be tested in rodents by evaluating their preference for sucrose solution over normal tap water. Studies have shown that hedonic behaviors might be influenced by many factors such as acute stress or nature of the stressor when one is present (Pucilowski *et al*, 1993), therefore it might be better to test for longer periods (>24 h) (Strekalova *et al*, 2004). The forced swim test (FST) is a common method to assess behavioral despair in rodents when they encounter an unescapable aversive situation, in this case a water-filled tank. Initially, animal try to escape the situation by struggling or swimming and eventually they learn that escape is not possible and give up (behavioral despair) (Porsolt *et al*, 1977a, 1977b). It has been originally proposed to test the preclinical effects of antidepressant drugs in rats (Porsolt *et al*, 1977b) and then modified for testing in mice (Porsolt *et al*, 1977a) as antidepressant treatment resulted in more struggling

behavior. One of the drawbacks of this approach has been suggested as different strains of animals having different baseline immobility behavior and responses to antidepressant treatment (Cryan *et al*, 2002). Another drawback is that even acute administration of antidepressants is suggested to be enough to observe the effect of treatment, however in humans it takes weeks to months for antidepressants to exert their effects (Cryan *et al*, 2002; Slattery and Cryan, 2017). Last but not least, not all tests validate the anti-depressive effects of these drugs leaving a blank in translational value of modeling multi-dimension side of depressive symptoms (Cryan *et al*, 2002; Krishnan and Nestler, 2008). Despite that, tests used to model depressive syndromes are still valuable to investigate neurobiological background of depression. For example, NMDA antagonists and NOS-I inhibitors have been shown to mimic the effects of clinically effective antidepressants in animal models (Harkin *et al*, 2004; Tang *et al*, 2015; Volke *et al*, 2003). Inhibition of NOS-I was even shown to increase the efficiency of antidepressants acting on serotonergic mechanisms (Harkin *et al*, 2004).

Impaired sensorimotor gating is a common endophenotype in many psychiatric conditions including schizophrenia and attention deficient disorders (Braff *et al*, 2001; Swerdlow and Geyer, 1998). Deficits in sensorimotor gating are result of impaired functioning in sensory, motor and cognitive levels engaging mostly the circuits between prefrontal cortex, nucleus accumbens and hippocampus (Geyer and Swerdlow, 2001). In rodents, the most common way to assess sensorimotor gating is to evaluate acoustic startle reflex (ASR). ASR is the stereotyped response given to a sudden and intense stimulus occurring as contraction of muscles leaving animal in a hunched position (Koch, 1999; Swerdlow and Geyer, 1998). This test has a high translational value across species due to fact that this has been long established in humans as eye blink reflex of startle measured by electromyography of the orbicularis oculi muscle (Braff *et al*, 2001). Prepulse inhibition (PPI) stand for an attenuation in ASR when startling stimulus was presented with a preceding weaker stimulus called prepulse (Swerdlow and Geyer, 1998). PPI deficits have been well established for many neuropsychiatric disorder especially for schizophrenia- spectrum patients (Braff *et al*, 2001). A previous study performed in rodents showed that chemical stimulation with glutamate agonist NMDA in ventral hippocampus disrupts PPI but not in dorsal hippocampus (Zhang *et al*, 2002a). Studies also showed that NOS-I inhibitor L-NAME reversed phencyclidine (NMDA receptor antagonist)-induced deficits in habituation to startle stimuli and PPI. It also reversed D-amphetamine (dopamine agonist) -induced deficits in habituation to startle

stimuli but not PPI (Klamer *et al*, 2004; Zhang *et al*, 2002a). Studies have shown inconsistent effects phencyclidine treatment in *NOS1* KO mice as resulting in either decreased or increased PPI (Klamer *et al*, 2005; Tanda *et al*, 2009). Altogether, findings indicate that both glutamatergic and dopaminergic neurotransmission is mediating habituation were affected by NOS-I activity.

Social interaction and recognition are important for normal psychosocial functioning of individuals to maintain their relationships and life quality. Sociability deficits such as increased social withdrawal are commonly observed in many psychiatric disorders including schizophrenia, depression, and autism (Wilson and Koenig, 2014). In rodents sociability is also important to maintain social hierarchy and it influences mate choices (Kaidanovich-Beilin *et al*, 2011). Studies have shown that psychotomimetic drugs such as NMDA receptor antagonists stimulate social interaction deficits similar to those observed in patients. On the other hand, there is a discrepancy between studies performed on *NOS1* KO mice. Tanda *et al*. (2009) and Weitzdoerfer *et al*. (2004) have shown normal social interaction behavior in novel environment in *NOS1* KO mice. However, in the home cage, Tanda *et al*. (2009) showed increased social interaction of *NOS1* KO mice compared to wild types. This difference might occur due to experimental design as being in novel environment might induce anxiogenic behavior as discussed above. It has been also shown that social interactions are under influence of many brain regions (e.g. hippocampus, frontal cortex) and neurotransmitter systems including glutamatergic, dopaminergic and GABAergic (reviewed in Wilson and Koenig, 2014).

Cognitive deficits in humans with psychiatric disorders are associated with impaired attention, information processing, thinking, perception and memory (Barch and Ceaser, 2012; Corvin *et al*, 2011).

Learning and memory is highly dependent on hippocampus and NMDA receptor functioning (Andrew Chambers *et al*, 1996; Driesen *et al*, 2013). Lesion studies performed on rodents revealed different roles for dorsal (spatial learning and memory) and ventral parts (emotion) of hippocampus (Bannerman *et al*, 2004). Studies performed by Moser and her colleges have shown that spatial reference memory (SRM; place learning) were highly dependent on dorsal hippocampus but not on ventral hippocampus (Moser *et al*, 1995). On the other hand, following studies have shown that spatial learning combining appetitive tasks (e.g. reward alternation task on T-maze – spatial working memory(SWM)) also required intact dorsal hippocampus activity but

barely dependent on ventral hippocampus (reviewed in Bannerman *et al*, 2004). Later studies also showed that animals with dorsal hippocampus lesions could still learn specific tasks requiring SWM, but not animals with whole hippocampus lesions which could be explained by the fact that ventral hippocampus also contain place cells involved in spatial learning (reviewed in Bannerman *et al*, 2004). Altogether these findings point out that dorsal and ventral hippocampus are not completely separated and they work together through specific projections for tasks requiring the function of both region (Bannerman *et al*, 2004; Sigurdsson and Duvarci, 2015). Later, lesion studies in rodents and imaging studies in humans have also shown that hippocampus work in high connectivity with prefrontal cortex for tasks such as SWM (Sigurdsson and Duvarci, 2015).

NMDA receptor dependent long term potentiation (LTP) has been long known to regulate learning and memory. Studies performed with transgenic animals or pharmacological intervention (with NMDA receptor antagonists) revealed that NMDA receptor activity is important for acquiring new memories but not for recall of these memories (Rezvani, 2006). AMPA receptor activity has been found to interfere with NMDA receptor activity on hippocampus dependent tasks as learning and memory (Wiltgen *et al*, 2010). Studies performed with GluA1 KO animals have shown that GluA1-dependent synaptic plasticity is critical for especially short term memory on SWM but not SRM (Sanderson and Bannerman, 2012; Sanderson *et al*, 2009). Another study suggested that AMPA receptor activity in prefrontal brain regions contribute to SWM more than hippocampus (Freudenberg *et al*, 2013b) supporting other studies emphasizing the importance of connectivity between hippocampus and prefrontal cortex in SWM (Sigurdsson and Duvarci, 2015). Studies performed with NOS-I knock out mice have also shown impaired spatial memory in rodents (Ohno *et al*, 1993; Zoubovsky *et al*, 2011). Altogether, it can be summarized as glutamate-dependent function of hippocampal and prefrontal regions is crucial for spatial learning.

Novel object recognition (NOR) test evaluates ability to remember a previously seen object and discriminate between a novel and a familiar objects based on rodent's innate exploratory behavior and recognition memory (Ennaceur, 2010). Studies have shown both perirhinal cortex and hippocampus are involved in object recognition memory (Antunes and Biala, 2012; Winters and Bussey, 2005). Both AMPA and NMDA receptor activity was found to be important for learning and recall of object recognition memory (Rezvani, 2006; Winters and Bussey, 2005).

Fear conditioning test is used to evaluate classical (Pavlovian) conditioning. In brief, rodents learn to associate when a neutral conditioned stimulus (CS; e.g. tone cue) is paired with an aversive unconditioned stimulus (US; e.g. foot shock). After a successful acquisition period, they develop conditioned fear and show increased freezing when they confronted to CS (Fanselow and Poulos, 2005; Perusini and Fanselow, 2015). Conditioning provides a rapid and long lasting effect which requires the involvement of somatosensory pathways and stress mechanism to develop learning and memory (Fanselow and Poulos, 2005; Kim and Jung, 2006). Lesion, neurophysiological and pharmacological studies have shown that amygdala is the major brain region mediating fear conditioning for both contextual and auditory conditioning, whereas hippocampus plays a role in mediating contextual conditioning but not auditory (Phillips and LeDoux, 1992). In addition, prefrontal cortex has been shown to play important roles in extinction of auditory conditioning memory but not contextual conditioning (Kim and Jung, 2006). NMDA receptor activity dependent LTP is also important for acquisition and recall of conditioning memory in amygdala, so far studies were able to show that NMDA receptor activity dependent LTP in hippocampus mediates only contextual conditioning (Kim and Jung, 2006). Interestingly, NOS-I activity has been also associated with contextual conditioning by mediating on hippocampal and amygdalar cGMP in rodent studies either using NOS-I inhibitor (S-methyl-L-thiocitrulline) or KO models (Kelley *et al*, 2009, 2010). These findings suggest that integrity of glutamatergic neurotransmission in hippocampus and amygdala is important for acquisition and consolidation of fear conditioning.

2. Materials and Methods

2.1. Molecular Methods

To investigate the role of NOS-I/ NOS1AP PDZ interactions in the development of morphological and behavioral phenotypes related to schizophrenia, recombinant adeno-associated virus (rAAV) based gene delivery was used to express different NOS1AP isoforms and NOS1AP deletion mutants, as well as the extended PDZ domain of NOS-I ([Figure 4](#)).

2.1.1. Plasmid cloning

To create the following constructs cDNA fragments were amplified by PCR (see [Table 1](#) for primers) 1) mCherry (737 bp), a stable red monomeric fluorophore, was used in this study to tag expressed proteins and as a control. 2) The long isoform of murine NOS1AP (NOS1AP-L, 1532 bp; NM_001109985.1; analogous to the human [NM_014697.2]). 3) The last 20 amino acids of murine NOS1AP-L (NOS1AP-L_{C20}, 60 bp), previously suggested to be necessary and sufficient for interaction with NOS-I (Jaffrey *et al*, 1998; Zhu *et al*, 2014). 4) Murine NOS1AP-L lacking the last 20 amino acids (NOS1AP-L_{ΔC20}, 1472 bp) which was expected to lack interaction with NOS-I (Jaffrey *et al*, 1998). 5) An artificial short isoform of NOS1AP that is analogous to the human short isoform (NOS1AP-S, 643 bp; NM_001126060.1). This short isoform lacks the PTB and CPE-binding domains and is expressed in humans and rats (Clattenburg *et al*, 2015; Xu *et al*, 2005), but there is no equivalent isoform expressed in mice. Therefore, the N-terminal domain of human NOS1AP-S (58 bp) and the C-terminal domain of murine NOS1AP (590 bp) were combined to create a mouse analogue of NOS1AP-S, which is different from the human NOS1AP-S by a G476S shift in proximity to the PDZ interaction motif. 6) N-terminal 133 amino acids of murine NOS-I (NOS-I₁₋₁₃₃, 426 bp) encoding for the extended PDZ domain. This construct was previously used to specifically disrupt NOS-I/PSD-95 interaction (Zhou *et al*, 2010). 7) Carboxyterminal 108 amino acids of murine NOS1AP-L (NOS1AP-L₃₉₆₋₅₀₃, 347 bp) was suggested to contain the required NOS-I binding motifs (Li *et al*, 2015). PCR reaction mix was prepared with iProof™ High Fidelity PCR Kit (Cat. No # 172-5330, BioRad, Munich). PCR thermal cycler protocol started with initial denaturation at 98°C for 30 s followed by 32 cycles of 10 s denaturation at 98°C, 30 s of annealing at 66°C, 30 s of elongation at 72°C, and ended with a final elongation at 72°C.

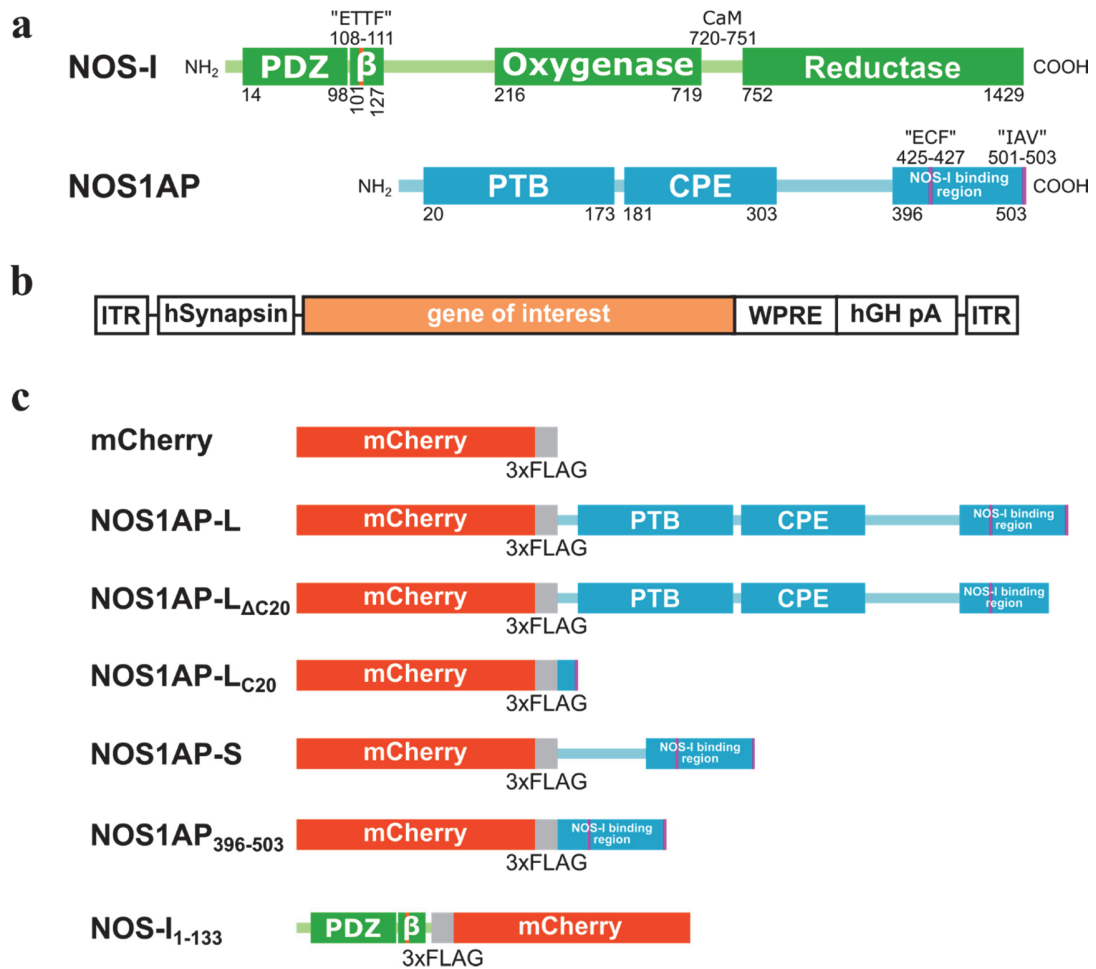


Figure 4. Recombinant adeno-associated virus (rAAV) vectors used in the study. a) Structure of the murine NOS-I and NOS1AP proteins. PDZ- β : core PDZ domain followed by the β -finger, containing an internal PDZ motif; PTB: Phosphotyrosine-binding domain; CPE: Carboxypeptidase E binding region. **b)** Exemplary representation of the used rAAV vector backbone. ITR: inverted terminal repeats; hSynapsin: human synapsin 1 gene promoter; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; hGH pA: human growth hormone polyadenylation signal. **c)** Schematic representation of the rAAV constructs expressing mCherry (molecular weight [MW]: 30 kDa), the long isoform of NOS1 AP (NOS1AP-L; MW: 86 kDa), NOS1AP-L lacking the last 20 amino acids (NOS1AP-L Δ C20; MW: 83 kDa), the last 20 amino acids of NOS1AP-L (NOS1AP-L $_{C20}$; MW: 32 kDa), the short isoform of NOS1AP (NOS1AP-S; MW: 53 kDa), residues between 396 and 503 of NOS1AP-L (NOS1AP $_{396-503}$; MW: 42 kDa) and the aminoterminal 133 amino acids of NOS-I (NOS-IN133; MW: 44 kDa). (adapted from Candemir *et al.*, 2016)

To create the vector backbone (pAAV-hSyn-mCherry.3xFLAG), plasmid pAAV-hSyn-hChr2(H134R)-mCherry (a gift from Karl Deisseroth, Addgene plasmid #26976), was enzymatically cut using Sall and EcoRI to remove the hChr2(H134R)-mCherry cassette. Amplified mCherry cDNA was also cut by restriction enzymes Sall and EcoRI to replace removed cassette. A linker encoding a 21 amino acid 3xFLAG epitope (Table 1) was inserted to the 3' end of mCherry at the BsrGI and HindIII sites. The resulting vector was used as the control vector (i.e.

mCherry) and as backbone to create other vectors. NOS1AP-L, NOS1AP-L_{C20}, NOS1AP-L_{ΔC20} and NOS1AP-L₃₉₆₋₅₀₃ were inserted 3' to the mCherry.3xFLAG fusion at the NheI and HindIII sites. To create NOS1AP-S, 5' 51 nucleotides (starting from the ATG) of the human NOS1AP-S ORF was ligated to the 3' 590 nucleotides of the mouse NOS1AP-L. To create NOS-I₁₋₁₃₃, EcoRI and Sall enzymes were used to cut out mCherry from backbone vector pAAV-hSyn-mCherry.3xFLAG and replace it with linker containing the Kozak sequence and start codon (Table 1). NOS-I₁₋₁₃₃ DNA was then inserted 5' of the 3xFLAG tag using SpeI and EcoRI sites in pAAV-hSyn.3xFLAG. All constructs (Figure 4) contained the human Synapsin-1 promoter, limiting expression to neurons (Kügler *et al*, 2003a). pAAV-6P-SEWB (a gift from Sebastian Kügler) (Kügler *et al*, 2003b) plasmid was used for viral expression of enhanced green fluorescent protein (eGFP). All AAV plasmids were amplified using One Shot® Stbl3™ Chemically Competent *E. coli* (Cat. No # C737303, Thermo Fisher Scientific Inc.). Plasmid DNA was purified with the PureYield™ Plasmid Midiprep System (Promega Corporation, USA). Part of liquid cultures were stored for long-term usage at -80°C (1:1 in 50% glycerol). Presence of correct inserts were confirmed by Sanger sequencing (Eurofins genomics sequencing department, Ebersberg, Germany).

Table 1. Oligonucleotides used in the study

Name	5'-3'	3'-5'
WPRE	TGCTCCCGTATGGCTTTTCAT	CATAGCGTAAAAGGAGCAACA
KozakATGLinker	TCGACactagtgccaccatgG	AATTCcatggtggcactagtG
3xFLAG	GTACaagGAATTCGACTACAAAGACCATGA CGGTGATTATAAAGATCATGATATtGATTA CAAGGATGACGATGACAAGctagecaTA	agctTAtgctagCTTGTCATCGTCATCCTTGTA TCaATATCATGATCTTTATAATCACCGTCA TGGTCTTTGTAGTCGAATTCctt
mCherry	CCCCGTAATGCAGAAGAAGA	TTGGTCACCTTCAGCTTGG
NOS1AP	AGTgctagetATGCCAGCAAACCAAGTAC	GTCaagettaCTACACGGCGATCTCATCATC
NOS1AP _{ΔC20}	AGTgctagetATGCCAGCAAACCAAGTAC	GTCaagettaCTAGCGTGGCAGCTCTTCCTG
NOS1AP _{CTD}	CAGGTACACTTACTGAAGGATCAG	GTCaagettaCTACACGGCGATCTCATCATC
hNOS1AP-S _{NTD}	ctagetATGTCCCTCTCTCTCTGTCTCTGTCTCTGTC TTCTCTGCCGCTGCCTCTTCTCTG	CAGAGAAGAGGCAGCGGCAGAGAAGACA GGACAGAGAGAAGAGAGGGACATag
NOS1AP ₃₉₆₋₅₀₃	AGTgctagetCACTCACCCTGCTGGGCGC	GTCaagettaCTACACGGCGATCTCATCATC
NOS-I ₁₋₁₃₃	GTCactagtGCCACCATGGAAGAGCACACGT TTGGG	GCCgaattcATCGACAGCTTTGGTGGG

2.1.2. Recombinant AAV production and titration

Recombinant AAV production was performed as described previously by McClure *et al* (2011). For each virus, five cultures of adherent AAV 293 cells (Agilent Technologies, Santa Clara, CA, USA) at 70-80% confluency in 150 mm diameter plates were transfected using calcium phosphate

transfection. A transfection mix containing the respective AAV plasmid (12.5 µg/plate), Adenovirus helper plasmid pFdelta6 (25 µg/plate), pH21 and pRV1 helper plasmids containing AAV1 and AAV2 Rep and Cap sequences respectively (each 6.25 µg/plate), and as a positive transfection control pEGFP-N3 (Clontech Laboratories Inc., Mountain View, CA, USA; 4 µg/plate) was prepared just before transfection and sterile filtered into a 50 ml tube. 2x HBS (HEPES buffered saline, pH 7.05) was prepared previously and stored at -20°C. On the day of transfection, 2x HBS was thawed at room temperature, sterile filtered into a 50 ml tube, and added to the transfection mix in a drop-wise fashion. This solution was mixed gently and added to AAV 293 cells (4 ml/plate). Six hours after transfection, the cell culture medium was replaced with fresh medium. Medium was removed from cells containing the viral particles ~48 hours after transfection. Cells were gently scraped in 18 ml 1x PBS and the suspension was collected in two 50 ml falcon tubes. Cells were centrifuged at 800 x g for 10 min, the pellet was resuspended in a solution containing 150 mM NaCl and 20 mM Tris (pH 8.0) and stored at -20°C. Before purification of rAAVs, suspensions were thawed at room temperature, treated with 0.5% NaDOC (Cat. No # D5670-5G, Sigma-Aldrich Chemie GmbH, Munich, Germany) and 50 U/ml benzonase nuclease (Cat. No # E1014-5KU, Sigma-Aldrich Chemie GmbH, Munich, Germany), incubated at 37°C for 1 hour. Cellular debris was removed by centrifugation at 3000 x g for 15 min. Supernatant was either stored at -20°C or immediately used for purification.

Viral particles were purified using 1 ml heparin affinity columns (GE Healthcare, Chalfont St Giles, UK). First, the column was equilibrated with 10 ml 150 mM NaCl and 20 mM Tris (pH 8.0), followed by ~50 ml virus-containing solution. Afterwards, the column was washed with 20 ml 100 mM NaCl and 20 mM Tris (pH 8.0), 1 ml 200 mM NaCl, 20 mM Tris, pH 8.0, followed by 1 ml 300 mM NaCl, 20 mM Tris, pH 8.0. Virus solution was eluted using 1.5 ml 400 mM NaCl 20 mM Tris pH 8.0, 3.0 ml 450 mM NaCl 20 mM Tris pH 8.0 and 1.5 ml 500 mM NaCl 20 mM Tris pH 8.0 and concentrated in 1x PBS (pH 7.4) using Amicon Ultra filter units (Merck Millipore, Billerica, MA, USA) by centrifugation at 2000 x g for 2 min (3 times) and sterile-filtered through a 0.22 µm syringe filter (Merck Millipore).

