Hippocampal overexpression of Nos1ap promotes endophenotypes related to mental disorders

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Article

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Abstract

Nitric oxide synthase 1 adaptor protein (NOS1AP; previously named CAPON) is linked to the glutamatergic postsynaptic density through interaction with neuronal nitric oxide synthase (nNOS). NOS1AP and its interaction with nNOS have been associated with several mental disorders. Despite the high levels of NOS1AP expression in the hippocampus and the relevance of this brain region in glutamatergic signaling as well as mental disorders, a potential role of hippocampal NOS1AP in the pathophysiology of these disorders has not been investigated yet. To uncover the function of NOS1AP in hippocampus, we made use of recombinant adeno-associated viruses to overexpress the murine Nos1ap isoform in the hippocampus of mice. We investigated these mice for changes in gene expression, electrophysiology, neuronal morphology, and relevant behavioral phenotypes. We found that overexpression of hippocampal Nos1ap markedly increased the interaction of nNOS with PSD-95, reduced dendritic spine density and changed dendritic spine morphology without affecting basic synaptic signaling properties at CA1 synapses. At the behavioral level, we observed an impairment in social interaction and social memory, as well as decreased spatial working memory capacity. Our data provide a mechanistic explanation for a highly selective and specific contribution of hippocampal NOS1AP and its interaction with the glutamatergic postsynaptic density to cross-disorder pathophysiology. Our findings allude to therapeutic relevance due to the druggability of this molecule.

1. Introduction

Nitric oxide synthase 1 adaptor protein (NOS1AP; previously named CAPON) is a scaffolding protein that has been linked to different mental disorders (reviewed in [1, 2]). For example, elevated NOS1AP mRNA and protein was found in blood [3], as well as in the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia [4, 5]. Increased NOS1AP immunoreactivity in the DLPFC, as well as the anterior cingulate cortex, was also described in patients with major depressive disorder [6]. Recently we found increased *NOS1AP* mRNA in hippocampus of schizophrenia patients (in preparation). Consistent with these changes in expression, *NOS1AP* variants have been associated with schizophrenia endophenotypes [7–9] and depression-related traits in schizophrenia patients [10]. Moreover, *NOS1AP* variants have been associated with symptom severity, and depression and anxiety symptoms in posttraumatic stress disorder (PTSD) [11, 12].

While it has not yet been shown whether increased NOS1AP expression directly contributes to psychopathology or endophenotypes, several preclinical studies hint at such a possibility. Overexpression of Nos1ap (i.e. the mouse ortholog of NOS1AP) in the mouse dentate gyrus was shown to have anxiogenic effects [13] and downregulation of Nos1ap in the medial prefrontal cortex reversed stress-induced depression-like behavior in mice [6]. Moreover, Nos1ap overexpression in neuronal cell cultures resulted in reduction of dendritic growth and the number of mature dendritic spines, and increased filopodia-like protrusions [14–18]. These findings are akin to observations in post-mortem studies of different mental conditions including schizophrenia, mood disorders, and intellectual disability (e.g.

NOS1AP is best known for its interaction with neuronal nitric oxide synthase (nNOS or NOS-I, encoded by the *NOS1* gene) by binding of an internal ExF motif [21] and a carboxyterminal PDZ-motif [22] to the PDZ domain of nNOS. In neurons, nNOS is linked to the postsynaptic density (PSD) of glutamatergic synapses [23, 24] through interaction with the PDZ2 domain of PSD-93 or -95 [25–28]. This interaction brings nNOS in proximity to NMDA receptors enabling NMDA receptor-dependent Ca^{2+} influx to activate nNOS [29, 30] resulting in further downstream effects that are involved in neuronal plasticity.

NOS1AP was originally described as an inhibitor of nNOS/PSD-95 interaction [22]. However, subsequent findings showed NOS1AP mediated NMDA receptor signaling through nNOS [31–33], which depends on nNOS/PSD-95 interaction [30], suggesting that NOS1AP may in fact mediate, not inhibit the function of the nNOS/PSD95/NMDA receptor complex. Models have been proposed to reconcile these apparently conflicting findings (discussed in [34]). In addition to nNOS, NOS1AP interacts with other proteins, including RasD1 (also known as DexRas1; encoded by *RASD1*) [31] and MKK3 (encoded by *MAP2K3*) [33], linking these proteins to nNOS and, thereby, mediating their activation (reviewed in [2, 34]).