Viral titers (number of viral genomes per ml) were quantified by qRT-PCR in 10 µl reaction mixes per sample consisting of 0.2 µmol primer pair (WPRE specific forward- and reverse-primers, [Table 1](#)), 1x SYBR Green Master Mix (Life Technologies, Darmstadt or Roche Molecular Systems, Inc.,

Mannheim, Germany), and template DNA. Amplification was performed using pre-incubation at 95°C for 5 min followed by 45 cycles of amplification as denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 10 s, followed by a melting curve.

All samples and negative controls were tested in triplicates in 384-well plates. To estimate the viral titers, crossing point values derived from AAV vector DNAs were compared to the standard curve generated from seven serial dilutions of a plasmid standard (10^3 - 10^9 copies).

2.1.3. *In vitro* experiments

2.1.3.1. Primary neuron cultures

Primary cultured neuron preparations were performed as described by Beaudoin et al. (2012) (Beaudoin *et al*, 2012) from the hippocampus and the cortex of the E18 mouse (C57BL/6J) embryos. Pregnant female mice were deeply anesthetized with isoflurane and killed by cervical dislocation. The abdomen was rinsed with 70% Ethanol and opened. Uteri containing the embryos were immediately removed and placed in a petri-dish. Embryos were decapitated, brains were removed and transferred into another dish containing Hank's buffered saline solution (HBSS, Ca^{2+} and Mg^{2+} free) and cleaned of meninges under a stereomicroscope. Hippocampal or cortical tissues were dissected and placed in a 15 ml centrifuge tube containing HBSS at room temperature. In a laminar flow hood, medium was aspirated and tissues were incubated with 0.05% Trypsin/0.02% EDTA in 1x PBS for 5 min at 37°C. After aspiration of Trypsin-EDTA, tissues were washed three times with HBSS and then triturated in 2 ml Neurobasal Medium (Life Technologies, Carlsbad, CA, USA) by pipetting up-down with fire-polished Pasteur-pipettes. When no more clumps of tissue were visible, 30 μl of sample was mixed with 30 μl of Trypan-blue and cell number was counted using an Auto T4 Cell Counter (Nexcelom Bioscience LLC., Lawrence, USA). Cells were then diluted accordingly and plated at a density of 10000 cells/well in 96-well plates, 25000 cells/well in 24-well plates containing 12 mm glass coverslips, or at 300000 cells/well in 6-well plates. All well-plates were pre-coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA) at least 2 h before plating and washed 3x times with warm 1x PBS right before plating. Primary neurons were allowed to grow in Neurobasal medium containing 0.4 mM L-glutamine, 1x B27 supplement (Life Technologies, Carlsbad, CA, USA), 2000 U/mL penicillin and streptomycin. Cells were infected with previously constructed recombinant AAVs 7 days after cells were plated (i.e. 7 days *in vitro*, DIV7) and further processed as indicated in section 2.1.4.

2.1.3.2. Western blot and co-immunoprecipitation

On DIV14, neurons were harvested at 4°C in 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 5% glycerol (300 µl/well) containing Protease inhibitors (Sigma Aldrich, St. Louis, MO, USA). Precision Red Advanced Protein Assay (Cytoskeleton Inc. Cytoskeleton, Inc., Denver, CO, USA) was used (at 1:100 ratio) to quantify protein concentrations at a wavelength of 600 nm and proteins lysates were stored at -20°C. Serial dilutions of Bovine serum albumin (BSA) in protein lysis buffer were used to generate a standard curve to compare sample protein concentrations.

For co-immunoprecipitation, 5 mg Dynabeads® M-280 Tosylactivated magnetic beads (Life Technologies) were linked to 100 µg monoclonal mouse anti-FLAG® M2 antibody (Sigma-Aldrich, catalogue number: F1804) as suggested by the manufacturer and incubated on a roller at 37°C for overnight. Next day, the supernatant was removed and sample was incubated in 1xPBS (pH 7.4) with 0.5% BSA at 37°C for 1 h. Beads were washed twice in 1xPBS (pH 7.4) with 0.1% BSA for 2 min and then resuspended in 1xPBS (pH 7.4) with 0.1% BSA. Afterwards, 15 µg of protein from each protein lysate was mixed with 1 mg bead/ligand solution and incubated at room temperature for 1 h in 500 µl reaction volume. Samples were washed three times in 1x PBS (pH7.4) with 0.1% BSA and bound proteins were eluted using 0.1 M Glycin-HCl (pH 3.0) which was neutralized by adding 0.5 M Tris-HCl (pH 7.4). Proteins were stored at -20°C until further use.

Proteins were separated using on 4-12% Bis-Tris Mini Gels in MOPS SDS Buffer for 32 min at 200 V and transferred to PVDF membranes for 1 h at 20 V. For immunoblotting, membranes were first blocked with 5% non-fat milk in TBS-T for 1 h and then incubated with primary antibodies (1:4000 mouse anti-FLAG® M2, Sigma-Aldrich, Cat. Nr: F1804; 1:1000 rabbit anti-NOS-I, Millipore, Cat. Nr: AB5380; 1:200 rabbit anti-NOS1AP, Santa Cruz, Cat. Nr: sc-9138) in 5% milk in TBS-T overnight at 4°C. Membranes were washed in TBS-T and incubated with horseradish peroxidase secondary antibodies (1:10000 anti-rabbit IgG from goat, Sigma Aldrich, Cat. Nr: A9169; 1:2000 anti-mouse IgG from goat, BD Pharmingen, Cat. Nr: 554002; 1:10000 anti-rabbit IgG from donkey, GE Healthcare, Cat. Nr: NA934) in 5% milk in TBS-T for 1h. After washing in TBS-T, membranes were incubated in Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and imaged using the ChemiDoc™ MP System (BioRad) or myECL™ Imager (Thermo Fisher Scientific).

2.1.4. Immunofluorescence and Microscopy

On DIV 7 primary cultured hippocampal and cortical neurons in 24-well plates were infected with 2×10^8 viral particles/well (3 wells/virus) and co-infected with $\sim 5 \times 10^7$ viral particles/well of the eGFP expressing 6P-SEWB rAAV (see 2.1.3.1). On DIV14 or 21, neuronal cells were fixed with pre-warmed solution including 4% paraformaldehyde (PFA) and 4% sucrose in 1x PBS for 10min, then treated with 0.1% Triton X-100 solution in 1x PBS for 10 min and pre-blocked with 5% fish gelatin (FG) and 0.3% BSA in 1x PBS for 1 h. Cells were incubated with primary antibody (monoclonal mouse anti-FLAG® M2, Sigma-Aldrich, Cat. No # F1804); polyclonal chicken anti-GFP, Abcam, Cat. No # ab13970) diluted 1:5000 in 2.5% FG and 0.3% BSA in 1x PBS overnight at 4°C. Next day, cells were washed three times in 1x PBS (pH 7.4) for 5 min and then incubated with fluorescent-conjugated secondary antibodies (goat anti-chicken Alexa Fluor 488; goat anti-mouse Alexa Fluor 555, both from Life Technologies) diluted 1:400 in solution 1% BSA in PBS at room temperature for 1 h. At the end, neuronal cells were washed three times with 1 x PBS (pH 7.4) and the coverslips were mounted on glass slides using Prolong Diamond Antifade with DAPI (Life Technologies). Immunofluorescence stainings were performed by a former Bachelor student Leonie Kollert.

Mounted cells were imaged (by Leonie Kollert) at 20x magnification on an Olympus IX81 Microscope (Olympus Microscopy, Tokyo, Japan). Images of individual neurons showing positive immunofluorescence for both eGFP and FLAG were saved in .tiff format and used to analyze dendritic branching and length via the Simple Neurite Tracer (Longair *et al*, 2011) and Sholl analysis plugins (Ferreira *et al*, 2014) for ImageJ/Fiji (Schindelin *et al*, 2012). In total, 30 individual neurons per viral construct were analyzed. For analysis of spine morphology, 27-30 secondary dendrites from different random neurons from at least three coverslips for each viral construct were imaged on a Zeiss Axio Observer.Z1 microscope using a 100x oil immersion objective (N.A.=0.35, Carl Zeiss) combined with 1.6x Tubulense optovar. Images were acquired as Z-stacks (10 Z-sections, 0.17 μm apart, 2752x2208 pixels, 14bit depth) and the number of protrusions extending at least 0.1 μm from a 15 μm segment were counted and classified manually for type of protrusion (i.e. cup-shaped, stubby, thin or filopodia-like as defined previously by Yuste and Bonhoeffer (2004)) using the Zen 2012-blue edition software (Carl Zeiss, Germany).

2.2. Behavioral Experiments

2.2.1. Animals and ethics

Animal experiments were performed in two rounds. In each round, 40 male wild type C57BL/6JRj mice were obtained from Janvier Labs (France) at 6 weeks of age and maintained at the Central Research Facility (University Hospital Frankfurt). Mice were housed in groups of four (12 h light/dark cycle, lights on: 07:00-19:00) under controlled ambient conditions ($21\pm 1^\circ\text{C}$, $55\pm 5\%$ humidity) with access to food and water ad libitum unless indicated otherwise. All experiments were performed with the experimenter blind to the treatment. Mice were tested during the light phase. They were brought in the experimental room at least 45min prior to testing to enable habituation. Unless otherwise stated, animals were exposed to 60 dB white noise while in the experimental room to reduce external noise. In each experiment, animals were tested in a randomized order to avoid any daytime-dependent activity differences. Prior to and between each test, equipment was cleaned with 20% Ethanol to reduce olfactory cues. All experiments were conducted according to Directive of the European Communities Council of 24 November 1986 (86/609/EEC) and German animal welfare laws (TierSchG and TSchV) and were approved by the Regierungspräsidium Darmstadt (approval ID: FK/1033).

2.2.2. Viral vector delivery

At 7 weeks of age, mice underwent stereotaxic surgery for delivery of viral vector constructs (Figure 4) chosen according to their efficiency and effects *in vitro* (see 2.1.3): (1) mCherry as control vector (2) NOS1AP-L, (3) NOS1AP₃₉₆₋₅₀₃ (4) NOS-I₁₋₁₃₃.

Mice were given Metamizol (Novaminsulfon-ratiopharm, Ratiopharm GmbH, Ulm, Germany) in drinking water (final concentration: 2 mg/ml) starting at least 48 h prior to surgery until at least 48 h after surgery. The surgical procedure was performed under isoflurane (AbbVie Inc., Wiesbaden, Germany) gas anesthesia through a precision vaporizer (Dräger Vapor[®] 3000, Drägerwerk AG & Co. KGaA, Lübeck, Germany). To avoid anesthesia-related heat-loss, the body temperature was maintained at 37°C by a thermostatically controlled pad. To reduce the pain of the surgical wound, 0.1 mL Ropivacaine (2 mg/ml) was administered subcutaneously at the incision site ~10 min before making the incision. Mice were mounted in the stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) and the pre-shaved skin above the head was incised using a scalpel. Viral constructs were injected through a small hole into dorsal hippocampus (coordinates

relative to bregma: AP: -2.1 mm, ML: ± 1.6 mm, DV: -1.25 mm, -2.0 mm) by 5 μ l Hamilton series NEUROS Model Syringe (33ga., Blunt; Catalog #53496, Stoelting Co., Wood Dale, IL, USA) which was mounted onto a motorized Quintessential Stereotaxic Injector (Stoelting Co., Wood Dale, IL, USA). Injections were performed as 2 times 1 μ l per hemisphere at a flow rate of 0.1 μ l/min and the needle was kept in place for another 4 min before it was withdrawn. After surgery, mice were placed in a cage under a heat lamp (Beurer, Ulm, Germany) for ~ 10 min. Mice were closely monitored on a daily basis for 1 week after surgery in order to verify proper recovery.

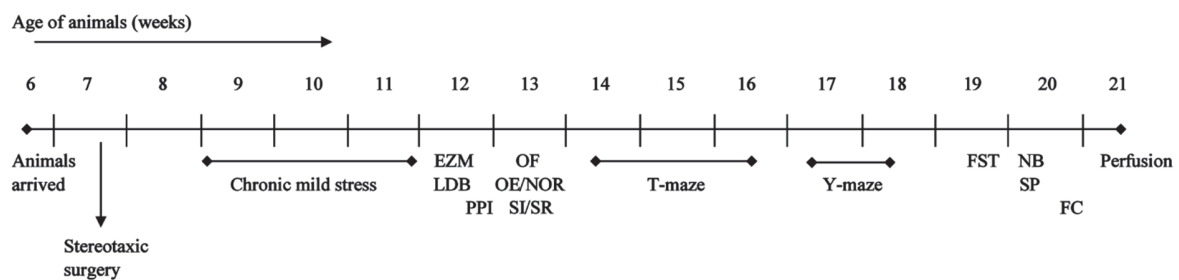


Figure 5. Representation of behavioral experiment schedule. Abbreviations: EZM: elevated zero maze, LDB: light-dark box, PPI: prepulse inhibition of startle reflex, OF: open field test, OE/NOR: object exploration and novel object recognition, SI/SR: social interaction and recognition, FST: forced swim test, NB: nesting behavior, SP: sucrose preference, FC: fear conditioning. Rewarded alternation task was performed on T-maze, spontaneous alternation task and spatial reference memory test were performed on Y-maze.

2.2.3. Chronic mild stress paradigm

As neuropsychiatric disorders arise from a complex interaction of genetic and environmental factors (Caspi and Moffitt, 2006; Homberg, 2012), we investigated the contribution of environmental stress in combination with the genetic intervention to development of behavioral alterations related to these disorders. Therefore, mice were randomly separated into two groups ($n=10$ /construct in total) at 8 weeks of age (\sim one week after surgery) and one group was exposed to a randomized three-week schedule of commonly used mild stressors (named CMS group, [Table 2](#), [Figure 5](#)) including continuous light exposure for 36 h, 3 h restraint in a 50 ml tube with small holes for ventilation, food or water deprivation (each for 15 h, starting 1 h before the dark phase), empty bottle (3 h), 30° tilted cage (3 h, without bedding) and wet bedding (15 h, starting 30 min before dark phase). To minimize impact of handling and habituation, non-stress group mice were also placed in the experimental room and handled the same number of times. To avoid stress-dependent olfactory and vocal cues, non-stressed mice and CMS mice were not handled at the same time.

Table 2. Exemplary schedule of chronic mild stress procedure.

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1 st week	Morning		Restraint	Tilted cage	Empty bottle	Empty bottle	Restraint	Tilted cage
	Afternoon	Overnight Illumination	Empty bottle	Wet bedding	Food deprivation	Tilted cage	Water deprivation	Restraint
2 nd week	Morning	Empty bottle	Tilted cage	Restraint	Tilted cage	Empty bottle	Restraint	Tilted cage
	Afternoon	Food deprivation	Empty bottle	Wet bedding	Overnight Illumination	Tilted cage	Water deprivation	Restraint
3 rd week	Morning	Empty bottle	Restraint	Empty bottle	Restraint	Tilted cage	Tilted cage	Restraint
	Afternoon	Food deprivation	Tilted cage	Wet bedding	Water deprivation	Restraint	Empty bottle	Overnight Illumination

2.2.4. Elevated zero maze (EZM)

The EZM apparatus is an elevated circular platform (diameter: 50 cm, lane width: 5 cm) including two opposite enclosed (wall height: 15 cm, inner walls: black infrared transparent perspex) and two opposite open compartments (with 5 mm high rim). It was placed on a table under an infrared (IR) illuminator and elevated 50 cm from the surface. Mice were placed on the EZM always facing the same enclosed compartment and their behavior was recorded for 10 min by use of an IR sensitive digital camera. Total distance travelled and time spent in open compartments were analyzed with ANY-maze video tracking system (v5.11b Beta, Stoelting Co., Wood Dale, IL, USA). Lighting levels were 120 lux in open compartments, 30 lux in enclosed compartments and consistent for all subjects.

2.2.5. Light-dark box (LDB)

The LDB apparatus consists of a dark chamber (with infrared transparent black Perspex walls, W 20 cm x L 40 cm x H 35 cm) and a brightly illuminated chamber (with clear acrylic walls, W 20 cm x L 40 cm x H 35 cm). The test was performed under constant light levels with 400 lux in the light compartment and 3 lux in the dark compartment. Each mouse was placed in the dark compartment facing the same corner and allowed to freely explore the arena for 10 min. Time

spent in the light compartment, the latency to enter to light compartment recorded with ANY-maze video tracking system equipped with IR sensitive digital camera.

2.2.6. Open field (OF)

The OF apparatus is a square chamber (40 cm x 40 cm) surrounded with black Perspex walls (H: 35 cm). Each mouse was placed into the OF facing one of the corners and allowed to explore the chamber for 10 min (Figure 6). Total distance travelled and time spent in the center of the arena (W 20 cm x L 20 cm) were recorded with the ANY-maze video tracking system equipped with a digital camera (The Imaging Source Europe GmbH, Bremen, Germany).

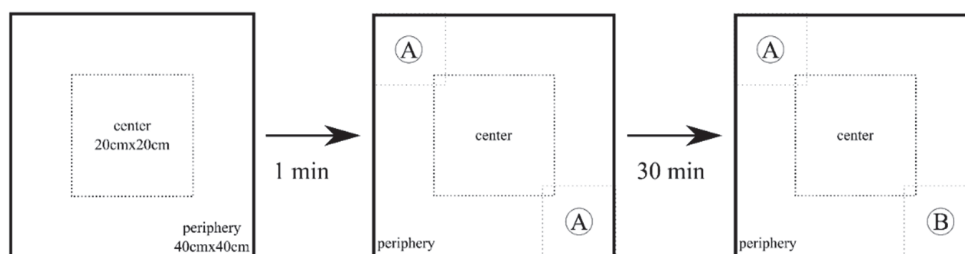


Figure 6. Schematic representation of experimental procedure for tests performed in OF. OF test was performed to assess locomotor activity and anxiety-related behaviors, which was followed by object exploration and novel object recognition tests.

2.2.7. Novel object exploration (OE) and recognition (NOR)

At the end of the OF test, the mouse was transferred to a holding cage for 1 min and then placed back into the OF containing two identical objects (A+A, Figure 6) positioned in opposite corners of the arena. Exploration of these objects (OE) was assessed for 5 min. At the end of the OE paradigm, mice were placed back to their home cage. 30 min later, mice were returned to the OF chamber containing two distinct objects, one was identical to the objects (A) used in the OE test and one is novel (B) (Ennaceur and Delacour, 1988; Hammond *et al*, 2004). Objects were positioned in the same corners of the arena and animals were allowed to explore for 5min. Novel object recognition (NOR) was defined as time spent exploring the novel over the familiar object. In these tests, exploration of an object was defined as time spent directing the nose at a distance shorter than 2 cm and/or touching the object with its nose, whereas sitting or turning around the object was not accepted as exploration.

Two kinds of objects were used in both tests: two white spheres and two grey cubes. Between animals, novel versus familiar objects and their positions were counterbalanced (Hale and Good, 2005; Hammond *et al*, 2004). Mice were placed in OF facing a corner not containing an object. Exploratory behavior was recorded by use of ANY-maze video tracking system and scored manually.

2.2.8. Social interaction and social novelty recognition

Deficits in social interaction and social recognition have been reported for a variety of neuropsychiatric disorders (Wilson and Koenig, 2014). In rodents, social interaction and recognition are especially critical for them to maintain their social hierarchy, mate choice and offspring recognition (Kaidanovich-Beilin *et al*, 2011). Therefore, we investigated exploration of novel conspecifics (4 weeks old male juveniles, [Figure 7](#)).

Social interaction (SI) paradigm: Experimental mice were placed into the OF arena (light level: 40 lux) and habituated for 1 min. Then, a pre-designated juvenile was placed into the OF arena with the experimental mouse. They were allowed to freely explore each other for 5 min. If an experimental mouse attacked a juvenile stimulus mouse the test was terminated prematurely. At the end, mice were returned to their home cage. SI was defined as time spent when experimental mice explored (e.g., sniffing, touching, licking) the juvenile; investigation of the test mouse by the juvenile was not included in the scoring. The first 3 min of interaction were manually evaluated using the ANY-Maze software.

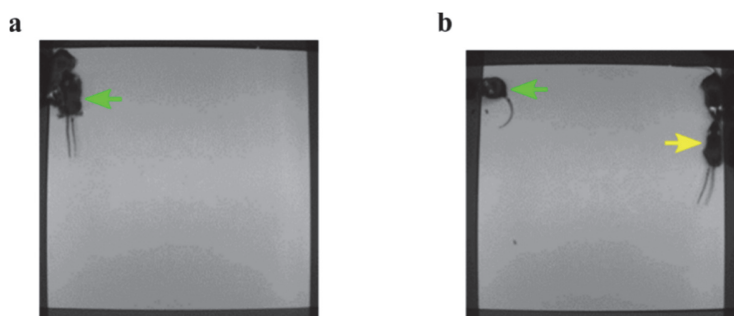


Figure 7. Social interaction procedure. a) Social interaction with an unfamiliar adolescent (4-week-old) male mouse (green arrow). **b)** After 30 min, social recognition was tested with the same (familiar, green arrow) mouse and a novel (yellow arrow) unfamiliar mouse.

Social novelty recognition (SR) paradigm: 30 min after SI, experimental mice were returned to the OF arena and habituated again for 1 min. Then, the same juvenile from the SI test and a novel juvenile were placed into the OF arena together with the experimental mice. They were allowed to freely explore each other for 5 min. SR was defined as time spent when only experimental mice exploring the novel over familiar juvenile. The first 3 min of SR was manually evaluated using the ANY-Maze software.

2.2.9. Prepulse inhibition (PPI) of the acoustic startle response (ASR)

Prepulse inhibition means an attenuation in startle reflex when a high/intense sensory stimulus (e.g. ASR) is presented right after a weaker non-startling pre-stimulus, and it is used as a measurement of sensorimotor gating (Swerdlow and Geyer, 1998). PPI deficits are commonly observed in patients with schizophrenia but also linked to cognitive and attentional deficits in other neuropsychiatric disorders (Geyer and Swerdlow, 2001). PPI of the ASR was measured as described previously (Chang *et al*, 2010) (Figure 8) by use of the SR-LAB™ startle response system (San Diego Instruments, Inc., USA). To avoid any vocal cues and habituation to the startle pulse, mice were kept in a separate room until testing and returned back to holding room afterwards.

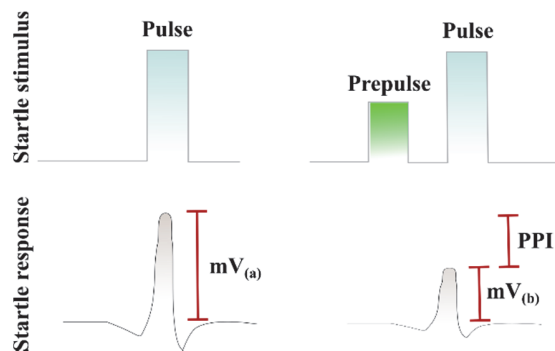


Figure 8. Schematic drawing of the protocol for PPI of the ASR. Presence of a weaker non-startling stimulus (prepulse) attenuates the magnitude of response (mV) to a startle stimulus.

The protocol started with 5 min of acclimation to constant background noise of 65 dB (continued throughout the test session) which was followed by six startle pulse only trials (40 ms burst of 120 dB) presented with an inter-trial interval (ITI) of 10 s. Then, mice received seven different trial types (10 trials each) presented at a pseudorandom order to assess PPI: 1) no-stimulus (i.e. only background noise was presented), 2) 40 ms of 120 dB startle pulse only, 3-6) 20 ms prepulse (69,

73, 77, 81 dB) + 40 ms of 120 dB startle pulse with 80ms interstimulus intervals, 7) 20 ms of 81 dB prepulse only. Trials were presented at an average ITI of 15 s which was pseudorandomized between 12 s – 30 s. The interstimulus intervals between prepulse and startle pulse were 80 ms. The test session ended with a final presentation of six startle pulse only trials separated by 10 s ITIs. Overall, this protocol lasted about 35 min. The magnitudes of ASR (whole body reflex) to pulse only trials were averaged for each mouse and defined as startle amplitude. PPI percentage was calculated as described in Geyer and Swerdlow (2001): $PPI = 100 \times [\text{startle pulse only} - (\text{prepulse} + \text{startle pulse})] / (\text{startle pulse only})$.

2.2.10. Novelty-induced suppressed feeding (Hyponeophagia)

Mice were given only 2.5 g/mouse regular diet on the evening (1 h prior to dark phase) before the test. On the test day, blocked right or left arm of a T-maze (W 5 cm x L 23 cm x H 10 cm) was used for test set-up. 40 μ l of sweetened condensed milk (Dovgan GmbH, Hamburg, Deutschland) diluted 50:50 with water was added into the food well in the maze arm. Each animal was placed in the arm facing away from the milk, given 2 min and the latency to proper drinking was recorded (Deacon, 2011). Animals, which did not consume the milk, were taken o homepage for 1 min and then tested again for another 2 min. This was repeated till they drank the milk (5 trials at most). Latency to consume the milk was calculated cumulatively.

2.2.11. Rewarded alternation task

The T-maze apparatus consisted of one longer start arm (35 cm x 5 cm) and two equal goal arms (28 cm x 5 cm; [Figure 9](#)) enclosed with 10 cm high walls. One day prior to hyponeophagia, animals were habituated to the maze for 10 min/cage to avoid novel environment induced anxiety. Mice were weighed daily and fed with chopped food pellets sufficiently to maintain their 80-85% free-feeding body weight until the end of the experiment. Small pieces of pellets were equally distributed in each cage to avoid one animal's dominance. Right after hyponeohagia test and during the first three days of forced alternation task, animals received additional 3 ml of sweetened condensed milk (50% diluted in water) to habituate them to the taste of the milk.

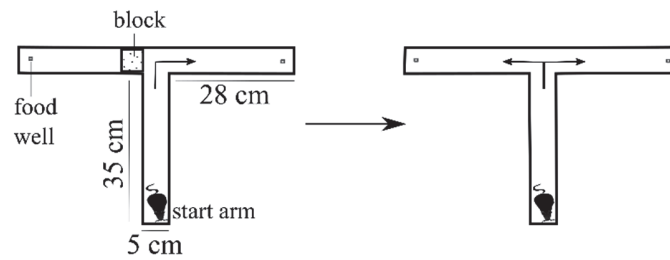


Figure 9. Rewarded alternation task was performed on T-maze.

For each trial, 20 μ l of sweetened condensed milk (50% diluted in water) was provided as a reward in food wells in both goal arms. Each mouse was placed in the start arm facing away from the goal arms. One of the goal arms was blocked forcing the mouse into the other goal arm. After mice consumed the milk in the unblocked arm, they were placed back to the start arm and the block was removed and were allowed to freely choose one of the goal arms. The correct choice (previously unvisited arm) was rewarded. Animals received one session (4 trials) per day for the first two days of learning and two sessions (4 trials each) per day for 11 test days. At the end of each day, percentage of correct choices from each session were scored. Each animal received an equal number of right and left arm runs to avoid any side-preferences. Animals were given ad libitum access to food at the end of the test.

2.2.12. Spatial novelty preference test

After T-maze, animals were given two resting days and then tested on a Y-maze for spatial working and reference memory respectively. The Y-maze apparatus consisted of three equal arms (35 cm x 35 cm x 35 cm, each has 5 cm run way enclosed with 10 cm high walls) at a 120° angle from each other. Mice were weighed daily and provided with external spatial cues to optimize cognitive performance on the Y-maze. For each mouse, one of the arms was randomly designated as novel arm and never changed throughout the experiment (Figure 10). The novel arm was blocked, the mouse was placed at the end of one of the unblocked arms (start arm) and allowed to freely explore both arms for 2 min after mouse left the start arm. This learning procedure was repeated 5 times in total (5 x 2 min trials with 1 min inter-trial intervals) by alternating the start arm. After the 5th trial, the mouse was taken back to the holding cage, the block was removed from the novel arm and the mouse was placed back to the start arm to explore the maze for a final 2 min. Time spent exploring the novel arm was recorded by ANY-maze video tracking system.

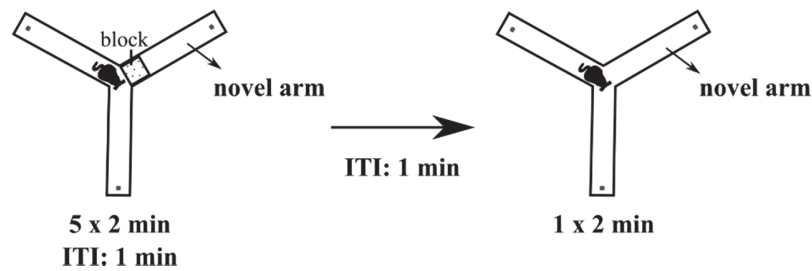


Figure 10. Schematic representation of novelty preference task on Y-maze

2.2.13. Spatial reference memory

After completion of the spatial novelty preference task, all food was taken away from mice. Mice were weighed daily and fed with chopped food pellets sufficiently to maintain their 80-85% free-feeding body weight starting from the first day of the experiment. For this test, each mouse was randomly assigned a goal arm (different than in 2.2.12) where 20 μ l of sweetened condensed milk (50% diluted in water) was received as a reward. A mouse was placed into the maze at one of the other two arms (i.e. start arm which was alternated in a pseudo-random order for each trial) and allowed to explore the maze until it consumed the milk. Then, the mouse was returned back to its home cage. Starting from the second day, mice were only rewarded if they chose the correct arm as first choice. Animals received two sessions (4 trials each) per day for the first two days and 3 sessions (4 trials each) for the following four days. At the end of the day, the percentage of correct choices were calculated per session.

2.2.14. Forced Swim Test (FST)

Forced swim test was performed to measure despair of rodents against an inescapable situation (Porsolt *et al.*, 1977a). Each animal was placed in a water-filled glass cylindrical tank (45 cm high, 17 cm in diameter, filled until 35 cm) and their behavior was recorded sideways for 6 min with a digital camera. At the end of each test, mice were kept in their cage in front of a 300 W ceramic infrared warming lamp (Beurer GmbH, Germany) at 30 cm distance in order to support animals' thermoregulation. Mice were kept in a separate room until testing and returned back to the holding room afterwards to avoid any olfactory or vocal cues. Experiments were performed in the afternoon towards the active phase. Water temperature was kept at 24-25°C. FST was repeated for each animal after 24 h under same conditions to test conditioning of mice.

2.2.15. Nest building behavior and Sucrose Preference

Building a nest is an important indicator of survival and well-being in rodents, because nests in nature function as shelters for protection from predators and harsh environmental conditions (Deacon, 2006). Therefore, each mouse was single-housed and given ~3 g of pressed cotton (Nestlets™, Ancare Corp., NY, USA) approximately 1.5 h before the dark phase. Next morning (~15 h), nests were also scored between 0-5 as suggested previously (Hess *et al*, 2008). The experiment was repeated for another 24 h using fresh nesting material.

Sucrose consumption, on the other hand, has been studied since years as an indicator of anhedonia, loss of ability to experience pleasure, to evaluate depressive-like state in rodents (Katz, 1981; Strekalova *et al*, 2004). For this purpose, mice were provided with two free-choice bottles, one filled with 1% sucrose in tap water and other with only tap water, for 48 hours in parallel to the nest building test. To avoid any side preferences, bottles with 1% sucrose were first positioned to the right of the feeder grid where mice were used to drink water and water bottles were positioned to left of the grid. 24 h later, their positions were switched.

2.2.16. Delay fear conditioning

In this study, fear conditioning was performed as suggested previously (Curzon *et al*, 2009) to evaluate the capability of associative learning which depends on Pavlovian conditioning ([Figure 11](#)). The ANY-maze controlled Ugo Basile fear conditioning system (Cat. No: 60461S, Ugo Basile SRL, Varese, Italy) was used to evoke and record fear learning. Animals were kept in a holding room with white noise at 60 dB to avoid any disruptions or influences (e.g. odor or sound). The experiment lasted 2 days. Freezing duration in each test was recorded with ANY-Maze software (v5.11b Beta, Stoelting Co., Wood Dale, IL, USA).

Conditioning: On day 1, each mouse was individually taken into the test room and placed in a chamber (17 cm x 17 cm x 25 cm, opaque walls) with electrified stainless steel grid floor inside of an isolation cubicle (lighting level = 100 lux). The test program started with 120 sec habituation period followed by 18 s of 1000 Hz – 85 dB tone (conditioned stimulus, CS) as cue which was repeated twice with 1 min interval. The tone was co-terminated with 3 s 0.3 A foot-shock (unconditioned stimulus, US). 30 s after the second foot-shock mice were returned to their home cage. Prior to and between each test, the chamber was cleaned with 20% ethanol.

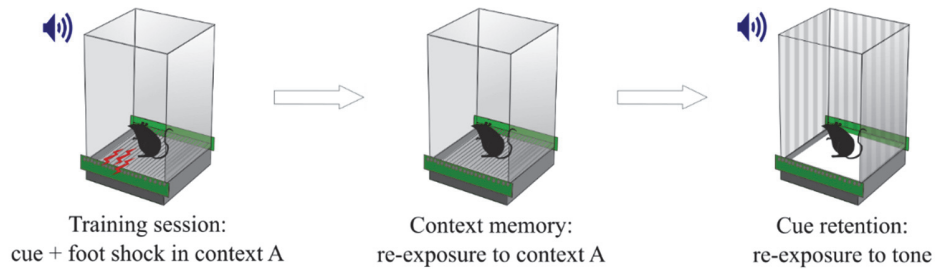


Figure 11. Representation of fear conditioning experimental design. Training session consisted of a 120 s habituation period followed by two trials of 18 s 85 dB tone cue paired with 0.3 A foot shock at its last 3 s. 24 h later, mice were tested in the same context without any cue or foot shock for context retention. At the end, mice tested for cue retention with presentation of 90 s of 85 dB tone after 120 s habituation period in a novel environment.

Context Memory: On day 2, mice were tested for contextual retention to evaluate whether they made an association with environmental context and therefore conditions (i.e. testing time of the day, odor, light, chamber design) were kept as similarly to the conditioning session. Mice were individually placed in test chamber for 4 min and returned back to their home cage afterwards. Prior to and between each test, the test chamber was cleaned with 20% ethanol solution to keep the same odor between conditioning session and contextual retention test.

Cue memory: 1 h after completion of contextual retention test for all animals, mice were tested for cue (tone) retention in a novel environment. Background of the chamber was replaced with vertically striped (black/white) acrylic plates and the floor was covered with white acrylic plate. 30 min before test, banana extract diluted 1:100 in 20 ml tap water was placed in isolation cubicle and left there throughout the experiment. Prior to and between each test, the test chamber was cleaned with Aerodesin 2000 Spray (Lysoform, Berlin, Germany). Mice were individually placed in test chamber and allowed to habituate for 120 s. Then, the cue (1000 Hz – 85 dB tone) was presented for 90 s. Afterwards mice were left undisturbed for 30 s before being returned to their home cage.

2.2.17. Perfusion of animals

Animals were deeply anesthetized with isoflurane and immediately fixed via transcardial perfusion with 6 ml 1x PBS solution followed by 30 ml 4% formaldehyde (FA) in 1x PBS with a perfusion speed of 3 ml/min. Brains were post-fixed in 4% FA in 1x PBS for 2 h, cryoprotected in 30% sucrose in 1x PBS for 48 h and then frozen in -20°C for further investigation.

2.2.18. Statistics

Behavioral measures were analyzed with two-way analysis of variance (ANOVA) (between-subjects factors: ‘stress’ and ‘injection’). Two way repeated measures (RM) ANOVAs were used for analysis of time-dependent tests. If assumption of sphericity was violated, Greenhouse-Geisser correction was used to correct the degrees of freedom of the *F*-distribution. When initial ANOVAs revealed any main effect for the corresponding factor(s), they were followed by two- or one-way ANOVAs. Multiple comparisons were performed with post hoc LSD test. The nonparametric Kruskal-Wallis “ANOVA by ranks” H-test and Mann-Whitney U-test employed for analyzing data with unequal variances. Data are presented as means \pm standard error of the mean (S.E.M.). Main effects were regarded as statistically significant with $p \leq 0.05$. A total of 80 mice were tested (n = 10 / group).

3. Results

3.1. *In vitro* results

3.1.1. Viruses and expression of virally encoded constructs

In order to investigate the role of disrupted NOS-I PDZ interactions on dendritic morphology, we constructed rAAV vectors expressing different isoforms or deletion mutants of NOS1AP as well as the aminoterminal 133 amino acids of NOS-I (NOS-I₁₋₁₃₃) ([Figure 4](#)). To test levels of transgene expression, immunoblotting against 3xFLAG tag was performed with protein lysates obtained seven days after infection (DIV14). Results revealed high levels of expression ([Figure 12](#)). Immunostainings for the FLAG-tag confirmed high expression levels and showed high rates of infectivity (>90% of all neurons infected; [Figure 12](#)). As viral overexpression of proteins may corrupt integrity and survival of neurons, we estimated the total number of cells by counting DAPI-positive nuclei and counted the number of eGFP-positive neurons. None of the constructs significantly changed the total number of cells ($F_{5,47}=0.699$, $p=0.627$ and $F_{5,38}=0.417$, $p=0.834$ at 1 or 2 weeks after infection respectively) or the number of eGFP-positive neurons ($F_{5,47}=1.343$, $p=0.263$ and $F_{5,47}=1.220$, $p=0.319$ at 1 or 2 weeks after infection respectively), suggesting that survival of neurons was not affected by overexpression of viral constructs, at least compared to neurons infected with the mCherry expressing rAAV.

3.1.2. Interaction of viral constructs with endogenous NOS-I

PDZ interaction of NOS-I has been proposed to be involved in the pathophysiology of schizophrenia (Freudenberg *et al*, 2015; Weber *et al*, 2014). To test whether our constructs interact with endogenous NOS-I, virally encoded proteins were isolated from primary cultured hippocampal neurons on DIV14 (i.e. 7 days after infection) and immunoprecipitated using the FLAG-tag ([Figure 12](#)). We then tested for co-precipitation of endogenous NOS-I by immunoblotting ([Figure 13](#)) and found that, as expected, both NOS1AP-L and NOS1AP-S strongly interacted with endogenous NOS-I, as both constructs contain the carboxyterminal domain shown to be required for interaction with NOS-I (Jaffrey *et al*, 1998; Li *et al*, 2015). Interestingly, endogenous NOS-I still co-precipitated with NOS1AP-L Δ C20, though co-precipitation of NOS-I with this construct was strongly decreased. Moreover, we found a lack of co-precipitation of endogenous NOS-I with NOS1AP-LC20, which is concordant with findings from a recent study (Li *et al*, 2015) suggesting that the NOS1AP C-terminus is not required for

interaction between NOS1AP and NOS-I but rather stabilizes this interaction. Finally, upon viral expression of NOS-I₁₋₁₃₃ I only found mild co precipitation of endogenous NOS-I. This is not surprising, as dimerization has been shown to be mediated by the oxygenase domain (Klatt *et al*, 1995, 1996).

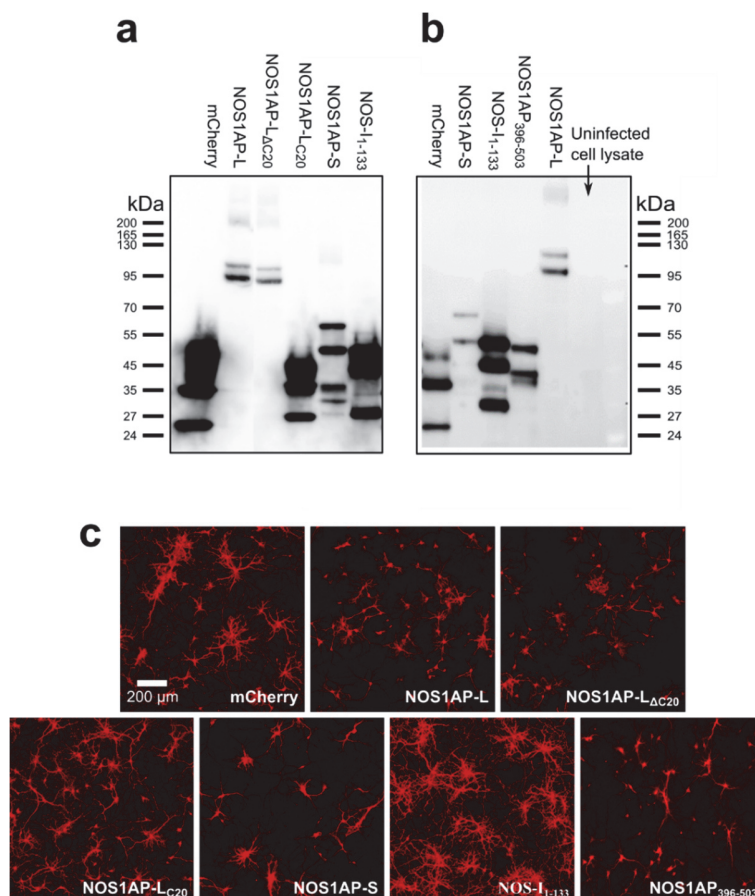


Figure 12. Expression levels in primary cultured hippocampal neurons 1 week after rAAV infection. a-b) Immunoblots against the 3xFlag tag reveals high expression levels for viral proteins in cell lysates. Smaller constructs had higher expression levels compared to larger constructs. For each sample, there is a smaller second band which is due to proteolysis of mCherry under denaturing conditions (Gross *et al*, 2000; Verkhusha and Sorkin, 2005). **c)** Immunofluorescence staining for α-FLAG confirmed high rates of infectivity.

Additionally, we tested for potential co-precipitation of endogenous NOS1AP (Figure 13) using a polyclonal antibody raised against amino acids 304-503 of rat NOS1APa (97% identity to amino acids 303-503 of mouse NOS1AP-L) (Clattenburg *et al*, 2015). Both, the short form (NM_027528.2; note that this isoform is different from the human short form used in this study) and the long form of NOS1AP were present in all protein lysates. For the long form of NOS1AP we identified two bands at ~55 and ~75 kDa, which have been suggested to reflect this protein in

unphosphorylated and phosphorylated state respectively (Xu *et al*, 2005). As expected, I also observed NOS1AP signal for virally expressed NOS1AP and deletion mutants at the appropriate molecular weights in protein and co-IP lysates. In addition, in protein lysates from neurons overexpressing NOS1AP-L, NOS1AP-L Δ C20 and NOS1AP-S we observed a relatively strong protein smear suggesting detection of relatively stable macromolecular protein complexes involving virally expressed NOS1AP. These smears were also observed in the corresponding co-IP lysates though at much weaker intensity. As described above, NOS-I₁₋₁₃₃ was shown to interact with endogenous NOS1AP (Jaffrey *et al*, 1998). However, we did not detect endogenous NOS1AP in co-IP lysates from neurons overexpressing NOS-I₁₋₁₃₃, suggesting that in our study design this construct may not have interacted with NOS1AP or that this interaction was either too moderate or too unstable for detection using co-IP.

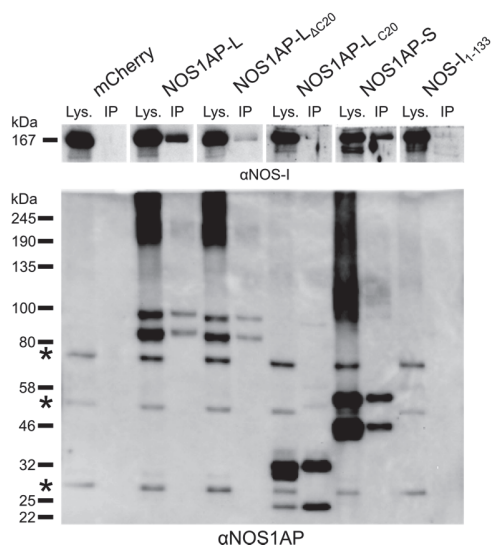


Figure 13. Co-immunoprecipitation of viral encoded proteins with endogenous NOS-I or NOS1AP. Immunoblotting was performed with protein lysates prepared from primary hippocampal neuron cultures one week after infection. Both endogenous NOS-I and NOS1AP did not co-precipitate with the control construct expressing mCherry. NOS1AP-L and NOS1AP-S strongly co-precipitated with endogenous NOS-I and NOS1AP-L Δ C20 showed strongly reduced co-precipitation. However, NOS1AP-L_{C20}, the residues claimed to be necessary for NOS-I binding, did not co-precipitate with endogenous NOS-I, supporting previous findings as NOS1AP's C-terminal 20 amino acids are only partially necessary and not sufficient for interaction with NOS-I (Li *et al*, 2015). Endogenous NOS1AP (at ~30, ~55 and ~75 kDa representing the murine short form, the unphosphorylated long form and the phosphorylated long form of NOS1AP respectively; indicated by the asterisks) was present in all protein lysates, but in none of the co-IP, suggesting that none of the constructs interacted with endogenous NOS1AP or only interacted at moderate levels.

NOS-I/PSD-95 interaction has been proposed to bring NOS-I in proximity to NMDA receptors, enabling NO production dependent on NMDA receptor activity (Freudenberg *et al*, 2015). Disruption of this interaction by overexpression of NOS1AP isoforms, its deletion mutants or NOS-I₁₋₁₃₃ might therefore result in reduced NOS-I activity. Thus, we tested for NO production by measuring DAF-FM fluorescence. Fluorescence of DAF-FM has been shown to be proportional to the amount of NO available (Kojima *et al*, 1998; Nakatsubo *et al*, 1998; Namin *et al*, 2013; Zhou and Zhu, 2009) allowing relative quantification of NO produced by neurons. After validation that DAF-FM can be used for quantification of NO levels in our system (Candemir *et al*, 2016), I

measured DAF-FM fluorescence in primary cultured hippocampal neurons under basal conditions (i.e. without stimulation of NMDA receptors) one week after infection (i.e. DIV14). There was no statistical difference in fluorescence ($p > 0.05$; [Figure 14](#)), suggesting that enzymatic activity of endogenous NOS-I was not affected by expression of the different constructs.

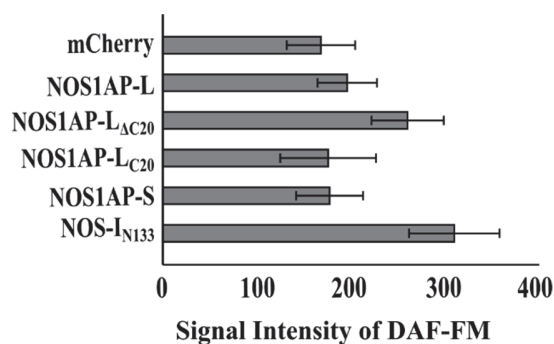


Figure 14. NOS-I enzymatic activity remained intact upon infection with different vectors. DAF-FM diacetate analysis was performed in primary cultured hippocampal neurons one week after infection (DIV14; ($F_{5, 37} = 2.000$, $p = 0.101$)). $n = 5-8$.