Together, these findings strongly argue that NOS1AP is a key effector component of glutamatergic pathways, and in doing so, it may have a role in psychiatric phenotypes across diagnostic boundaries. This is in good agreement with the current understanding about the involvement of the glutamatergic system in the pathophysiology of several mental disorders (see e.g. [35–38]) as also suggested by cross-disorder genetics [39, 40].

The hippocampus, a highly interconnected glutamatergic brain region, has been suggested as an important brain structure for mental disorders and related phenotypes (see e.g. [41–45]) as also supported by cross-disorder brain imaging studies [46]. Despite the important role of nitric oxide signaling in the hippocampus [47] and the above-described increase of *NOS1AP* mRNA in patients suffering from schizophrenia, a potential involvement of hippocampal NOS1AP and its interaction with the nNOS/PSD95/NMDA receptor complex to different endophenotypes of severe mental disorders has not been investigated yet. Thus, to further clarify the neural and behavioral circuits that are affected by NOS1AP and thus to disentangle the psychiatric phenotypes linked to disturbed NMDA/nNOS/NOS1AP signaling, we overexpressed Nos1ap in the hippocampus of wild type mice and studied the resulting changes in gene expression, electrophysiology, neuronal morphology, and behavior.

2. Materials And Methods

Viral vectors

Cloning of the pAAV plasmids coding for mCherry (pAAV-hSyn-mCherry.3xFLAG-WPRE) and Nos1ap (pAAV-hSyn-mCherry.3xFLAG.Nos1ap-WPRE), was previously described [14]. The pAAV-hSyn-mCherry.3xFLAG.$Nos1ap_{396-503}$-WPRE plasmid was cloned analogous to the Nos1ap plasmid [14] See Supplemental Material for additional details. All constructs contained the human *Synapsin-1* promoter,

limiting expression to neurons [48]. Recombinant adeno-associated viruses (rAAVs) were generated and titrated to $2x10^9$ viral genomes/µl using WPRE-specific primers (Table S1) as described [14].

All virus plasmids are available from Addgene (https://www.addgene.org/Florian_Freudenberg/).

Mice

Wild-type male C57BL/6JRj mice (Janvier Labs) were maintained under controlled conditions (lights on 0700–1900; 21 ± 1°C; 55 ± 5% humidity) *ad libitum* access to food and water. Mice were tested during the light-phase by evaluators blinded to the treatment. Mice were habituated to the experimental room > 45 min before testing and exposed to 60 dB white noise while staying in the experimental room. All experiments were conducted according to the *Council Directive 86/609/EEC of 24 November 1986* and German animal welfare laws (*TierSchG* and *TSchV*) and were approved by the Regierungspräsidium Darmstadt (approval ID: FK/1033) and the Landesuntersuchungsamt Koblenz (approval ID: G 17-1-060).

Stereotaxic surgeries

Mice were stereotaxically injected with the rAAVs in the dorsal hippocampus (ventral hippocampus for electrophysiological experiments) as described [49] and allowed to recover for 4–5 weeks before starting experiments. See Supplemental Material for details.

Molecular analyses of mouse brain tissue

Quantitative PCR (qPCR)

Whole RNA was isolated from dorsal hippocampus of mice with virus-mediated overexpression of mCherry, Nos1ap, and $Nos1ap_{396-503}$ and reverse transcribed into cDNA. Target-specific quantitative PCR (qPCR) was performed using target specific primers (Table S2). Relative gene expression was analyzed using GenEx6 v3.1.3 (MultiD Analyses AB). Data were statistically analyzed by one-way ANOVAs. The significant P-value (i.e. significance threshold) was Bonferroni-corrected for the number of tested targets ($P_{Bonf}=\frac{0.05}{17\ \text{target genes}}$ =0.00294117). See Supplemental Material for details.