3.1.3. Regulation of dendritic development and spinogenesis

3.1.3.1. Dendritic branching

Overexpression of NOS1AP has previously been shown to reduce dendritic branching in primary neurons (Carrel *et al*, 2009a; Richier *et al*, 2010). Therefore, we traced individual neurons and calculated their dendritic branching pattern by Sholl analysis ([Figure 15](#)). In contrast to previously published findings (Carrel *et al*, 2009a; Richier *et al*, 2010), upon overexpression of NOS1AP-L in primary cultured cortical neurons one week after infection (DIV14) we only observed a mild reduction of dendritic length and branching with a trend for significance ($p = 0.056$ and $p = 0.057$ respectively), while no alterations in dendritic branching or length were observed two weeks after infection (i.e. DIV21) in cortical neurons and one or two weeks after infection (i.e. DIV14 and DIV21 respectively) in hippocampal neurons ([Figure 16](#)). Despite reduced interaction of NOS1AP-L Δ C20 with endogenous NOS-I, overexpression of this construct resulted in significant reduction of dendritic length and branching in primary cultured hippocampal neurons one week after infection (i.e. DIV14; $p < 0.001$ and $p = 0.002$ respectively). This reduction was also apparent two weeks post-infection (DIV21) though this effect was not significant ($p = 0.414$ and $p = 0.156$ respectively).

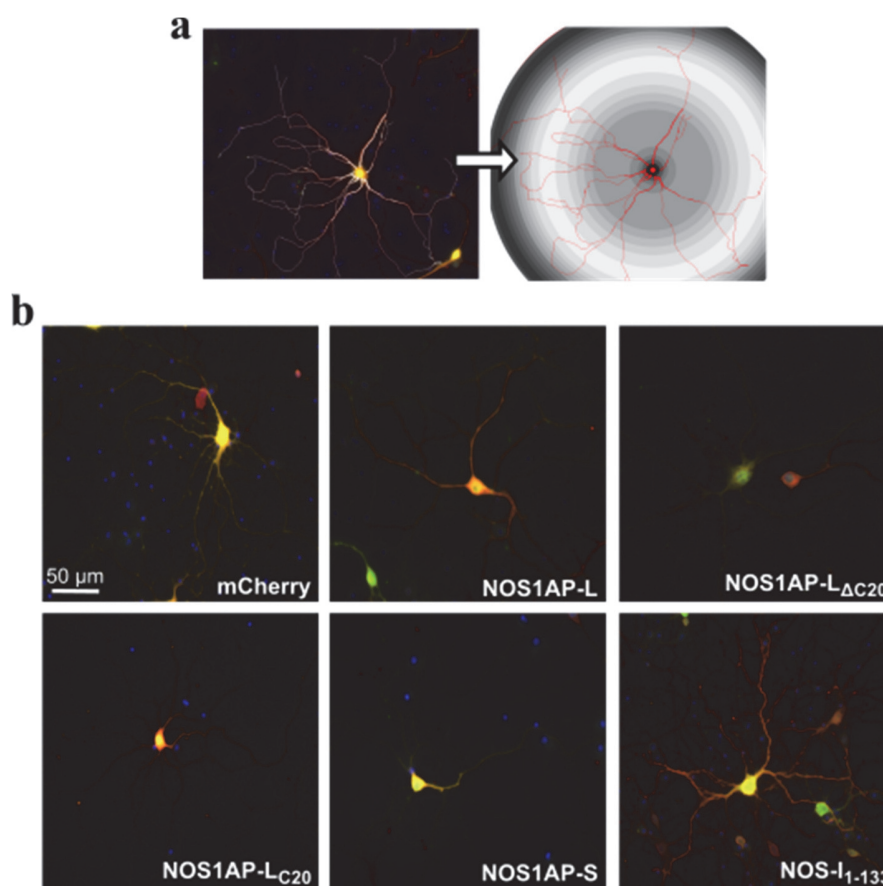


Figure 15. Representative images of dendritic branching in primary cultured neurons. **a)** Dendritic arborization was traced with Simple Neurite Tracer plugin and the intersection mask colored due to number of intersections with Sholl Analysis plugin in ImageJ/Fiji. **b)** Illustrations of dendritic branching and growth in the primary cultured cortical neurons seven days after infection.

In cortical neurons expressing NOS1AP-L Δ C20 we only found a mild, non-significant ($p=0.298$) reduction in the sum of intersections one week after infection (DIV14) and no effect in any other condition. Viral expression of NOS1AP-LC20, hypothesized to disrupt NOS-I/NOS1AP interaction did not cause significant alterations in dendritic length or branching. Moreover, overexpression of NOS1AP-S, the isoform lacking the PTB and carboxypeptidase E (CPE) binding domains, did not have an effect on ending radius or sum of intersections of infected primary cultured neurons, suggesting that interaction of NOS1AP with the PDZ domain of NOS-I may not be involved in the regulation of dendritic patterning. Further supporting this hypothesis, disruption NOS-I PDZ interaction by NOS-I₁₋₁₃₃ expression did not reduce length or branching of dendrites in primary cultured neurons. In fact, expression of NOS-I₁₋₁₃₃ in hippocampus had a beneficial effect on dendritic growth to an extent, making Sholl analysis of these neurons impossible.

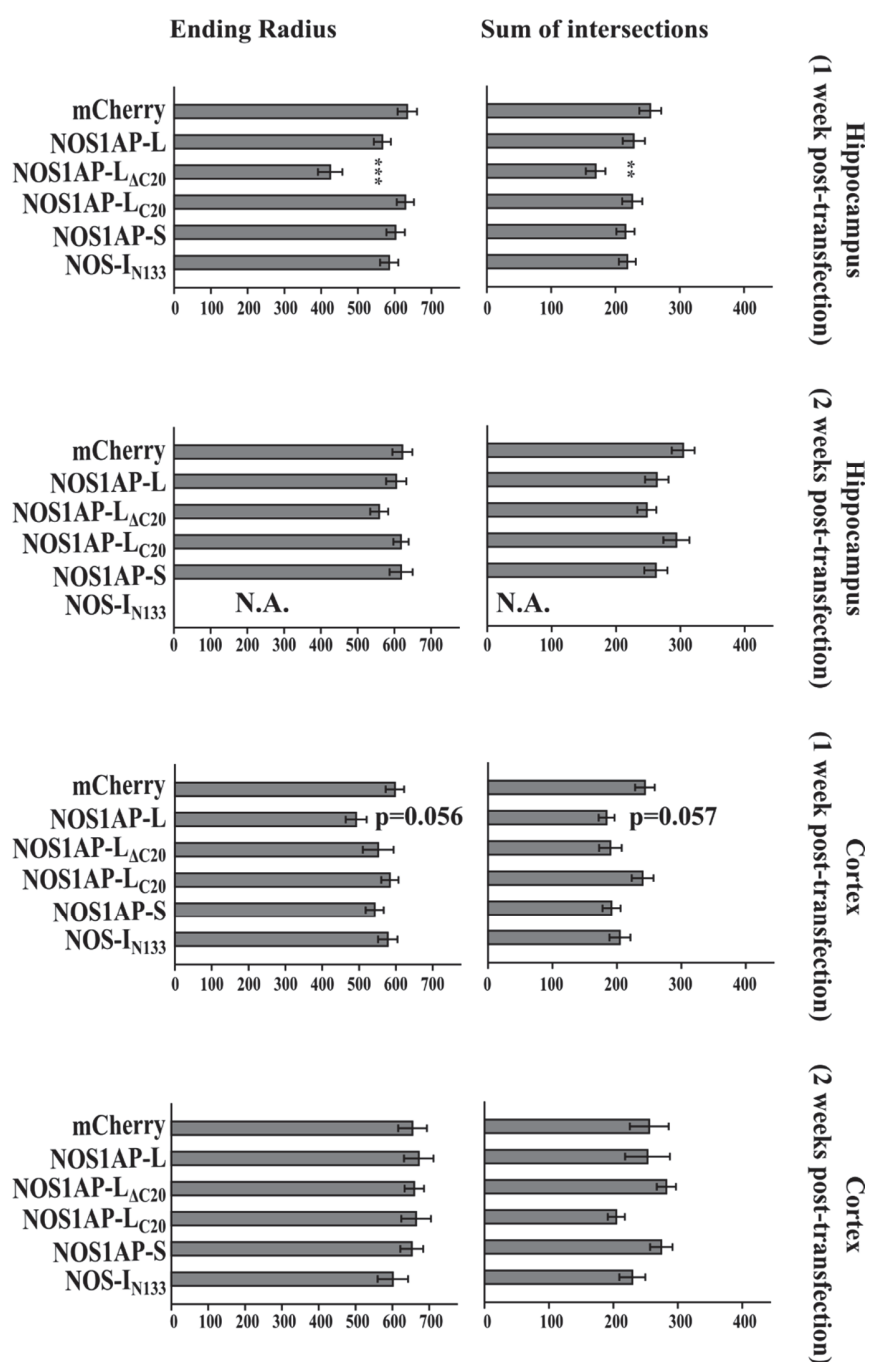


Figure 16. Dendritic branching was mildly reduced by overexpression of NOSA1P isoforms or deletion mutants. Sholl analysis was used to determine branching (sum of intersections) and dendritic length (ending radius) with a “radius step size” of 15 px (~5 μ m) represented as mean \pm S.E.M (in pixels). Hippocampus, 1 week post-infection: ending radius $F_{5,169}=8.849$, $p<0.001$; sum of intersections $F_{5,157}=3.350$, $p=0.007$. Hippocampus, 2 weeks post-infection: ending radius $F_{4,125}=1.086$, $p=0.367$; sum of intersections $F_{4,125}=1.792$, $p=0.135$. Cortex, 1 week post-infection: ending radius $F_{5,157}=2.124$, $p=0.065$; sum of intersections $F_{5,157}=2.965$, $p=0.014$. Cortex, 2 weeks post-infection: ending radius $F_{5,66}=0.147$, $p=0.98$; sum of intersections $F_{5,66}=1.657$, $p=0.157$. post-hoc Tukey’s t-test: # $p=0.056$, † $p=0.057$, ** $p<0.005$ and *** $p<0.001$ vs. control vector. N.A.= not available, $n=30$.

3.1.3.2. Plasticity of dendritic spines

Reduced synaptic activity and dendritic spine density have been described as a pathological feature of schizophrenia (reviewed in Moyer *et al*, 2014). Overexpression of NOS1AP has been previously reported to increase the amount of dendritic protrusions (Richier *et al*, 2010; Zhu *et al*, 2014). Therefore, we investigated whether overexpression of NOS1AP alters dendritic spine plasticity by quantification of dendritic spines according to their morphologies as stubby, thin, mushroom and filopodia-like ([Figure 17](#)) (Yuste and Bonhoeffer, 2004).

We found that overexpression of NOS1AP isoforms, NOS1AP deletion mutants and NOS-I₁₋₁₃₃ resulted in highly altered dendritic spine morphology and caused an increased amount of long, thin filopodial dendritic protrusions ([Table 2, Figure 17](#)). Specifically, we observed a significant decrease in the total number of spines (particularly thin and mushroom spines) in neurons overexpressing NOS1AP-L. Additionally, we found a significantly increased number of filopodia. As postsynaptic signaling is crucial for stabilization and maintenance of dendritic processes, increased filopodia growth might point towards a disruption of spine maintenance or reduced glutamatergic neurotransmission, both of which are important pathophysiological features observed in schizophrenia (Moyer *et al*, 2014). In neurons overexpressing NOS1AP-L Δ C20 we found a decrease in the number of mature spines (thin and mushroom spines) and significantly increased filopodia-like structures in primary cultured neurons of the hippocampus (one week post-infection only; DIV14) and cortex (one and two weeks after infection; DIV14 and 21). Analogous to our findings on dendritic patterning this may suggest that NOS1AP exerts its effects on spine plasticity through interactions not involving NOS-I. However, overexpression of NOS1AP-LC20 resulted in a significant decrease in the number of mature spines and neurons overexpressing NOS1AP-S displayed a reduced number of mature spines in all conditions and increased number of filopodial structures in primary cultured cortical (one week post-infection only; DIV14) and hippocampal (two weeks post-infection only; DIV21) neurons. While these results suggest an important involvement of NOS1AP carboxyterminal interaction (potentially involving NOS-I) in spine plasticity, general disruption of NOS-I PDZ interaction by overexpression of NOS-I₁₋₁₃₃ only had mild effects on spine development, with neurons overexpressing NOS-I₁₋₁₃₃ showing a reduction in mature spines but an unaltered filopodia number.

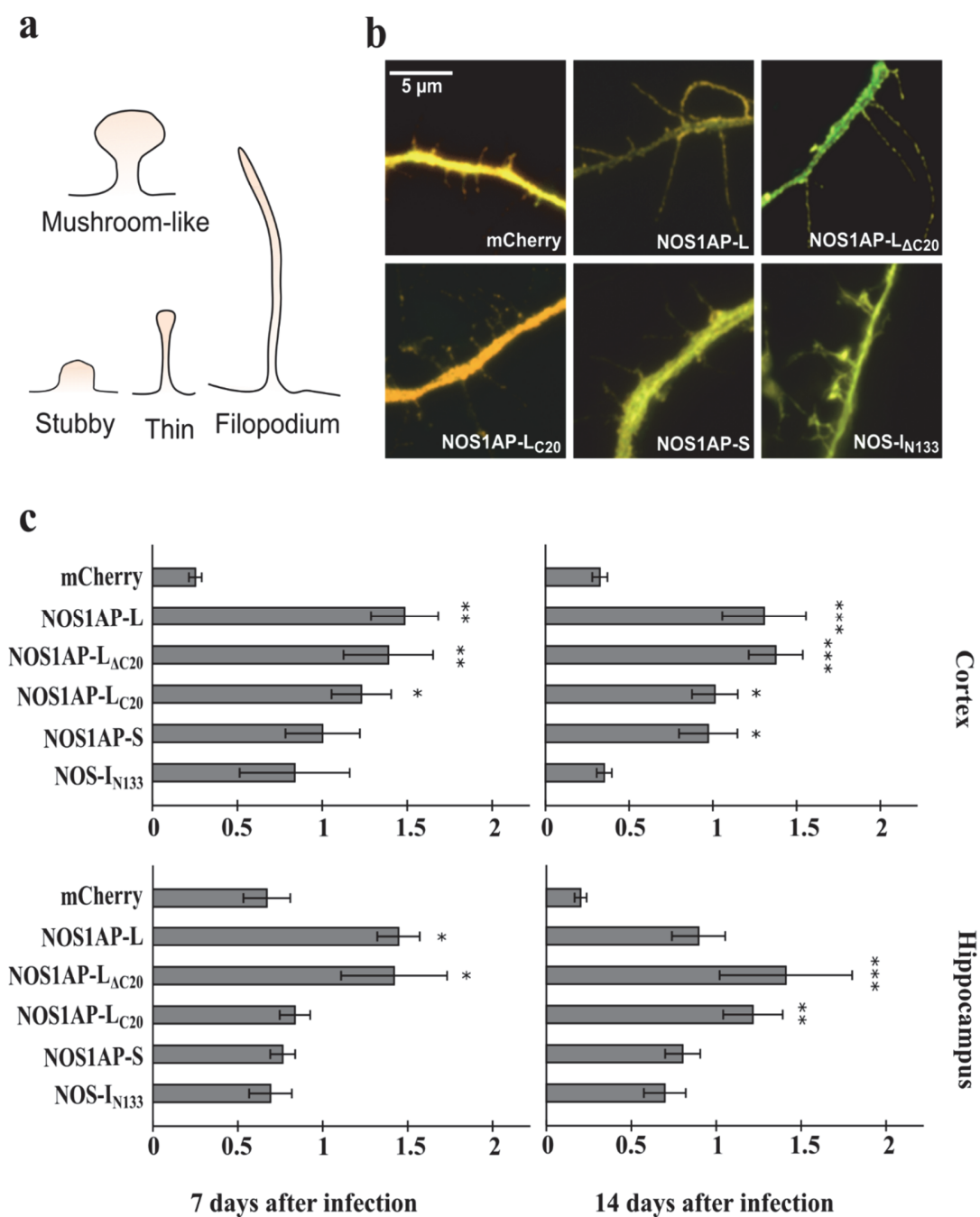


Figure 17. Disruption of NOS-I PDZ interactions resulted in increased filopodia-like dendritic protrusions. **a)** Illustration of dendritic spine classes (Yuste and Bonhoeffer, 2004). **b)** Representative images of dendritic spine morphology on primary cultured cortical neurons 7 days post-infection. **c)** Ratio of filopodia-like structures over total mature spines per 15 μ m dendrite. Cortex, 7 days after infection: $F_{5, 156}=4.156$, $p=0.001$; Cortex, 14 days after infection: $F_{5, 174}=8.800$, $p<0.001$; Hippocampus, 7 days after infection: $F_{5, 174}=4.871$, $p<0.001$; Hippocampus, 14 days after infection: $F_{5, 174}=4.567$, $p=0.001$. Data is represented as mean \pm S.E.M. $n=27-30$.

Table 3. Count of dendritic spine subgroups per 15 µm dendritic segment in primary neuronal cultures transfected with different viral vectors

		mCherry	NOS1AP-L	NOS1AP-L _{ΔC20}	NOS1AP-L _{C20}	NOS1AP-S	NOS-I ₁₊₁₃₃	Statistics
Primary Cortex Neuronal Culture (1 week after infection; DIV14) (n=27)								
Thin	5.19 ± 0.29	2.07 ± 0.18***	2.44 ± 0.27***	3.15 ± 0.22***	2.89 ± 0.31**	3.26 ± 0.25**	F _{5, 156} =9.863, p<0.001	
Stubby	2.78 ± 0.24	2.11 ± 0.19	2.48 ± 0.30	1.96 ± 0.14	4.04 ± 0.35	2.67 ± 0.30	F _{5, 156} =5.153, p<0.001	
Mushroom-like	1.59 ± 0.22	0.89 ± 0.22	1.07 ± 0.17	1.07 ± 0.16	0.56 ± 0.12*	1.96 ± 0.22	F _{5, 156} =4.269, p=0.001	
Filopodia	2.33 ± 0.24	5.89 ± 0.38***	5.15 ± 0.35***	5.85 ± 0.27***	4.63 ± 0.42**	4.04 ± 0.46	F _{5, 156} =8.639, p<0.001	
Total Spine (exc. Filopodia)	9.56 ± 0.38	5.07 ± 0.36***	6.00 ± 0.53**	6.19 ± 0.41**	7.48 ± 0.57	7.89 ± 0.48	F _{5, 156} =6.959, p<0.001	
Primary Cortex Neuronal Culture (2 weeks after infection; DIV21) (n=30)								
Thin	6.03 ± 0.32	3.33 ± 0.32***	3.67 ± 0.30**	4.87 ± 0.41	3.67 ± 0.26**	6.17 ± 0.38	F _{5, 174} =8.267, p<0.001	
Stubby	3.03 ± 0.19	2.70 ± 0.23	3.00 ± 0.32	1.97 ± 0.15	2.40 ± 0.23	2.47 ± 0.22	F _{5, 174} =1.751, p=0.126	
Mushroom-like	2.87 ± 0.22	1.30 ± 0.19**	1.13 ± 0.16***	1.43 ± 0.17**	1.27 ± 0.16***	3.90 ± 0.25	F _{5, 174} =18.881, p<0.001	
Filopodia	3.63 ± 0.40	6.67 ± 0.52**	8.00 ± 0.43***	5.97 ± 0.43*	5.13 ± 0.36	3.97 ± 0.35	F _{5, 174} =9.518, p<0.001	
Total Spine (exc. Filopodia)	11.93 ± 0.54	7.33 ± 0.56***	7.80 ± 0.62**	8.27 ± 0.57**	7.33 ± 0.48***	12.53 ± 0.55	F _{5, 174} =11.034, p<0.001	
Primary Hippocampal Neuronal Culture (1 week after infection; DIV14) (n=30)								
Thin	4.30 ± 0.38	2.60 ± 0.28*	2.67 ± 0.20*	3.90 ± 0.23	3.37 ± 0.22	3.40 ± 0.34	F _{5, 174} =3.366, p=0.006	
Stubby	2.83 ± 0.33	2.03 ± 0.15	1.97 ± 0.15	2.40 ± 0.22	2.87 ± 0.21	2.47 ± 0.22	F _{5, 174} =1.947, p=0.089	
Mushroom-like	2.60 ± 0.23	1.13 ± 0.18**	1.07 ± 0.16**	2.00 ± 0.17	1.23 ± 0.17**	2.70 ± 0.33	F _{5, 174} =7.668, p<0.001	
Filopodia	4.67 ± 0.45	7.37 ± 0.34**	6.13 ± 0.35	6.23 ± 0.45	5.20 ± 0.35	4.30 ± 0.42	F _{5, 174} =5.273, p<0.001	
Total Spine (exc. Filopodia)	9.73 ± 0.72	5.77 ± 0.34***	5.70 ± 0.30***	8.30 ± 0.39	7.47 ± 0.31	8.57 ± 0.64	F _{5, 174} =7.417, p<0.001	
Primary Hippocampal Neuronal Culture (2 weeks after infection; DIV21) (n=30)								
Thin	6.03 ± 0.51	3.80 ± 0.27**	2.97 ± 0.31***	2.67 ± 0.26***	3.30 ± 0.30***	4.07 ± 0.32*	F _{5, 174} =7.815, p<0.001	
Stubby	4.13 ± 0.29	3.60 ± 0.25	3.27 ± 0.18	3.23 ± 0.26	3.07 ± 0.18	2.43 ± 0.22**	F _{5, 174} =3.245, p=0.008	
Mushroom-like	2.80 ± 0.30	0.90 ± 0.14***	1.13 ± 0.12**	1.47 ± 0.21*	1.40 ± 0.15**	2.20 ± 0.29	F _{5, 174} =6.456, p<0.001	
Filopodia	2.47 ± 0.28	5.97 ± 0.36***	6.63 ± 0.50***	7.20 ± 0.55**	5.60 ± 0.42*	4.67 ± 0.47	F _{5, 174} =9.843, p<0.001	
Total Spine (exc. Filopodia)	12.97 ± 0.82	8.30 ± 0.40***	7.37 ± 0.45***	7.37 ± 0.40***	7.77 ± 0.35***	8.70 ± 0.57***	F _{5, 174} =10.865, p<0.001	

Counting and classification were performed manually based on dendritic spine classifications as previously described (Yuste and Bonhoeffer, 2004). Values are represented as mean ± S.E.M. post-hoc Tukey's t-test: *p<0.05, **p<0.005 and ***p<0.001 vs. control vector expressing mCherry. DIV = days *in vitro*

3.2. Behavioral consequences of disrupted NOS-I PDZ interactions

Given the disrupted dendritic growth and branching *in vitro*, the next step was to investigate whether disruption of NOS-I PDZ interactions results in behavioral deficits associated with neuropsychiatric conditions such as schizophrenia, anxiety or depression. To this end, rAAVs (Figure 4) expressing full length murine NOS1AP-L, NOS1AP₃₉₆₋₅₀₃ encoding the NOS-I interaction motif, NOS-I₁₋₁₃₃ containing the PDZ domain, and mCherry control vector were stereotaxically delivered to the dorsal hippocampus of adult male C57Bl/6J mice. Expression of virus-encoded proteins was verified by immunofluorescent staining against the FLAG-tag and showed relatively high levels of expression, which is largely restricted to the dorsal hippocampus (Figure 18).

One week after recovery from surgery, mice were exposed to three weeks of CMS and afterwards were subjected to a comprehensive analysis to monitor behaviors relevant to human neuropsychiatric conditions.

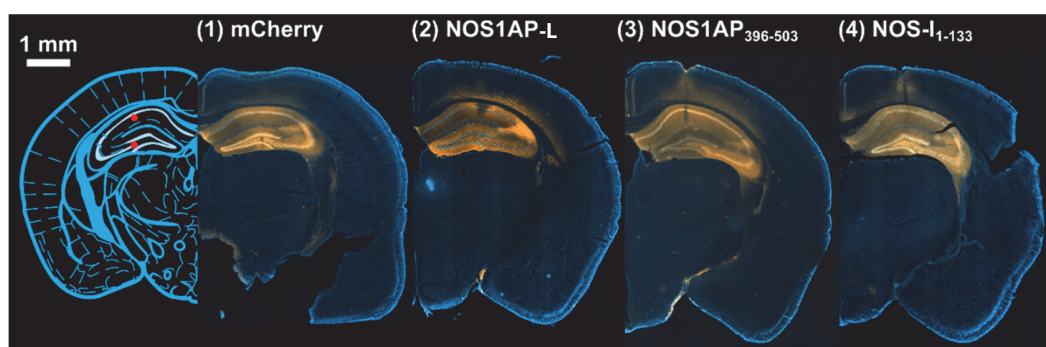


Figure 18. Exemplary images of viral vector expression in mice stereotaxically injected in dorsal hippocampus (dHPC). Injections sites are indicated by the red dots in the left-hand schematic. Expression of virally expressed proteins is largely limited to the targeted brain areas and little ectopic expression in cortical areas surrounding the injection site. Images are overlays of DAPI epifluorescence (blue) and mCherry enhanced by immunostaining for the 3xFLAG tag (orange).

3.2.1. Body weight of mice

Body weights of mice was monitored regularly to ensure healthy development. Mice were weighed at six specific time points: (1) just before the surgery (at age of 7 weeks old), (2) after the end of the CMS protocol (at age of 11 weeks old), as well as at (3) 12, (4) 14, (5) 17, and (6) 21 weeks of age. Changes in weight were analyzed by RM ANOVA with two between-subjects factors (stress x injection). Experimental mice from all groups significantly increased their body weight over time ($F_{1,24, 88.9}=42.48, p<0.001$) as they developed from adolescence to adulthood (data not shown).

However, the weight was not effected by treatment ($F_{1, 72}=2.232$, $p=0.14$), injection ($F_{3, 72}=2.251$, $p=0.9$) or the interaction of treatment and injection ($F_{3, 72}=0.758$, $p=0.522$). Body weights of mice were also closely monitored during the tests, which required food restriction. Obtained data will be mentioned in the relevant sections.

3.2.2. Locomotor Activity

Stereotypic behaviors (Gainetdinov *et al*, 2001) which can be modeled in rodents as increased locomotor activity has been previously shown in *NOS1* knock-out (KO) mouse models (Tanda *et al*, 2009; Weitzdoerfer *et al*, 2004). Therefore, mice were tested for locomotor activity which was defined as total distance travelled in the OF arena. Locomotor activity was significantly reduced over time ($p<0.001$) in all groups, as they habituate to the OF. Non-stressed mice overexpressing NOS-I₁₋₁₃₃ showed increased activity compared to mCherry expressing mice between 6th – 8th min ($p=0.04$), however this effect was lost after 8 min ($p=0.31$). Increased locomotion upon overexpression of NOS-I₁₋₁₃₃, encoding for the extended PDZ domain of NOS-I, might indicate an involvement of NOS-I PDZ domain in regulation of stereotypic behaviors observed in many psychotic disorders. On the other hand, non-stressed NOSIAP₃₉₆₋₅₀₃ showed much lower activity compared to mCherry overexpressing mice between 8th - 10th min ($p=0.008$) suggesting that this construct might exert a therapeutical effect resembling the effects of antipsychotics those reducing activity in mice (Freed *et al*, 1984).

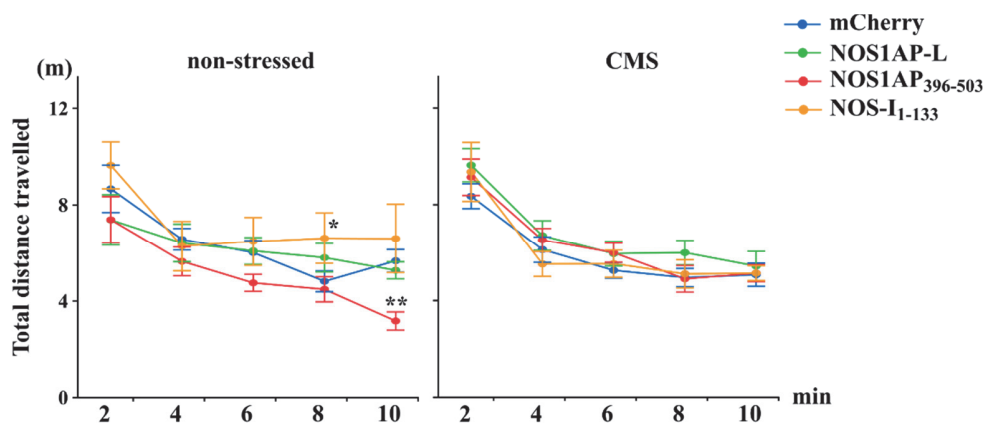


Figure 19. Locomotor activity was mildly altered by expression of different viral vectors. Data is shown as mean \pm SEM. Two way RM ANOVA with Greenhouse-Geisser correction (Mauchly's Test of Sphericity, $p>0.05$), for time: $F_{2,488, 179.112}=76.970$, $p<0.001$; time x injection: $F_{7,463, 179.112}=1.419$, $p=0.196$; time x stress: $F_{2,488, 179.112}=1.704$, $p=0.177$; time x stress x injection: $F_{7,463, 179.112}=0.767$, $p=0.624$. post-hoc LSD test, * $p<0.05$ vs mCherry. $n=10$ per group.

3.2.3. Anxiety-Related Behaviors

A recent study has shown that increased formation of the NOS1AP/NOS-I complex in the hippocampus of mice resulted in anxiogenic-like behavior which was reversed by disruption of this complex (Zhu *et al*, 2014). To monitor whether mice in this study would show the same phenotype, I have tested mice overexpressing different interaction domains in OF, LDB, hyponeophagia and EZM (Figure 20). Overall, CMS mice spent more time in the center of the OF arena ($p=0.046$) and displayed a lower latency to enter to the center ($p=0.01$). Also in LDB, CMS mice spent more time in the light compartment compared to non-stressed mice ($p<0.001$) and they started to explore the light compartment earlier than non-stressed mice ($p=0.004$).

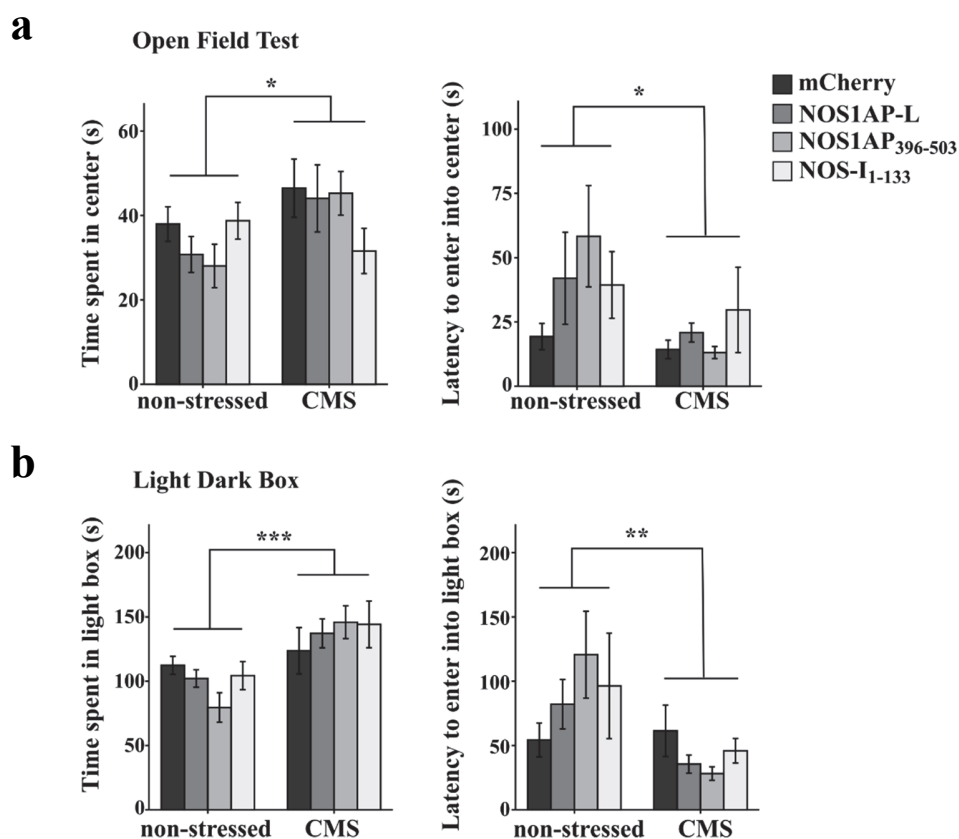


Figure 20. CMS induced anxiolytic phenotype regardless of viral constructs in both OF and LDB tests.

a) Open field test. Two way ANOVA for time spent in center: stress, $F_{1, 72}=33.603$, $p=0.046$; injection, $F_{3, 36}=0.603$, $p=0.615$; interaction stress x injection, $F_{3, 36}=1.856$, $p=0.145$. Nonparametric tests for latency (Levene's test, $p<0.05$): stress, Mann-Whitney U test, $U= 531$, $p=0.01$; injection, Kruskal-Wallis H Test, $\chi^2(3)=4.205$, $p=0.240$. **b)** Light-dark box test. Two way ANOVA for time spent in center: stress, $F_{1, 72}=18.083$, $p<0.001$; injection, $F_{3, 72}=0.282$, $p=0.838$; interaction stress x injection, $F_{3, 72}=1.581$, $p=0.201$. Nonparametric tests for latency (Levene's test, $p<0.05$): stress, Mann-Whitney U test, $U= 504.5$, $p=0.004$; injection, Kruskal-Wallis H Test, $\chi^2(2)=0.040$, $p=0.998$. Data is shown as mean \pm SEM. * $p<0.05$, ** $p<0.005$, *** $p<0.001$ for CMS vs non-stressed mice. $n=10$ per group.

Mice were tested for novelty-induced suppressed feeding with a novel food (i.e. sweetened milk) which was not significantly altered by targeting NOS-I PDZ interaction in hippocampus. Finally, mice were tested for anxiety against elevated and open novel environment in EZM and neither stress treatment nor the constructs had an effect on their behavior. In summary, data revealed that mice undergoing three weeks of CMS showed lower anxiety levels compared to non-stressed mice in OF and LDB tests regardless of the overexpressed viral construct. However, this anxiolytic phenotype is an overall group effect which seems to be dependent on the alterations in behavior of mice overexpressing NOS1AP-L or NOS1AP₃₉₆₋₅₀₃, but this was failed to be proven by post-hoc tests.

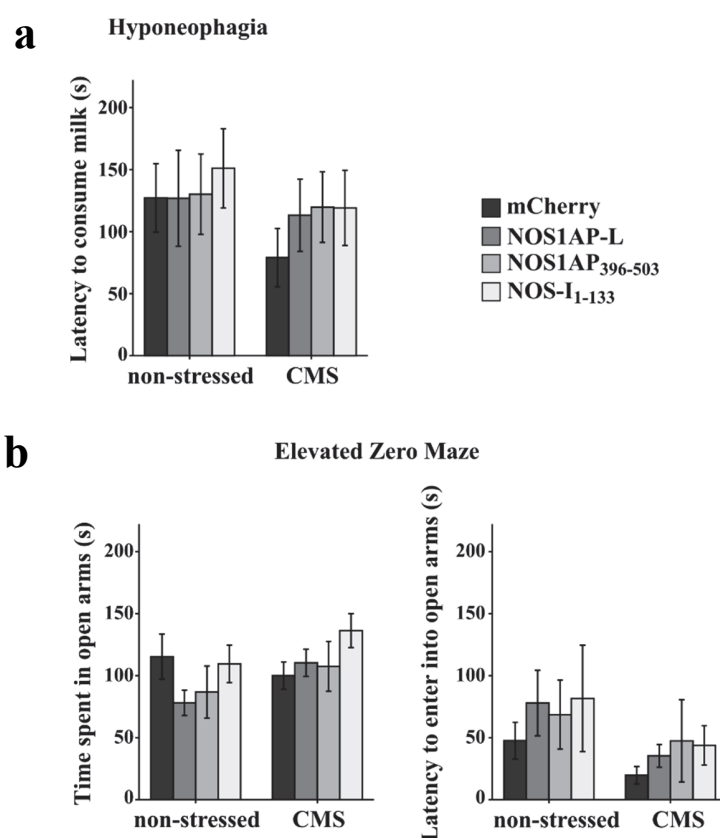


Figure 21. Targeting NOS-I PDZ interactions did not alter behavior in hyponeophagia and EZM tests. a) Hyponeophagia. Two way ANOVA: stress, $F_{1,70}=0.789$, $p=0.378$, injection, $F_{3,70}=0.593$, $p=0.622$; interaction stress x injection, $F_{3,70}=0.475$, $p=0.701$. b) Elevated zero maze. Levene's test indicated unequal variances for time spent in open arms ($F=2.164$, $p=0.048$) and latency to enter into open arms ($F=3.178$, $p=0.006$) in two way ANOVA. Kruskal-Wallis H Test revealed neither stress nor injection factors had any effect on time spent in open arms ($\chi^2(2)=2.618$, $p=0.106$ and $\chi^2(2)=4.603$, $p=0.203$ respectively) and latency ($\chi^2(2)=1.328$, $p=0.723$ and $\chi^2(2)=1.899$, $p=0.168$ respectively). Data is shown as mean \pm SEM. $n=10$ per group.

3.2.4. Novel object recognition

NOR is a memory task based on the rodent's innate exploratory behavior because they tend to explore more when they confronted novelty (Ennaceur, 2010). Studies have shown that hippocampus plays a key role to process contextual information (Antunes and Biala, 2012). Therefore, mice were tested for NOR (Figure 22). During the familiarization period, two identical objects (A+A) were given to mice to explore. In the non-stressed group, mice overexpressing NOS1AP-L ($p=0.036$) and mice overexpressing NOS-I₁₋₁₃₃ ($p=0.025$) spent significantly less time to explore the object compared to mCherry mice suggesting exploratory behavior deficits in these mice. Despite the reduced exploration, all mice displayed comparable recognition memory of a novel object indicating unaltered short-term context memory in these mice.

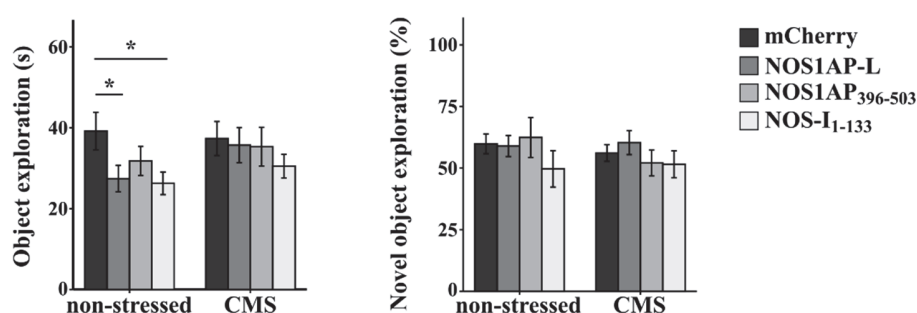


Figure 22. Disrupted NOS-I PDZ interactions impaired exploratory behavior in object exploration test but did not altered novelty preference. Familiarization period, two way ANOVA: stress, $F_{1, 71}=1.640$, $p=0.204$; injection, $F_{3, 71}=2.216$, $p=0.094$; interaction of stress x injection, $F_{3, 71}=0.573$, $p=0.635$. Novelty preference test, two way ANOVA: stress, $F_{1, 72}=0.473$, $p=0.494$; injection, $F_{3, 72}=1.017$, $p=0.39$; interaction of stress x injection, ($F_{3, 72}=0.523$, $p=0.668$). * $p<0.05$ vs mCherry (pairwise comparisons). Data is shown as mean \pm SEM. $n=10$ per group.

3.2.5. Depressive-like behaviors

Disrupting the interaction between NOS-I and PSD-95 leading to reduced formation of the NOS-I/PSD-95/NMDA receptor complex has been shown to result in antidepressive-like behavior in rodents (Doucet *et al*, 2013). Therefore, endophenotypes of depression such as behavioral despair (by FST), anhedonia (inability to enjoy pleasure, i.e. sucrose preference) and well-being (nesting behavior) were evaluated in this study.

In the FST, immobility (i.e. floating) and latency to reach the first immobile episode is suggested to reflect behavioral despair against an inescapable situation (Porsolt *et al*, 1977a). In repeated FST, rodents tend to show increased immobility and reduced latency which may reflect a conditioned behavioral response (Freudenberg *et al*, 2013a). Levene's test indicated homogeneity of variances for time spent immobile (day1: $F=0.475$, $p=0.850$, day2: $F=1.485$, $p=0.187$) but not for latency to reach to immobility (day1: $F=1.182$, $p=0.324$, day2: $F=3.526$, $p=0.003$) in two way RM ANOVA (stress x injection as two between-subjects factors and repeated measures for 2 trial days, [Figure 23](#)). Therefore, latency data was log-transformed and analyzed again with two way RM ANOVA. One of the NOS-I₁₋₁₃₃ overexpressing CMS mice was removed from analysis as its activity deviated from group average by more than 40 SD (mean=293.4).

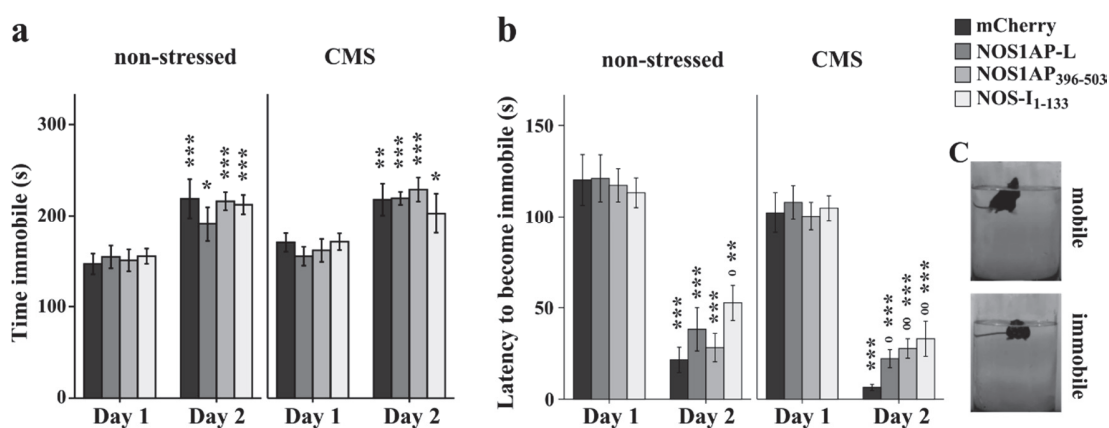


Figure 23. Performance of mice in forced swim test. Mice from all groups increased their immobility on 2nd day, suggesting that they were able to show an adaptive behavioral response. **a)** Immobility time, two way RM ANOVA: time, $F_{1,71}=103.533$, $p<0.001$; time x stress, $F_{1,71}=0.220$, $p=0.64$; time x injection, $F_{3,71}=0.810$, $p=0.492$; time x stress x injection, $F_{3,71}=1.393$, $p=0.252$. **b)** latency to become immobile, two way RM ANOVA: time, $F_{1,70}=244.311$, $p<0.001$; time x stress, $F_{1,70}=0.843$, $p=0.362$; time x injection, $F_{3,70}=4.884$, $p=0.004$; time x stress x injection, $F_{3,70}=1.030$, $p=0.385$. **c)** Representative images showing mobile and immobile behavior in mice. post-hoc LSD test for 'day 1 vs day 2': * $p<0.05$, ** $p<0.005$, *** $p<0.001$ and for injection groups vs mCherry in corresponding day and stress group: ^o $p<0.05$, ^{oo} $p<0.005$. Data is shown as mean \pm SEM. $n=9-10$ per group.

As expected, time spent immobile was significantly increased ($p<0.001$) and latency to immobility was significantly reduced ($p<0.001$) on the 2nd test day compared to 1st day, suggesting that all mice showed an adaptive behavioral response. On both testing days, time spent immobile was comparable in all groups ($p>0.05$). Conversely, on 2nd day the latency was significantly higher for mice overexpressing NOS1AP-L ($p=0.023$), NOS1AP₃₉₆₋₅₀₃ ($p=0.005$) and NOS-I₁₋₁₃₃ ($p=0.003$) compared to mCherry expressing mice in the CMS group and NOS-I₁₋₁₃₃ ($p=0.003$) compared to mCherry expressing mice in the non-stressed group. Higher latency observed in these test groups

on 2nd day indicates an impairment in their memory in terms of late implementation of experience-dependent adaptive changes, even though they showed overall reduced struggling in water.

Mice were tested for anhedonia by monitoring their preference of 1% sucrose solution over tap water for 48 h. Regardless of viral construct or stress treatment, all mice showed comparable sucrose preference indicating unchanged hedonic behavior (Figure 24a; overall $p > 0.1$). Nesting behavior was monitored for two consecutive days. Each day mice received a new pressed cotton block and next morning nests were scored (Figure 24b). On the 1st day, two way RM ANOVA revealed that nest quality was reduced in CMS mice compared to non-stressed mice ($p = 0.006$). Post-hoc tests revealed that nest quality in non-stressed and CMS mice overexpressing mCherry was comparable ($p = 0.586$). Therefore, this effect seems to be dependent on the lower nest scores of mice overexpressing NOS1AP-L (non-stressed 4.4 ± 0.13 , CMS 3.78 ± 0.23 , $p = 0.091$), NOS1AP₃₉₆₋₅₀₃ (non-stressed 4.15 ± 0.27 , CMS 3.61 ± 0.33 , $p = 0.088$) and NOS-I₁₋₁₃₃ (non-stressed 4.6 ± 0.15 , CMS 3.98 ± 0.29 , $p = 0.091$). However, differences between these groups did not reach statistically significant levels. On the second day of testing, all mice built nests of comparable quality (overall $p > 0.2$).

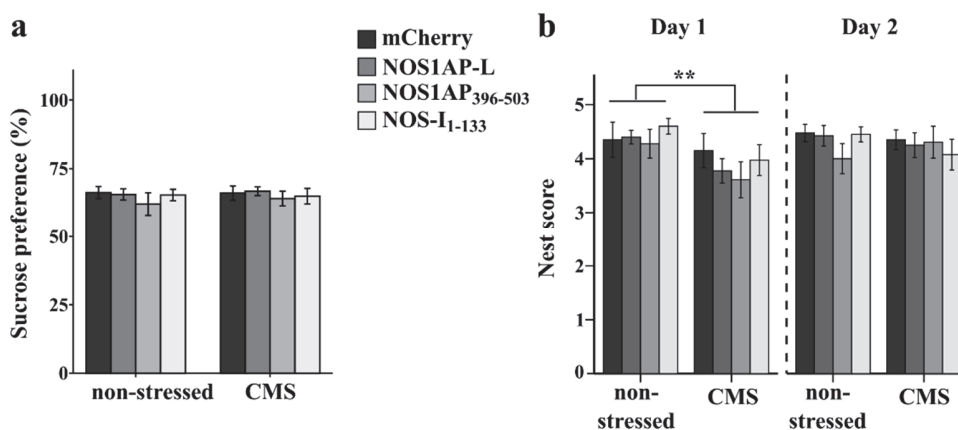


Figure 24. Disruption of NOS-I PDZ interactions in hippocampus did not cause anhedonia or self-neglect. a) Sucrose preference was comparable in all mice. Two way ANOVA: stress, $F_{1, 70} = 0.123$, $p = 0.727$; injection, $F_{3, 70} = 0.610$, $p = 0.611$; interaction stress x injection $F_{3, 70} = 0.095$, $p = 0.963$. **b)** Viral construct expression did not affect nest building, however non-stressed mice built better nests on first day. When repeated, all mice built equally successful nests. Two way RM ANOVA: time, $F_{1, 70} = 2.252$, $p = 0.138$; time x stress, $F_{1, 70} = 4.840$, $p = 0.031$; time x injection $F_{3, 70} = 0.381$, $p = 0.767$, time x stress x injection, $F_{3, 70} = 0.920$, $p = 0.436$. post-hoc LSD, $**p < 0.05$. Data is shown as mean \pm SEM. $n = 9-10$ per group.

3.2.6. Spatial working memory

Working memory is an endophenotypic marker for many psychiatric disorders such as schizophrenia (Barch and Ceaser, 2012). Impaired NOS-I signaling has also been associated with deficits in SWM in previous studies (Sandra P. Zoubovsky, 2011; Tanda *et al*, 2009; Zoubovsky *et al*, 2011). Therefore, I have tested mice for SWM in a rewarded alternation task in T-maze (Figure 9) and for spatial novelty preference in Y-maze (Figure 10) to evaluate how targeting different interactions of NOS-I would alter or regulate working memory in mice. In the rewarded alternation task in T-maze, mice were presented two runs (sample run vs choice run) per trial, and they were expected to learn to make correct choices. They received in total 24 session and each session consisted of 4 trials. In order to keep mice motivated to reward, mice underwent food restriction, but they maintained 80-85% of their free-feeding weight during the experiments (Figure 25). The percentage alternation was analyzed and presented in 6 blocks (4 session per block, Figure 26). mCherry overexpressing mice started to perform around 60% (non-stressed 63.75 ± 4.04 , CMS 59.38 ± 4.19) and increased the successful alternation up to 75% (non-stressed 63.75 ± 4.04 , $p=0.041$; CMS 59.38 ± 4.19 , $p=0.031$) in the final block. Stress treatment did not alter working memory ($p=0.911$). Mice overexpressing the NOS-I₁₋₁₃₃ construct, which was shown to disrupt NOS-I/PSD-95 interaction, performed significantly lower (on 6th block: non-stressed $p=0.001$; CMS $p=0.032$) compared to mCherry mice. Mice overexpressing NOS1AP-L (on 6th block compared to corresponding mCherry: non-stressed $p=0.255$; CMS $p=0.755$) and

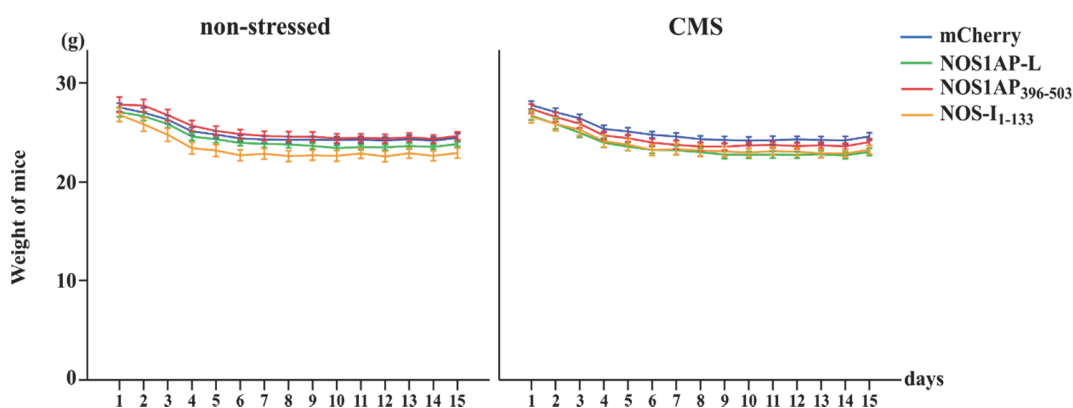


Figure 25. Mice were underwent food restriction during rewarded alternation task. They were fed with chopped food pellets to maintain 80 – 85% of their free feeding weight. Two way RM ANOVA with a Greenhouse-Geisser correction (Mauchly's sphericity test, $p<0.05$): time (i.e. test days), $F_{1,235, 88.927}=42.476$, $p<0.001$; time x stress, $F_{1,235, 88.927}=0.960$, $p=0.492$; time x injection $F_{3,705, 88.927}=1.390$, $p=0.246$; block x stress x injection, $F_{3,705, 88.927}=0.376$, $p=0.811$. Data is shown as mean \pm SEM. $n=10$ per group.

NOS1AP₃₉₆₋₅₀₃ (on 6th block compared to corresponding mCherry: non-stressed $p=0.3$; CMS $p=0.677$) performed rather comparable to mCherry mice. It can be suggested considering data that NOS1AP-L overexpression or its interactions does not regulate SWM in rewarded alternation task.

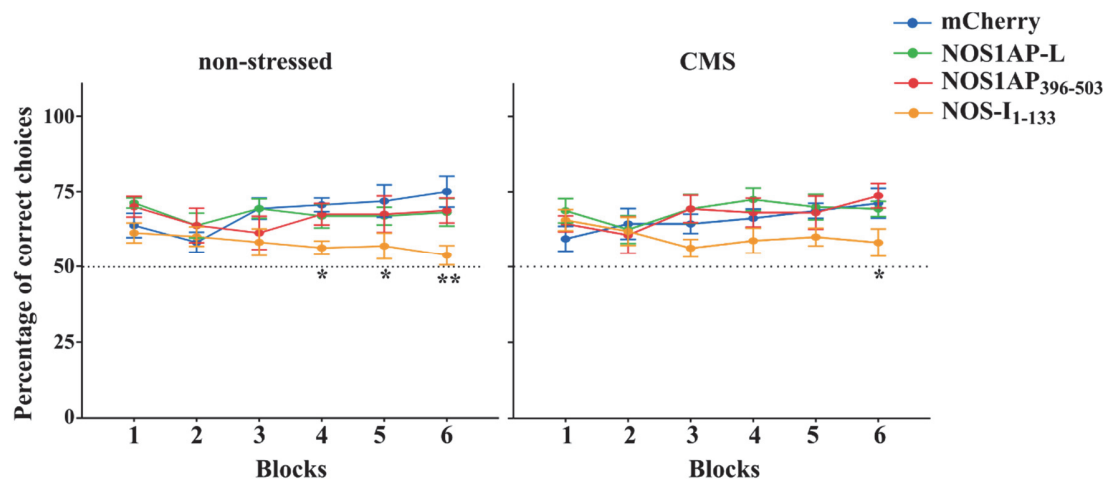


Figure 26. Rewarded alternation task was performed on T-maze. Successful alternation was defined by percentage of correct choices given by mice. Only NOS-I₁₋₁₃₃ mice had significantly less correct arm choices. The dashed lines show chance level performance (50 %). Two way RM ANOVA with a Greenhouse-Geisser correction (Mauchly's sphericity test, $p<0.05$): block, $F_{4,337, 312.277}=2.130$, $p=0.071$; block x stress, $F_{4,337, 312.277}=0.268$, $p=0.911$; block x injection $F_{13,012, 312.277}=1.661$, $p=0.068$; block x stress x injection, $F_{13,012, 312.277}=0.66$, $p=0.802$. post-hoc LSD test: * $p<0.05$, ** $p<0.005$. Data is shown as mean \pm SEM. $n=10$ per group.

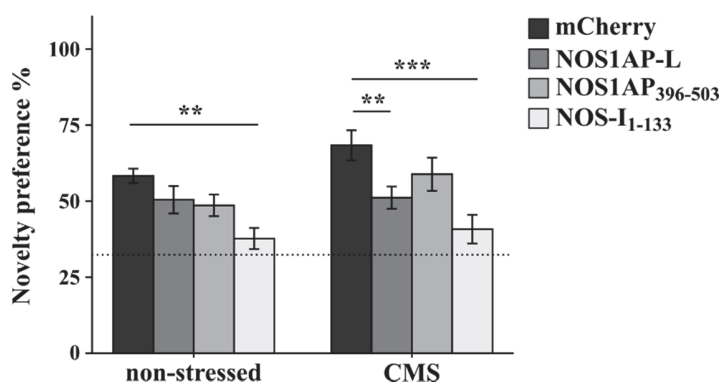


Figure 27. Spatial novelty preference was impaired in mice with envisaged disruption of NOS-I/PSD-95 interactions in hippocampus. Two way ANOVA: stress, $F_{1, 72}=4.133$, $p=0.046$; injection, $F_{3, 72}=11.189$, $p<0.001$; interaction of stress x injection, $F_{3, 72}=0.672$, $p=0.572$. post-hoc LSD test: ** $p\leq 0.005$, *** $p<0.001$. The dashed lines show chance level performance (33 %). Data is shown as mean \pm SEM. $n=10$ per group.

In spatial novelty preference test in Y-maze (Figure 27), spatial learning and memory are assessed by measuring rodent's exploration of a novel arm. Two way ANOVA suggested significantly increased novelty preference in CMS compared to non-stressed mice ($p=0.046$). However, this

effect seems to occur due to increased novelty preference of mice overexpressing mCherry (non-stressed 58.3 ± 2.4 , CMS 68.4 ± 4.95 , $p=0.094$) and mice overexpressing NOS1AP₃₉₆₋₅₀₃ (non-stressed 48.6 ± 3.5 , CMS 58.9 ± 5.5 , $p=0.088$) and not due to overexpression of NOS1AP-L (non-stressed 50.5 ± 4.5 , CMS 51.2 ± 3.7 , $p=0.906$) or NOS-I₁₋₁₃₃ (non-stressed 37.7 ± 3.5 , CMS 40.8 ± 4.7 , $p=0.602$). On the other hand, impaired novelty preference in CMS mice overexpressing NOS1AP-L ($p=0.005$) and mice overexpressing NOS-I₁₋₁₃₃ ($p<0.001$) might be also caused by this higher preference of CMS mice overexpressing mCherry or might occur due to disrupted interaction of NOS-I with PSD-95 as both constructs were shown to lead reduced NOS-I/PSD-95 formation (Jaffrey *et al.*, 1998; Zhou *et al.*, 2010). Involvement of NOS-I PDZ interactions in novelty preference is also supported by the fact that in the non-stressed group all test constructs showed impaired novelty preference. However, a significant impairment was only observed in mice overexpressing NOS-I₁₋₁₃₃ ($p=0.001$).

Altogether, data from the rewarded alternation task in the T-maze and spatial novelty preference test in the Y-maze suggest that disruption of NOS-I PDZ interactions, especially overexpressing NOS-I PDZ domain, in dorsal hippocampus results in impaired SWM in mice.

3.2.7. Spatial reference memory

NOS1 KO mice has been previously reported to show impaired spatial reference memory (Tanda *et al.*, 2009). Therefore, I have assessed SRM in Y-maze across four blocks of four sessions (4 trials each). Mice were confronted food restriction during SRM task, but they maintained 80-85% of their free-feeding weight during the experiments (Figure 28). Control mice overexpressing mCherry started to perform around 40% (non-stressed 42.5 ± 6.8 , CMS 47.5 ± 5.6) and increased the performance up to 87.5% (non-stressed 87.5 ± 3.23 , $p<0.001$; CMS 82.5 ± 4.8 , $p<0.001$) in the final block, suggesting successful learning of the fixed goal arm position in the maze (Figure 29). In fact, mice from all groups showed performance over 70% in the final block ($p<0.001$) suggesting that SRM was mainly intact regardless of construct or stress treatment. However, the performance of non-stressed mice overexpressing NOS-I₁₋₁₃₃ ($p=0.016$) was lower compared to non-stressed mice overexpressing mCherry in the final block. Even though they succeeded 71.9% (± 6.1) performance on this task, this still indicates an impaired SRM upon overexpression of NOS-I₁₋₁₃₃ construct.

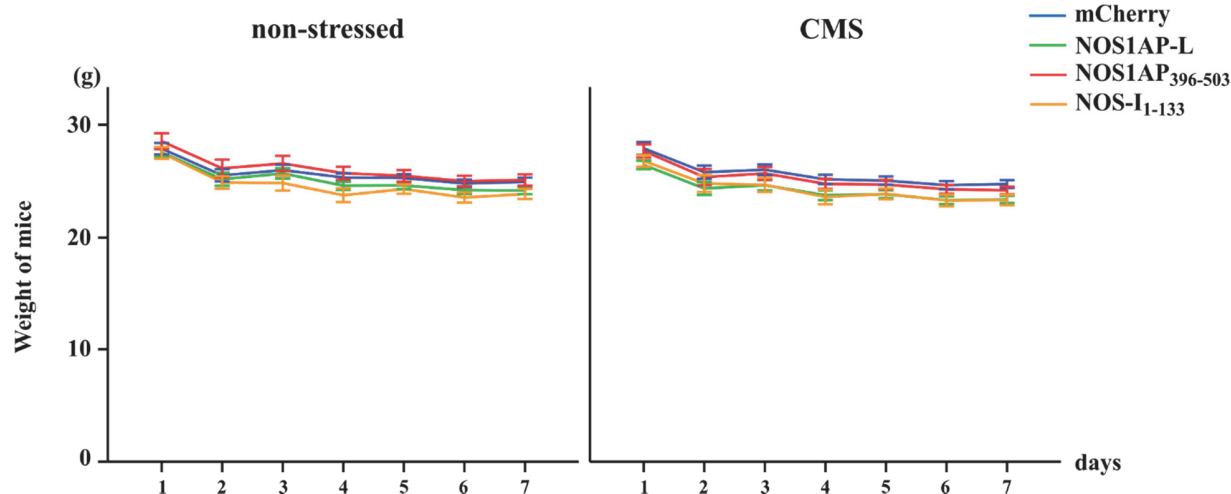


Figure 28. Mice were underwent food restriction during spatial reference memory task. Mice were fed with chopped food pellets to keep 80 – 85% of their free feeding weight. Two way RM ANOVA with a Greenhouse-Geisser correction (Mauchly's sphericity test, $p < 0.05$): time (i.e. test days), $F_{2,025, 143.780} = 42.476$, $p < 0.001$; time x stress, $F_{2,025, 143.780} = 0.699$, $p = 0.5$; time x injection $F_{6,075, 143.780} = 1.482$, $p = 0.187$; block x stress x injection, $F_{6,075, 143.780} = 0.44$, $p = 0.853$. Data is shown as mean \pm SEM. $n = 10$ per group.

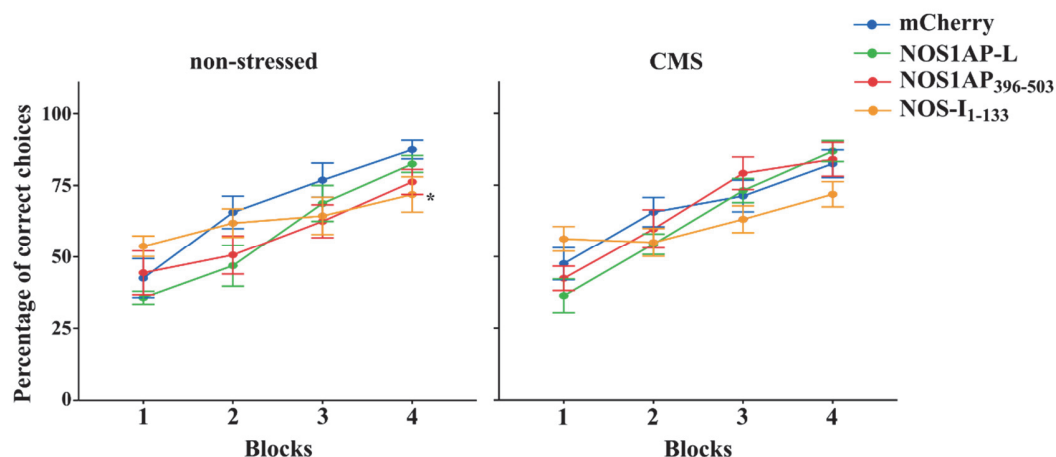


Figure 29. Spatial reference memory was remained mainly intact upon disruption of NOS-I PDZ interactions in hippocampus. Two way RM ANOVA: Two way RM ANOVA with a Greenhouse-Geisser correction (Mauchly's sphericity test, $p < 0.05$): block, $F_{2,640, 187.446} = 130.153$, $p < 0.001$; block x stress, $F_{2,640, 187.446} = 0.11$, $p = 0.939$; block x injection $F_{7,920, 187.446} = 5.679$, $p < 0.001$; block x stress x injection, $F_{7,920, 187.446} = 1.174$, $p = 0.317$. post-hoc LSD test, $*p < 0.05$. Data is shown as mean \pm SEM. $n = 9-10$ per group

3.2.8. Sensorimotor gating deficits

Impaired sensorimotor gating is a common endophenotype for many psychiatric disorder such as schizophrenia. PPI of ASR (Figure 8) is a validated measure of sensorimotor gating in rodents and can also be translated to humans (Fendt and Koch, 2013; Swerdlow and Geyer, 1998). PPI of a 120 dB stimulus was measured using prepulses ranging from 69 to 81 dB (Figure 30).

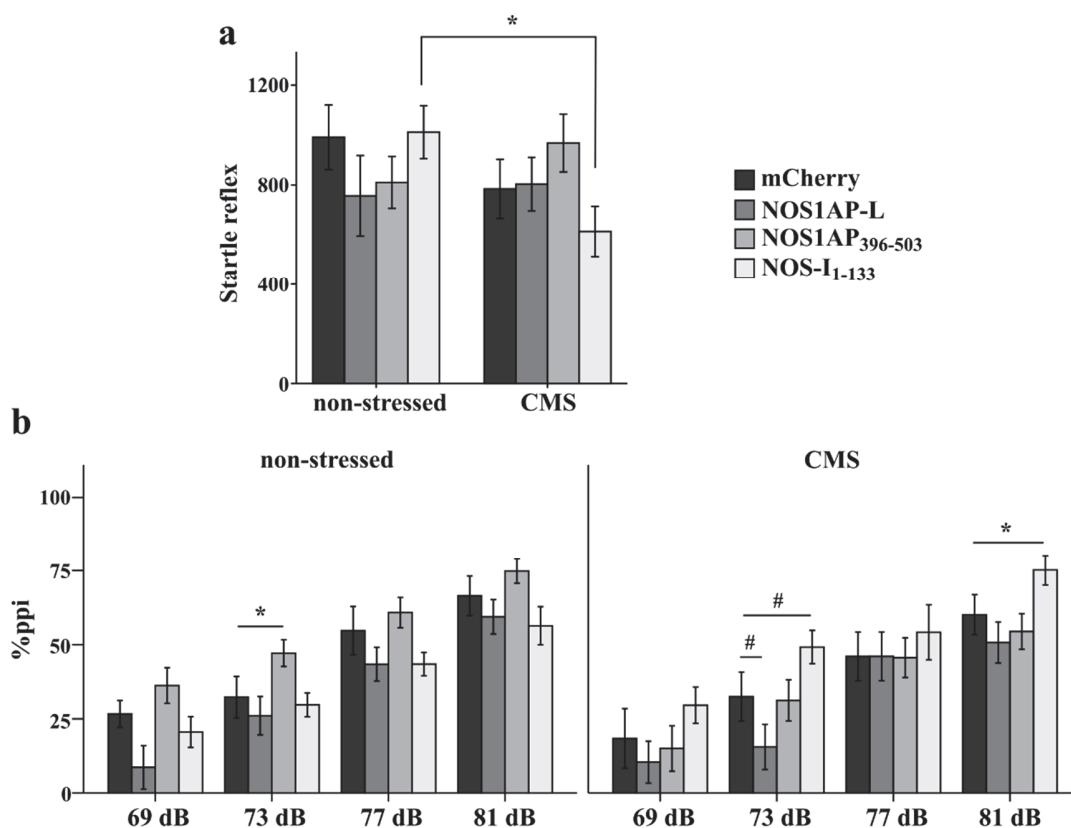


Figure 30. NOS1AP₃₉₆₋₅₀₃ overexpression results in enhanced prepulse inhibition. **a)** Basal startle reflex was comparable in all groups except NOS-I₁₋₁₃₃. Two-way ANOVA: stress, $F_{1,72}=1.408$, $p=0.239$; injection $F_{3,72}=0.424$, $p=0.736$; interaction of stress x injection, $F_{3,72}=2.207$, $p=0.095$. **b)** Representation of prepulse inhibition as percentages. 69 dB, two way ANOVA: stress, $F_{1,72}=0.123$, $p=0.727$; injection $F_{3,72}=1.859$, $p=0.144$; interaction of stress x injection, $F_{3,72}=1.408$, $p=0.248$. 73 dB, two way ANOVA: stress, $F_{1,72}=0.004$, $p=0.947$; injection $F_{3,72}=3.588$, $p=0.018$; interaction of stress x injection, $F_{3,72}=2.926$, $p=0.039$. 77 dB, two way ANOVA: stress, $F_{1,72}=0.104$, $p=0.748$; injection $F_{3,72}=0.472$, $p=0.702$; interaction of stress x injection, $F_{3,72}=1.189$, $p=0.32$. 81 dB, two way ANOVA: stress, $F_{1,72}=0.712$, $p=0.402$; injection $F_{3,72}=1.286$, $p=0.286$; interaction of stress x injection, $F_{3,72}=3.755$, $p=0.015$. post-hoc LSD test: * $p<0.05$, # $p\leq 0.08$. Data is shown as mean \pm SEM. $n=10$ per group.

ASR levels were comparable in all groups, except NOS-I₁₋₁₃₃ overexpressing mice in CMS group showed significantly lower baseline startle reflex compared to non-stressed NOS-I₁₋₁₃₃ ($p=0.021$), which is a phenotype observed in bipolar patients (Giakoumaki *et al*, 2010). The PPI was calculated as the percent reduction in ASR following prepulses at 4, 8, 12 and 16 dB above background (Geyer and Swerdlow, 2001). Results were analyzed using two way RM ANOVA Greenhouse-Geisser correction (Mauchly's sphericity test, $p<0.05$) which revealed a significant effect for factor prepulse ($F_{2,547,183.370}=157.223$, $p<0.001$), suggesting that inhibition of the ASR was increased as the prepulse intensity increased. Therefore, the rest of the analysis was performed for each prepulse individually. All mice showed comparable PPI levels at both 4 dB and 12 dB

above background. When prepulse was presented at 8 dB above background, mice overexpressing NOS1AP₃₉₆₋₅₀₃ ($p=0.028$) construct showed enhanced prepulse inhibition compared to mCherry mice in non-stressed group. NOS1AP-L overexpressing mice produced overall reduced PPI compared to mCherry mice, however this was not proven statistically ($p>0.05$). Moreover, CMS mice overexpressing NOS-I₁₋₁₃₃ showed a higher rate of PPI compared to non-stressed mice overexpressing NOS-I₁₋₁₃₃ (8 dB: $p=0.080$ and 16 dB: $p=0.005$). However, this might be dependent on low basal ASR in these mice.

3.2.9. Social interaction and social novelty recognition

Deficits in social life is a common feature observed in patients with neuropsychiatric disorders. To evaluate this in mice, we introduced the experimental mice to 4-weeks old juvenile congenics of the same sex (Figure 7), scored the time spent sniffing the juveniles during the first 3 min as sociability in rodents declines with familiarization and analyzed the results with two way ANOVA (Figure 31a). In the non-stressed group, overexpression of all test constructs resulted in lower interaction time with the juvenile during the familiarization period (NOS1AP-L: 14.64 ± 3.17 , $p=0.029$; NOS1AP₃₉₆₋₅₀₃: 14.71 ± 3.74 , $p=0.03$; NOS-I₁₋₁₃₃: 11.6 ± 2.25 , $p=0.006$) compared to mCherry overexpressing mice (25.95 ± 5.2). This difference was not present in the CMS group as even mCherry overexpressing mice showed a reduction in interaction time (CMS mCherry:

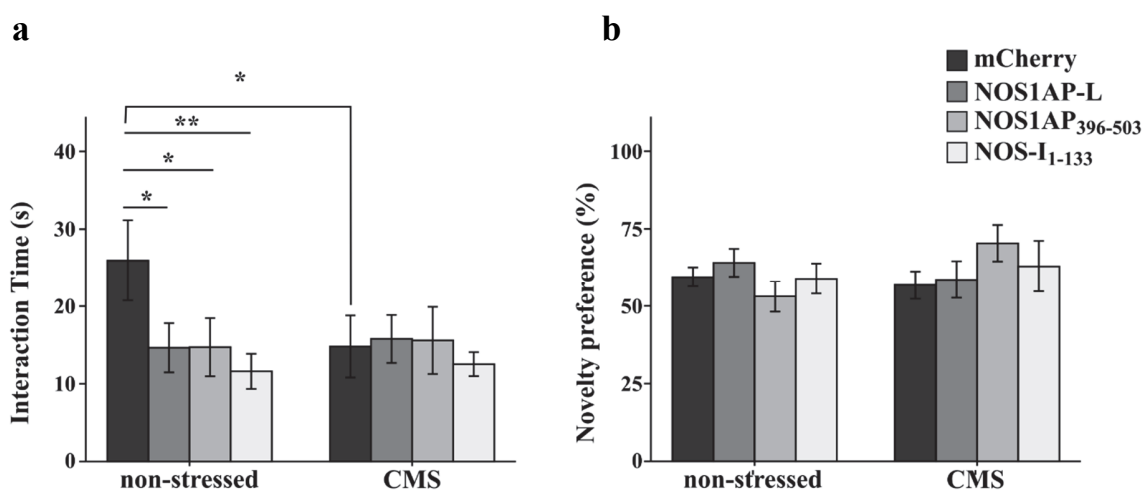


Figure 31. Disruption of NOS-I PDZ interactions resulted in social deficits. a) Interaction time with a 4-weeks old juvenile was reduced in mice which overexpresses viral constructs targeting NOS-I PDZ domain in hippocampus. Two-way ANOVA: stress, $F_{1, 72}=0.657$, $p=0.42$; injection $F_{3, 72}=1.847$, $p=0.146$; interaction of stress x injection, $F_{3, 72}=1.430$, $p=0.241$. b) Social novelty preference was not markedly altered by interactions of NOS-I PDZ domain. Two-way ANOVA: stress, $F_{1, 72}=0.732$, $p=0.395$; injection $F_{3, 72}=0.180$, $p=0.91$; interaction of stress x injection, $F_{3, 72}=1.751$, $p=0.164$. * $p<0.05$, ** $p<0.005$. Data is shown as mean \pm SEM. $n=10$ per group.

14.8±4, $p=0.031$ vs non-stressed mCherry. NOS1AP-L: 15.77±4, $p=0.849$; NOS1AP₃₉₆₋₅₀₃: 15.58±4.3, $p=0.878$; NOS-I₁₋₁₃₃: 12.53±1.6, $p=0.855$ vs mCherry, CMS). For social novelty preference (Figure 31b), mice were presented with the familiar and an additional novel juvenile, 30 min after familiarization. The first 3 min were scored and the percentages of novelty preference were analyzed by two way ANOVA. Results revealed that none of the constructs altered social novelty preference.

3.2.10. Delay fear conditioning

NOS1 KO mice has been previously suggested to show impaired contextual fear conditioning memory in a Pavlovian conditioning paradigm (Kelley *et al*, 2009, 2010). To monitor whether mice in this study would reveal complementary results, conditioned freezing and activity of mice were evaluated either in the training context for contextual memory or to the tone cue in a novel context for auditory conditioning. Acquisition trial consisted of 120 s habituation period followed by 120 s of CS-US pairings, hence I have separated the data from acquisition trial into two blocks (each 120 s) to compare initial freezing to freezing behavior after onset of first CS. Results revealed significantly increased post-shock freezing duration ($F_{1,70}=121.097$, $p<0.001$) and decreased activity ($F_{1,70}=38.809$, $p<0.001$) regardless of injected construct or stress paradigm which indicates a successful US–CS conditioning (Figure 32a). Next day, mice were tested in the same environmental context, at around the same time of the day but without tone in order to assess contextual conditioning (Figure 32b). Results were split in two time blocks (each 120 s) which was comparable to pre-tone vs tone phases of acquisition trial. In this test, all groups showed overall comparable freezing between time blocks to environmental (spatial) context. Even mice with mCherry overexpression showed weak reconsolidation of context memory (non-stressed mCherry, pre-shock: 39.28±3.6, post-shock: 40.78±5.4; CMS mCherry, pre-shock: 45.07±3.2, post-shock: 42.53±7) which might compromise the behavioral effects of other constructs. It is still noteworthy to report that only CMS mice overexpressing NOS-I₁₋₁₃₃ showed reduced freezing in the first 120 s of test ($p=0.013$), which might indicate an increased locomotion upon induced emotional stress (fear against foot shock). In the cue retention test (Figure 32c), mice were allowed to habituate to new context for 120 s and then presented 90 s of the same cue tone from acquisition trial in a novel environment to assess auditory conditioning. Overall, mice showed significantly higher freezing upon presentation of tone cue regardless of injection, stress or their interaction (overall $p<0.001$) suggesting intact auditory conditioning. Interestingly, NOS-I₁₋₁₃₃ overexpression

resulted in increased freezing in non-stressed group ($p=0.016$) during the first 120 s, which might suggest reduced locomotion because of the new environmental context. However, this contradicts the data from locomotion and anxiety-related tests and might require further investigation.

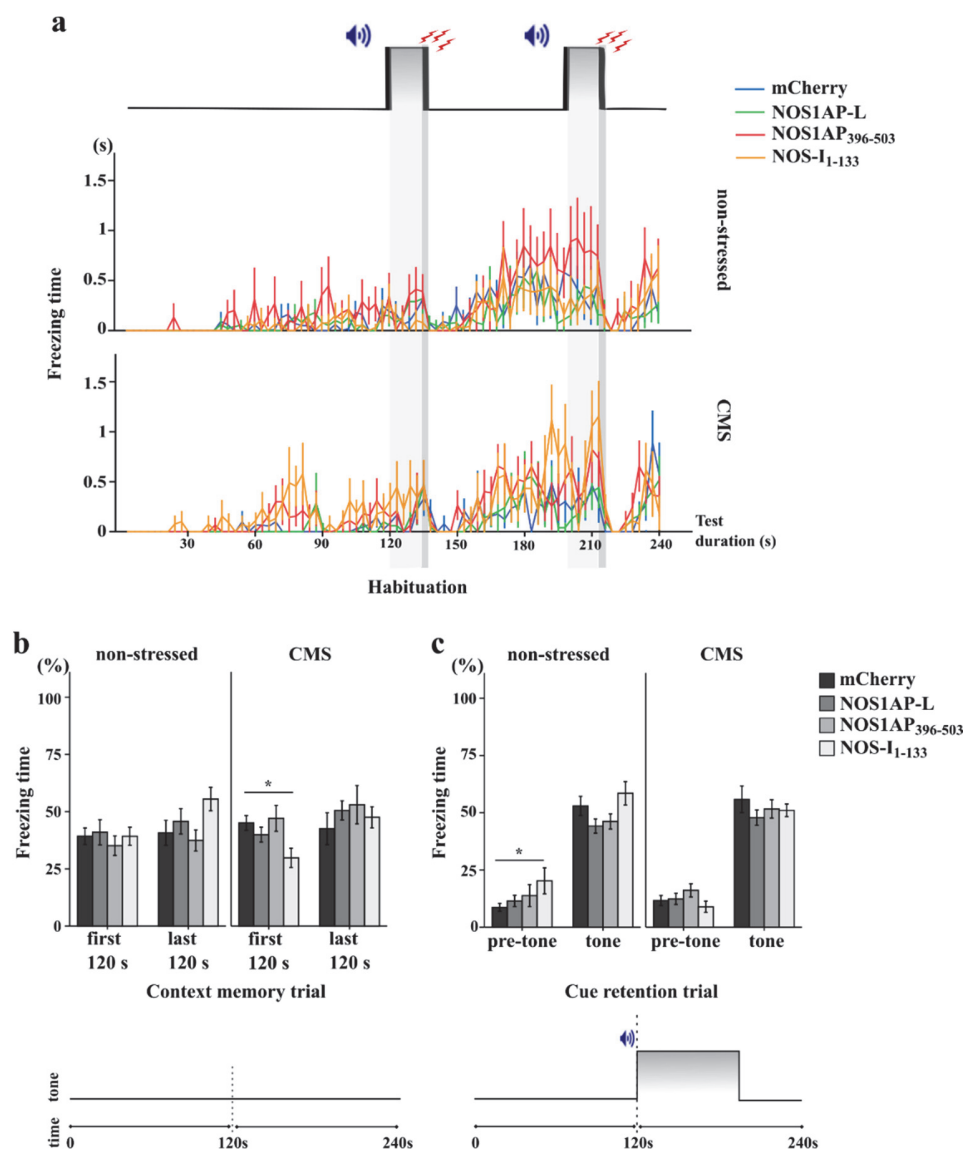


Figure 32. Delayed fear conditioning resulted in increased freezing duration and reduced activity. **a)** All mice showed successful US–CS conditioning. **b)** Overexpression of viral constructs has no effect on freezing behavior in context retention test. Two-way RM ANOVA: time, $F_{1,71}=17.115$, $p<0.001$; time \times stress, $F_{1,71}=0.260$, $p=0.612$; time \times injection, $F_{3,71}=4.758$, $p=0.004$; time \times stress \times injection, $F_{3,71}=0.388$, $p=0.762$. **c)** Intact auditory conditioning was observed in cue-retention test. Two-way RM ANOVA: time, $F_{1,71}=416.415$, $p<0.001$; time \times stress, $F_{1,71}=0.429$, $p=0.515$; time \times injection, $F_{3,71}=1.800$, $p=0.155$; time \times stress \times injection, $F_{3,71}=0.055$, $p=0.983$. post-hoc LSD test: $*p<0.005$.vs. corresponding mCherry mice. Data is shown as (mean \pm SEM). $n=9-10$ per group

4. Discussion

In many human and animal studies, NOS-I has been associated with a wide range of neuropsychiatric disorders such as ADHD (Gao and Heldt, 2015; Reif *et al*, 2009; Weber *et al*, 2015), depression (Doucet *et al*, 2012; Luciano *et al*, 2012) and schizophrenia (O'Donovan *et al*, 2008; Reif *et al*, 2006). Despite the fact that NOS-I involvement in endophenotypes of these disorders (e.g. working memory, social interaction...) has been well established, the exact mechanisms of NOS-I involvement remain to be resolved. NOS-I exerts its effects through either NMDA receptor-NO-sGC pathway (Burette *et al*, 2002) or S-nitrosylation of proteins bearing thiol groups (Shahani and Sawa, 2012). NOS1AP interacts with the core PDZ domain of NOS-I and disrupts the integrity of NOS-I/PSD-95/NMDA receptor complex (Jaffrey *et al*, 1998) mediating NMDAR/NOS-I dependent excitotoxic signaling (Li *et al*, 2013). As stated in previous sections, elevated levels of NOS1AP have been shown in post-mortem brain samples of bipolar and schizophrenia patients (Hadzimidichalis *et al*, 2010; Xu *et al*, 2005). Altogether it is plausible to assume that targeting protein interactions of NOS-I and NOS1AP will eventually help for a better understanding of psychopathology.

Here, the aim of the project was to assess how disrupted NOS-I interactions affect dendritic morphology and result in behavioral phenotypes observed in patients with neuropsychiatric disorders, especially those related to schizophrenia and its comorbid disorders. To this end, rAAV vectors expressing different isoforms / deletion mutants of NOS1AP and the N-terminal 133 amino acids of NOS-I were created and used to evaluate alterations at morphological and behavioral levels.

4.1. Interaction of virally expressed proteins with endogenous proteins

It has been initially proposed that the C-terminal 20 amino acids of NOS1AP are necessary and sufficient for interaction with the PDZ domain of NOS-I (Jaffrey *et al*, 1998). This has been supported by another recent study (Zhu *et al*, 2014). In contrast, Li *et al*. (2015) showed that NOS1AP residues 400-431 (396-427 in murine NOS1AP-L), containing an internal ExF motif at residues 429-431 (425-427 in murine NOS1AP-L), are mainly mediating the interaction between NOS-I and NOS1AP, while the C-terminus of NOS1AP, encoding the IAV PDZ-motif, stabilizes this interaction (Figure 4). The authors of this study showed that the NOS1AP carboxyterminal domain excluding the ExF motif does not bind to the NOS-I PDZ-domain in a GST-pulldown

assay. Likewise, in my study I was not able to show interaction of NOS1AP-L_{C20} with endogenous NOS-I whereas (Figure 13). Jaffrey *et al.* (1998) have shown in their study that the C-terminus of NOS1AP only binds to the first 100 amino acids of NOS-I (encoding the PDZ domain). However, in this study I was not able to show an interaction between NOS-I₁₋₁₃₃ and endogenous NOS1AP in co-immunoprecipitation assay (Figure 13). While it appears unlikely that there is no interaction between NOS-I₁₋₁₃₃ and NOS1AP, this interaction may be too moderate to be detected. Alternatively, it might be possible that NOS-I₁₋₁₃₃/NOS1AP interaction is not stable enough under certain experimental conditions (e.g. denaturation). However, in the study by Li *et al.* (2015) the aminoterminal 155 amino acids of NOS-I were sufficient for stable interaction with NOS1AP. Therefore, unless the interaction between NOS-I and the ExF motif of NOS1AP requires amino acids 134 and 155 of NOS-I, this explanation appears unlikely. In either case the interpretation of NOS-I₁₋₁₃₃/NOS1AP interaction have to be treated with caution.

Considering that both NOS1AP and NOS-I₁₋₁₃₃ were expected to disrupt interaction of NOS-I with PSD-95, potentially preventing NMDA receptor dependent activation of NOS-I, it was surprising to find that none of the constructs used in this study had an effect on NOS-I catalytic activity (Figure 14). Disruption of the NOS-I/PSD-95 interaction using the small molecule inhibitor ZL006 has been shown to result in strongly reduced NO production, at least when stimulating neurons with glutamate (Zhou *et al.*, 2010). In contrast, another study showed that NO production under basal conditions was unaffected by treatment with IC87201, another small molecule inhibitor of NOS-I/PSD-95 interaction (Florio *et al.*, 2009). Therefore, it is very well possible that NMDA receptor activation under basal conditions is not sufficient to detect a potential disruption of NOS-I catalytic activity due to overexpression of NOS-I₁₋₁₃₃. Alternatively, it is possible that a reduction of the DAF-FM signal was masked by unspecific signal from DiI, which was used for visualization of the neurons in culture. With the used filter kit DiI is only excited at ~25% intensity (~99% for DAF-FM) and only ~28% of DiI emission is picked up (~73% for DAF-FM). Therefore, this explanation seems unlikely. Finally, though it has been shown that the fluorescence of DAF-FM is proportional to the NO concentration (Kojima *et al.*, 1998; Nakatsubo *et al.*, 1998; Namin *et al.*, 2013; Zhou *et al.*, 2010), it is very well possible that the relatively sparse number of neurons that produce high amounts of NO is not sufficient to detect more moderate changes in NOS-I catalytic activity. Taken together, though data in this study indicate that none of our constructs caused significant changes in NO production, there are several potential technical limitations that may

have caused us to miss a potential impact of our constructs (in particular NOS-I₁₋₁₃₃) on NOS-I catalytic activity.

4.2. Disruption of NOS-I PDZ interactions alters neurite growth

Within this study, I was able to show that overexpression of NOS1AP isoforms / deletion mutants in primary cultured hippocampal and cortical neurons resulted a mild reduction in dendritic branching ([Figure 16](#)) and a strong reduction in amount of mature dendritic spines ([Figure 17](#), [Table 3](#)).

Increased NO levels were shown to stimulate dendritic branching via activity of guanylyl cyclase in primary hippocampal neurons (Audesirk *et al*, 2003). Dendritic branching was also shown to be mediated by NMDA receptor activity and PSD-95 interaction (Kulkarni and Firestein, 2012; Sweet *et al*, 2011). Disruption of NOS-I/PSD-95 interaction, as a consequence of NOS1AP overexpression, may therefore be responsible for the observed effects on dendritic growth and branching. However, we found that only constructs carrying the aminoterminal domain of NOS1AP-L (i.e. NOS1AP-L and NOS1AP-L Δ C20; containing the PTB- and CPE-binding domains) had an effect on dendritic growth and branching. Furthermore, disruption of NOS-I/PSD-95 interaction by NOS-I₁₋₁₃₃ overexpression had no negative effect on dendritic branching. These findings suggest that NOS1AP affects dendritic patterning through interactions with proteins other than NOS-I. This is in agreement with findings showing that the NOS1AP PTB-domain influences a wide range of neurodevelopmental processes including NMDA receptor activity induced neurotoxicity, iron uptake and dendritic development (Carrel *et al*, 2009a; Courtney *et al*, 2014; Li *et al*, 2015). Moreover, NOS1AP interaction with CPE has been suggested to regulate dendrite morphology (Carrel *et al*, 2009). In a recent study performed in neuronal progenitor cells of the embryonic rat neocortex, a study from the same group showed that the PTB-domain of NOS1AP has an important role in mediating migration of cortical neurons through signaling pathways which may not involve NOS-I (Carrel *et al*, 2015) and are different from the mechanisms regulating dendritogenesis. On the other hand, Richier *et al*. (2010) showed that the PTB domain of NOS1AP was sufficient to induce growth of dendritic protrusions in cultured hippocampal neurons by influencing the activation of Rho family GTPases through a complex involving Scrib.

Although our and previously published (Carrel *et al*, 2009a) findings suggest that the effect of NOS1AP-L on dendritic patterning requires aminoterminal interaction (i.e. through the PTB and/or

CPE binding domains) this does not exclude the possibility that NOS-I and interaction between NOS-I and NOS1AP may contribute to NOS1AP's effects on dendritic patterning. NOS-I interacts with a wide range of proteins via direct binding or S-nitrosylation and some of these interactions (e.g. with DISC1, mNudE-L) have been shown to be involved in the control of neurite outgrowth (Kamiya *et al*, 2006; Zoubovsky *et al*, 2011). Importantly, NOS-I indirectly interacts with DISC1 and mNudE-L through a complex involving synapsin and NOS1AP. Therefore, it is possible that NOS1AP affects dendritic patterning by linking NOS-I to these proteins thereby regulating growth and patterning of dendrites. The importance of NOS-I for dendritic growth is also supported by findings showing that NOS1AP-L dependent reduction in dendritic growth is recovered by NOS-I blockade using L-NAME (Carrel *et al*, 2009a). However, it remains unclear whether these effects are due to NOS-I enzymatic activity directly affecting NOS1AP function or by preventing S-nitrosylation of downstream targets (e.g. mNudE-L) involved in the control of neurite outgrowth.

As an alternative model to NOS1AP disrupting the NOS-I/PSD-95/NMDA receptor complex, Li *et al*. (2013) suggested that NMDA receptor activation actually induces the interaction of NOS-I with NOS1AP which mediates interaction with downstream signaling proteins (e.g. p38MAPK and DexRas). In this case, these downstream signaling proteins might be regulating dendritic development through NMDA receptor induced NOS1AP/NOS-I interaction.

In this study, the effect of NOS1AP on length and branching of dendrites was very mild ([Figure 16](#)) compared to previously published findings. Carrel *et al*. (2009) found a much stronger effect on dendrite number upon overexpression of NOS1AP-L in primary cultured rat hippocampal neurons. However, they transfected the cultures between DIV2-10 and dendritic patterning was investigated two days after transfection. Dendritic maturation and spinogenesis occur towards the end of the second week in culture and continue throughout the third week (Kaech and Banker, 2006). Therefore, analysis of cultured neurons on DIV12 or earlier might give insufficient results for the detection of mature dendritic patterning.

NOS-I¹⁻¹³³ overexpression caused excessive branching in primary cultured hippocampal neurons two weeks after infection (on DIV21) to an extent rendering an appropriate analysis of separate neurons impossible. Under normal cell culture conditions, cultured neurons start to die around 3-4 weeks. Therefore, it was quite unexpected to see this increased branching. Expression of

NOS-I₁₋₁₃₃ has been previously shown to prevent NMDA receptor-dependent excitotoxicity by disruption of the NOS-I/PSD-95 complex (Zhou *et al*, 2010). NOS-I₁₋₁₃₃ mediated neuroprotection might therefore explain the excessive neuronal growth observed after three weeks *in vitro*.

Dendritic spines receive excitatory information to form synapses, hence their amount and morphology indicates the neuron's connectivity (Glausier and Lewis, 2013; Hering and Sheng, 2001). Altered spine development and increased filopodia-like long protrusions are important neurophysiological features observed in psychiatric disorders including schizophrenia (De Bartolomeis *et al*, 2014; Penzes *et al*, 2011). In this study, NOS1AP-L overexpression had a strong effect on dendritic spine development, reducing the number of mature spines and increasing the number of filopodia in both hippocampal and cortical neurons (Table 3). Overexpression of NOS1AP-L_{C20} was sufficient to disrupt spine development and increase growth of filopodia-like structures.

It should be noted that interactions of NOS1AP (e.g. through the NOS1AP PTB and CPE domains) with proteins other than NOS-I are involved in its effect on dendritic development (Carrel *et al*, 2009a; Richier *et al*, 2010). This appears to be supported by our finding that NOS1AP-L_{ΔC20} still affected dendritic spine development. However, in agreement with the recent findings suggesting that the carboxyterminal 20 amino acids stabilize, but not mediate NOS-I/NOS1AP interaction (Li *et al*, 2015), we found that NOS1AP-L_{ΔC20} still interacted with endogenous NOS-I (though at strongly reduced levels). A study by Richier *et al*. (2010) reported that overexpression of the NOS1AP PTB-domain alone was sufficient to increase the number of dendritic processes (processes extending at least 0.1 μm from the dendrite, i.e. including spines and filopodia) and NOS1AP lacking the PTB-domain had no effect on the number of processes. However, the authors of this study did not distinguish between spines and filopodia, making a direct comparison of these findings impossible. In our study, we still observed significant reductions in mature spine amount by overexpression of NOS1AP-S lacking the N-terminal domains (i.e. PTB- and CPE binding domains). Therefore, the specific effect of the PTB-domain on spine development and maturation remains unclear and will require further investigation.

4.3. Behavioral consequences of disrupted NOS-I PDZ interactions

Despite the evidence linking NOS-I/PSD-95/NMDA receptor complex integrity with impaired psychiatric phenotypes (i.e. depression, anxiety...), the exact molecular mechanism mediating

these phenotypes still remain to be understood. Therefore, I aimed to investigate the contribution of specific NOS-I PDZ interactions in these phenotypes related to different neuropsychiatric conditions. To this end, rAAV vectors expressing NOS1AP-L, NOS1AP₃₉₆₋₅₀₃, NOS-I₁₋₁₃₃ and mCherry were stereotaxically delivered to the dorsal hippocampus of adult male C57Bl/6J mice. One week after recovery, half of the mice were exposed to 3-weeks of CMS and afterwards all mice were subjected to a comprehensive behavioral analysis.

4.3.1. NOS-I interaction partners have different effects on locomotion

Previous findings for contribution of NOS-I in locomotor activity are inconsistent. Studies showed that *NOS1* knockdown mice, lacking expression of the PDZ domain containing NOS-I isoform, had increased locomotor activity in open field arena (Gao and Heldt, 2015; Weitzdoerfer *et al*, 2004), whereas one study showed unaltered activity in an NOS-I knockdown model (Wultsch *et al*, 2007). In this study increased activity in OF upon overexpression of NOS-I₁₋₁₃₃ (Figure 19), encoding the extended PDZ domain of NOS-I, suggests a role for PDZ interactions in mediation of locomotion. This construct was suggested to disrupt the integrity of NOS-I/PSD-95 interaction (Zhou *et al*, 2010) disrupting the association of NOS-I with NMDA receptors, which might underlie its effects on locomotion. This sounds plausible as previous studies showed that NMDA receptor hypoactivity results in increased stereotypic behaviors so called psychomotor agitation (Gainetdinov *et al*, 2001; Mohn *et al*, 1999). Moreover, increased locomotion was reversed by antipsychotic treatment in rodents (Freed *et al*, 1984). Studies performed with NOS-I inhibitors also revealed attenuated locomotor activity in rodents (Volke *et al*, 2003), suggest that an interplay between NOS-I and glutamatergic post-synaptic components might be contributing to regulation of locomotor activity.

Unaltered locomotion by overexpression of the NOS1AP-L construct is in concordance with the findings of a previous study, in which viral expression of NOS1AP in hippocampus had no effect on locomotor activity (Zhu *et al*, 2014). The same study also showed that overexpression of the C-terminal 125 amino acids of NOS1AP in hippocampus of mice resulted in unaltered locomotor activity, while impairing formation of the NOS-I/NOS1AP complex. In the present study, overexpressing the C-terminal 108 amino acids of NOS1AP (i.e. NOS1AP₃₉₆₋₅₀₃) resulted in reduced locomotor activity in non-stressed group. There might be two explanations for this contradiction. First, the function of the structure between the CPE and PDZ binding domains of

NOS1AP still unknown. Therefore, the difference of 18 amino acids between these two constructs may exert different effects. Second, locomotor activity was measured differently in both studies. While, Zhu *et al* (2014) counted the number of squares crossed on a grid arena, I used an automated video tracking system to analyze the total distance travelled in the OF arena. Therefore, it is very likely that overexpression of NOS1AP₃₉₆₋₅₀₃, which expresses NOS1AP's NOS-I PDZ binding region but not PTB and CPE regions, has an attenuating effect on locomotor activity resembling those induced by NOS-I inhibitors and antipsychotic drugs (Volke *et al*, 2003; Wiley, 2008).

4.3.2. Anxiety-related behaviors upon disrupted NOS-I PDZ interactions

In a previous study, increased NOS1AP/NOS-I coupling, due to lentiviral overexpression of full length NOS1AP in mouse hippocampus, had anxiogenic effects, whereas disruption of this interaction by lentiviral overexpression of the NOS-I binding region of NOS1AP (C-terminal 125 residues) induced anxiolytic-like behavior (Zhu *et al*, 2014). In contrast to this, I found that both NOS1AP-L and NOS1AP₃₉₆₋₅₀₃ overexpressing mice in the non-stressed group showed higher anxiety-like behavior in the OF and LDB, which however was not statistically significant (Figure 20). There were no behavioral alterations in the EZM or novelty-induced suppressed feeding test (Figure 21).

In a previous report, CMS was shown to enhance anxiety, as well as NOS1AP/NOS-I coupling in the mouse hippocampus and disruption of this interaction prevented stress-induced anxiogenic behaviors (Zhu *et al*, 2014). In contrast in the present study, CMS mice showed reduced anxiety-like behaviors compared to non-stressed mice in both OF and LDB, which was largely independent of the injected viral construct. Moreover, non-stressed mice overexpressing NOS1AP₃₉₆₋₅₀₃ showing increased anxiogenic behavior compared to their CMS counterparts suggests that disruption of NOS-I/NOS1AP interaction might contribute to anxiogenic behaviors. However, this does not eliminate a potential involvement of N-terminal domains of NOS1AP in regulation of anxiety-like behavior. In their study, Zhu *et al*. (2014) have also claimed that NOS1AP/NOS-I coupling exerted its anxiolytic effects via Dexras1-ERK signaling, implicated in depression and synaptogenesis, and this was reversed by overexpression of Dexras1 in hippocampus. Therefore, further investigation on protein interactions of NOS1AP might be useful to understand its involvement in anxiety-related symptoms.

4.3.3. Disruption of NOS-I PDZ interaction does not induce depressive-like behavior

Small-molecule inhibitors of the PSD-95/NOS-I interaction have been shown to induce antidepressant like effects in forced swim test without altering locomotor activity or retention of fear memory in mice (Doucet *et al*, 2013). In this study, mice with disrupted NOS-I PDZ interactions did not show any major changes in immobility in the FST (Figure 23), nor did they display changes in hedonic or nest building behaviors (Figure 24). This contradiction to previous findings is most likely due to differences in the experimental design. The small molecules inhibiting NOS-I/PSD-95 interaction used by Doucet *et al* (2013) were administered intraperitoneally, tested for acute response, and were never tested specifically in hippocampus (Doucet *et al*, 2013). Moreover, a recent study implicated that these molecules may not target the extended NOS-I PDZ domain, nor directly target NOS-/PSD-95 interaction (Bach *et al*, 2015). Yet, strong evidence for an involvement of NOS-I in depressive-like behavior remains (Harkin *et al*, 2004; Luciano *et al*, 2012; Wegener and Volke, 2010). In previous studies, both *NOS1* KO mice or mice receiving NOS-I inhibitors mimicked the effects of antidepressants (Volke *et al*, 2003; Zhou *et al*, 2007).

During the repetition of FST on 2nd day, mice overexpressing NOS-I₁₋₁₃₃ showed increased latency to become immobile compared to mCherry mice regardless of the stress treatment, suggesting that these mice might have a disrupted memory for learned helplessness. These mice also showed impaired memory in other tests, which is discussed in section 4.3.6. However, decreased latency might be confounded by the increased locomotor activity in these mice (Figure 19). However locomotor activity was altered only in non-stressed mice, therefore this argument is not sufficient to explain increased latency in FST which was apparent regardless of stress treatment. In addition, mice overexpressing NOS1AP-L and mice overexpressing NOS1AP₃₉₆₋₅₀₃ also showed increased latency to become immobile in FST test on 2nd day and this was apparent only in the CMS group, implicating an interaction of disrupted NOS-I/NOS1AP interaction with stress in learned behavioral despair.

4.3.4. Sensorimotor gating remain substantially intact upon disruption of NOS-I PDZ interaction

Sensorimotor gating deficits have been reported for many neuropsychiatric disorders (Braff *et al*, 2001) and they have a high translational value across species (Geyer and Swerdlow, 2001). In this study (Figure 30), reduced PPI levels in mice overexpressing NOS1AP-L, which was shown to be increase in schizophrenia and bipolar disorder patients (Hadzimichalis *et al*, 2010; Xu *et al*, 2005), suggests a prominent value for NOS1AP-L overexpression in rodents to model positive symptoms of these disorders. While the effect of NOS1AP-L overexpression was not statistically significant, this might be explained by small sample size and high variability between sample means. Moreover, there might be also effects of the ventral hippocampus involvement and/or other mechanisms mediating startle response (Zhang *et al*, 2002b) as mentioned before.

On the other hand, NOS1AP₃₉₆₋₅₀₃ overexpression in hippocampus resulted in enhanced PPI in non-stressed mice, indicating similar effects observed in schizophrenia patients treated with antipsychotics (Swerdlow *et al*, 2006). Considering the fact that overexpression of the PDZ binding region of NOS1AP was associated with reduced NOS-I/NOS1AP formation (Zhu *et al*, 2014), it is plausible to assume that disrupting NOS-I/NOS1AP interaction in hippocampus of mice exerts antipsychotic-like effects. Nevertheless, it should not be disregarded that the interaction of NOS1AP₃₉₆₋₅₀₃ construct with other proteins, such as NOS1AP and NOS-I in hippocampus has not been confirmed yet. Therefore, further investigation will be valuable to develop treatment strategies targeting NOS1AP interactions.

4.3.5. Social interaction is dependent on NOS-I PDZ interactions

Social withdrawal is another common endophenotype observed in patients with neuropsychiatric disorders such as schizophrenia. Studies performed in rodents produce results which are highly comparable to phenotypes observed in patients (Kas *et al*, 2007; Wilson and Koenig, 2014). When rodents are tested for social interaction, it is important to keep anxiogenic conditions to a minimum such as testing them in a familiar arena, otherwise the scored interaction may be compromised by the animal's increased anxiety. Therefore, I have tested mice in the OF arena where they have been already habituated.

Although studies performed in rodents using NOS-I inhibitors revealed inconsistent data on sociability, overall impairment in NOS-I signaling seems to reduce sociability (Tanda *et al*, 2009;

Trainor *et al*, 2007), which is consistent with data from this study (Figure 31). Mice overexpressing NOS1AP-L, NOS1AP₃₉₆₋₅₀₃ or NOS-I₁₋₁₃₃ showed strongly reduced social interaction. One commonality in these construct is their potential to disrupt NOS-I interactions, especially the interaction between NOS-I and PSD-95 (Jaffrey *et al*, 1998; Zhou *et al*, 2010; Zhu *et al*, 2014), indicating that disrupted interaction between NOS-I and PSD-95 might be contributing to impaired sociability. However, this hypothesis still needs to be experimentally confirmed. Moreover, both mice overexpressing NOS1AP₃₉₆₋₅₀₃ and NOS1AP-L constructs showing impaired social interaction suggest a role for the C-terminus of NOS1AP in the control of social interaction and not for the PTB- or CPE domain interaction of NOS1AP. Again, this needs to be confirmed experimentally. Moreover, while CMS resulted in much lower sociability in only mCherry expressing mice, it did not induce any effect on other groups. Therefore, NOS-I might be regulating sociability via PDZ interactions regardless of stress conditions, which would explain altered levels of interaction only in mCherry mice.

Social recognition (i.e. social novelty preference), on the other hand, was not affected by alterations in NOS-I related mechanisms and this will be further discussed in the next session focusing on cognitive deficits.

4.3.6. Learning and memory is mildly impaired by disrupted NOS-I PDZ interactions

Recognition memory was tested in both social (Figure 31) and non-social (object recognition, Figure 22) context. In both tests, all experimental groups showed comparable novelty preference performance compared to mCherry mice regardless of stress treatment. Recent studies revealed that NMDA receptor dysfunction results in impaired object recognition memory in both humans and rodents and the hippocampus is especially important for the formation of object recognition memory (Goulart *et al*, 2010; Rezvani, 2006). Even though the constructs used in this study might interfere with the integrity of the NOS-I/PSD-95/NMDA receptor complex, they do not interfere directly with NMDA receptor function or PSD-95/GluN2B interaction. On the other hand, NOS-I knockout mice showed reduced social recognition in previous studies (Tanda *et al*, 2009; Walton *et al*, 2013) suggesting that NOS-I plays an important role in social recognition. However, in this study, NOS-I activity was not completely abolished, though PDZ interactions of NOS-I were disrupted. It appears that NOS-I interaction with neither NOS1AP nor PSD proteins played a role

in novelty discrimination. Alternatively, other factors might have compensated disrupted NOS-I interactions. For example, recognition memory is also under influence of several factors e.g. functioning of perirhinal cortex (Winters and Bussey, 2005), involvement of other genes associated with activity of NOS-I (Dachtler *et al*, 2016) or intact NMDA receptor activity as mentioned before. In short, results from this study reveal that NOS-I PDZ interactions in hippocampus do not appear to contribute to mediating short-term memory dependent novelty preference.

In this study, SWM tasks were also designed to evaluate ability of mice to process information in short term memory. SWM deficits were tested by using a rewarded alternation paradigm on T-maze (Figure 26) and spatial novelty preference task on Y-maze (Figure 27). On T-maze mice from all groups showed intact SWM starting from the beginning of test and all except mice overexpressing NOS-I₁₋₁₃₃ completed the test without impaired working memory. On Y-maze, NOS-I₁₋₁₃₃ also showed impaired SWM. These mice also showed impaired SRM on Y-maze (Figure 29). Altogether, data suggest an important involvement of NOS-I PDZ interactions in spatial memory. Impaired spatial memory upon disruption of NOS-I interactions is consistent with other human and rodent studies associating interrupted activity of NOS-I with impaired SWM (Donohoe *et al*, 2009; Zoubovsky *et al*, 2011) and SRM (Zou *et al*, 1998). SWM deficits were also present in NOS1AP-L overexpressing mice at significant level only in the group that underwent CMS. It should be noted that the NOS-I₁₋₁₃₃ construct could disrupt the interaction between both NOS-I/PSD-95 and NOS-I/NOS1AP as it contains required PDZ structure for both protein-protein interactions. However, overexpression of the NOS1AP-L construct disrupts interaction of NOS-I/PSD-95 receptor complex (Jaffrey *et al*, 1998), and it also resembles the increased NOS1AP expression in brains of patients with schizophrenia and bipolar disorder. Moreover, increased NOS1AP-L expression might induce effects also through its interactions with other proteins. For example, increased expression of Dexas1 has been also associated with impaired working memory (Carlson *et al*, 2016). In addition, NOS1AP₃₉₆₋₅₀₃ overexpressing mice showed deficits neither in SWM nor SRM tasks. This may suggest that NOS-I/NOS1AP interactions may not directly be involved in the regulation of spatial memory. Therefore, further analysis of NOS1AP protein interactions could provide a better understanding of its involvement in the regulation of spatial memory. It should also be noted that NOS1AP-L overexpression resulted in impaired working memory only on the Y-maze but not the T-maze. One explanation might be that the

rewarded alternation task in T-maze involves goal directed navigation as mice receive the award (milk in the goal arm) when they show successful alternation. However, spatial novelty preference in Y-maze is largely based on habituation and exploratory behavior of mice to the visited arms of the maze (Leising and Blaisdell, 2009). This suggest that NOS1AP-L overexpression might disrupt SWM when recall of the memory is not dependent on emotional stimuli (reward), but rather leads to impaired short-term habituation. SWM is also under the influence of medial prefrontal cortex in rodents (Bannerman. *et al*, 2001). For example, deletion of NMDA receptor subunit NR2B from forebrain in mice was shown to induce more prominent working memory deficits then deletion in hippocampus (von Engelhardt *et al*, 2008). Therefore, further studies investigating the effect of NOS1AP overexpression in prefrontal cortex might shed more light on specific effects of NOS-I PDZ interactions in neuropsychiatric disorders.

To assess short-term associative learning, mice were tested for delayed fear conditioning ([Figure 32](#)). Previous studies have implicated the importance of NOS-I activity in fear conditioning, as *NOS1* KO mice showed impaired contextual memory in short term, but not auditory memory (Kelley *et al*, 2009). In another study, administration of NOS-I inhibitors (N ω -propyl-L-arginine and NO scavenger carboxy-PTIO) in ventral medial prefrontal cortex resulted in impaired contextual fear conditioning (Moraes Resstel *et al*, 2008). The same study also showed that using NMDA receptor antagonist revealed similar effects suggesting a role for NMDA/NO neurotransmission in contextual conditioning. In the present study, I did not find any apparent differences in contextual fear conditioning. One potential reason for this discrepancy might be differences in the fear induction. For example, I have used two CS-US pairings with 60 min ITI and tested mice for context memory 24 h later. Kelley *et al*. (2009) performed 4 training trials (ITI 10-12 min) for acquisition of conditioning each consisting one CS-US pairing and they tested mice for contextual conditioning between each training trial. Moraes Resstel *et al*. (2008), on the other hand, performed two 10 min acquisition trials (morning and afternoon trials) each consisting 6 foot shocks at 20 s to 1 min ITIs. While the mice that I tested in the present study did show successful fear acquisition, increasing the intensity of fear acquisition may still result in a differential contribution of NOS-I PDZ interactions to fear memory.

In the cue retention test, all mice showed intact auditory conditioning. Previous studies have also shown that auditory conditioning is more of a amygdala-dependent tasks, because it involves processing an emotional stimulus (CS: tone cue – US: foot shock) such as fear (Phillips and LeDoux, 1992). In addition previous studies also showed that auditory conditioning was not dependent on the activity of NOS-I (Kelley *et al*, 2009, 2010).

4.4. Limitations of the study

One important limitation to this study is that proper targeting and expression of the injected viral constructs has not yet been completed for all mice. So far, the brains assessed for viral expression revealed stable expression levels which were largely confined to the dorsal hippocampus. However, there might be mice which failed to express the injected construct due to surgery-dependent complications (e.g. mistargeting) and these mice would have to be excluded from the analysis. In addition, animal sample size in this study is rather small. Considering high variance within the groups in most behavioral tests, increased number of mice and final confirmation of construct expressions in dorsal hippocampus would provide statistically more powerful data.

Second, localization and co-localization analysis of the constructs in the brains of mice is missing. Constructs used in the study have been associated with crucial pathways both at the pre- and postsynaptic terminals, as well as in intracellular pathways. Therefore, a detailed analysis of virally expressed proteins will be very valuable for more reliable interpretation of the data.

Last but not least, NOS-I PDZ domain is very critical for the Ca^{2+} -influx dependent production of NO. Therefore the consequences of virally overexpressing the constructs from this study on functionality of ionotropic glutamate receptors could be tested using electrophysiological methods.

5. Conclusion

In this study, I have investigated the consequences of disrupted NOS-I PDZ interactions at morphological and behavioral levels. Within the first part of the study, neuronal morphology alterations which resemble the alterations in post-mortem brain tissues from patients with psychiatric disorders were shown to be regulated by NOS1AP overexpression. I showed here that dendritic morphology was mildly altered, on the other hand dendritic spine maintenance was highly influenced by overexpression of NOS1AP isoforms/deletion mutants. This study also links NOS-I and its interaction partners to the developmental theory of disorders such as schizophrenia by providing clues on spine regulation throughout developmental stages. Involvement other pre-/postsynaptic proteins influencing microtubule organization and physiological integrity in cells overexpressing NOS1AP isoforms/deletion mutants can be further investigated.

In the second part of the study, I have investigated whether NOS-I and its interaction partners are involved in development of neuropsychiatric disorders as previous studies linked these interactions to anxiety and depression. Even though, this study did not provide a strong link between NOS-I PDZ interactions and disorders such as anxiety or depression, brain region specific effects of these interactions might be helpful to further investigate in prefrontal cortex and ventral hippocampus. Results reveal that lack of exploratory behavior, impaired social interaction and working memory together with partially altered sensorimotor gating in mice with disrupted NOS-I PDZ interactions indicate translational relevance to the symptoms observed in patients with psychotic symptoms including patients with schizophrenia. These findings also suggest that NOS-I PDZ interactions and elevated levels of NOS1AP contribute to the development of schizophrenia and fits well with the glutamatergic theory of schizophrenia. Moreover, overexpression of NOS1AP₃₉₆₋₅₀₃, which would disrupt the interaction between endogenous NOS1AP and NOS-I, in dorsal hippocampus of C57BL/6J mice largely resembled the effects of antipsychotic treatments suggesting that NOS1AP/NOS-I interaction might be a potential target to develop novel treatment strategies.

Further studies are required to complement the results from this study. First of all, increasing the animal number used for behavioral testing or eliminating the seasonal effects (winter-summer) might reduce the high variances within groups. Second, NOS-I localization and functioning is altered due to its interaction with NOS1AP as previously mentioned. Therefore, localization / co-localization analyses (i.e. immunofluorescence stainings, protein and co-immunoprecipitation

analyses by Western blotting for NMDA receptor subunits, PSD proteins, NOS-I and NOS1AP) in brains of these mice will provide a clear understanding of the molecular mechanisms associating NOS-I and its interactions with either NOS1AP or PSD proteins with the development of neuropsychiatric disorders. In addition, I was able to show dendritic arborization alterations similar to those observed in patients with psychiatric disorders. However, cultured neurons do not represent same neuronal wiring patterns as *in vivo*. Therefore, observed dendritic alterations in primary cultured neurons should also be replicated in brains of mice injected with the viral vectors from this study. These investigations are currently ongoing and performed by students contributing to this study.

6. References

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7. Abbreviations

AAV	adeno-associated virus
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	Anterior – Posterior
ASR	acoustic startle response
BH4	(6R)-5,6,7,8-tetrahydrobiopterin
BSA	Bovine serum albumin
CaM	calcium-calmodulin complex
CAPON	NOS-I adaptor protein, carboxy-terminal PDZ ligand of NOS-I
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CMS	chronic mild stress
co-IP	co-immunoprecipitation
CPE	carboxypeptidase E binding region
CS	conditioned stimulus
CTD	carboxy terminal domain
DAF-FM	4-Amino-5-Methylamino-2',7'-Difluorofluorescein
DAPI	4',6-diamidino-2-phenylindole
dHPC	dorsal hippocampus
DISC1	Disrupted In Schizophrenia 1
DIV	days in vitro
DiI	1,1'-Diiododecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DNA	deoxyribonucleic acid
DV	Dorsal - Ventral
E	glutamic acid
<i>E. coli</i>	Escherichia coli
E18	embryonic day 18
EDRF	endothelial derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescence protein
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
EZM	elevated zero maze
F	Phenylalanine
FAD	flavin adenine dinucleotide
FC	fear conditioning
FG	fish gelatine
FMN	flavin adenine mononucleotide

FST	forced swim test
GFP	green fluorescent protein
GluN2	N-methyl-D-aspartate receptor subunit
GMP	guanosine monophosphate
GST	glutathione S-transferase
HBS	HEPES buffered saline
HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGH pA	human growth hormone polyadenylation signal
HIF	hypoxia-inducible factor
HSP90	heat shock protein 90
hSyn	human synapsin 1 gene promoter
hSynapsin	human synapsin 1 gene promoter
IAV	Isoleucine-Alanine-Valine
IgG	Immunoglobulin G
ITI	inter-trial interval
ITR	inverted terminal repeat
iNOS	inducible nitric oxide synthase
LDB	light-dark box
MAGUK	membrane associated guanylate kinase
ML	Medial – Lateral
mNudE-L	mouse NudE-like protein
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
N.A.	not available
NA	numerical aperture
NaCl	sodium chloride
NaDOC	sodium deoxycholate
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NB	<i>nesting behavior</i>
NDEL1	NudE Neurodevelopment <i>Protein</i> 1 Like 1
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
<i>NOS1</i>	neuronal nitric oxide synthase gene
NOS1AP	NOS-I adaptor protein, carboxy-terminal PDZ ligand of NOS-I
NOS-I	neuronal nitric oxide synthase protein
NOS-II	inducible nitric oxide synthase

NOS-III	endothelial nitric oxide synthase
NP40	nonyl phenoxyethoxyethanol
NR2A	N-methyl D-aspartate receptor subtype 2A
NR2B	N-methyl D-aspartate receptor subtype 2B
NTD	amino terminal domain
OE/NOR	object exploration / novel object recognition
OF	open field
ORF	open reading frame
pAAV	AAV plasmid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ	postsynaptic density protein 95 (PSD95) /discs large 1/zona occludens 1
PDZ- β	core PDZ domain followed by β -finger including internal PDZ motif
PFA	paraformaldehyde
pFdelta6	adenovirus helper-plasmid
PGC	Psychiatric Genomics Consortium
pH21	plasmid expressing cap genes of AAV1
PPI	prepulse inhibition of startle reflex
pRV1	plasmid expressing rep and cap genes of AAV2
PSD	post-synaptic density
PSD-93	post-synaptic density protein 93 kDa
PSD-95	post-synaptic density protein 95 kDa
PTB	phosphotyrosine binding domain
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real time PCR
rAAV	recombinant adeno-associated virus
RM	repeated measures
RNA	ribonucleic acid
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulfate
sGC	soluble guanylyl cyclase
SI	social interaction
SNP	single nucleotide polymorphism
SP	<i>sucrose preference</i>
SR	social recognition
SWM	spatial working memory
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween20
Tris	trishydroxymethylaminomethane

US	unconditioned stimulus
WB	western blot
WHO	World Health Organization
WMH	World Mental Health
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element

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