Protein isolation and co-IP

Hippocampal tissue from dorsal hippocampus of uninjected mice, and from mice with virus-mediated dorsal hippocampal overexpression of mCherry, Nos1ap, and $Nos1ap_{396-503}$ was dissected and snap-frozen. Co-immunoprecipitation was performed as described [33] (see Supplemental Material for details).

Electrophysiology

For electrophysiological recordings horizontal brain slices from 2-month-old C57BL/6JRj mice injected with mCherry, Nos1ap or $Nos1ap_{396-503}$ expressing rAAVs were made and processed for electrophysiological recordings in the hippocampal CA1 region as detailed in the Supplemental Material.

AMPA/NMDA ratios and paired-pulse ratios from CA1 evoked excitatory postsynaptic currents (EPSCs), and CA1 miniature excitatory postsynaptic currents (mEPSCs) were recorded and analyzed as described [50] (see Supplemental Material for a detailed description).

Dendritic spine analysis

One month after rAAV delivery to the dorsal hippocampus, mice were killed, and brains were removed and stained using the FD Rapid GolgiStain kit (FD NeuroTechnologies). For each viral vector, 25 secondary dendrites from 5 different mice (i.e. 5 dendrites/mouse) from pyramidal neurons of the dorsal CA1 *stratum radiatum* were randomly selected and imaged. Dendritic spines were analyzed using the trainable classifier in NeuronStudio (version 0.9.92). Output classes were defined as stubby, mushroom, long-thin, thin, filopodia and branched. For analysis, thin and long-thin spines were combined. Branched spines were excluded from analysis. The total number of mature spines was calculated as the sum of all spine types excluding branched or filopodia. See Supplemental Material for details.

Analysis of behavior

Mice were tested behaviorally for anxiety in the light dark box and the elevated zero maze, for locomotor activity in the open field, for sensorimotor gating by measuring the PPI of the ASR, for anhedonia by measuring sucrose preference, for spatial working memory (SWM) in the rewarded alternation task on the T-maze and the spatial novelty preference paradigm in the Y-maze, and for spatial reference memory in the Y-maze. A detailed description of the behavioral tests can be found in the Supplemental Material.

Within two weeks after testing, mice were killed, and brains were analyzed (see Supplemental Material).

Statistical analyses

Data were statistically analyzed using JASP (0.11.1 and above) and Jamovi (v1.1.4.0 and above). Unless described otherwise, data were analyzed by ANOVA followed by LSD post hoc testing.

3. Results

Hippocampal overexpression of Nos1ap increases the nNOS/PSD-95 interaction

For the experiments described in this study, we stereotaxically delivered rAAVs encoding murine Nos1ap tagged with 3xFLAG and mCherry [14] or the Nos1ap carboxyterminus ($Nos1ap_{396-503}$), required for nNOS interaction [21] to the dorsal hippocampus of wild-type C57BL/6JRj mice. An rAAV expressing mCherry and 3xFLAG was used as a control (Fig. 1A,B).

Analysis of mCherry fluorescence (Fig. 1C) confirmed high expression levels in all subregions of the dorsal hippocampus (Figure S1). Congruent with previous findings [14] the Nos1ap rAAV had the lowest expression of the viruses. Ectopic expression was limited to the deep cortical layers above the injection site, comparable to previous observations [49]. Immunoblot analysis indicated that expression of the

virally-encoded proteins was substantially higher than endogenous Nos1ap levels (Fig. 1D), though these blots were not readily quantifiable due to signal saturation. Expression analysis using qPCR (Fig. 1E, Table 1) showed ~ 294-fold increase of *Nos1ap* in Nos1ap rAAV injected mice ($P < 0.001$). As the primers targeted the aminoterminal region of *Nos1ap*, no expression changes were detected in $Nos1ap_{396-503}$ mice ($P = 0.593$).

Table 1
Gene expression levels of Nos1ap and associated genes (see Figure S3 for graphical representation)

Gene	mCherry	Nos1ap	$Nos1ap_{396-503}$	Statistics[1]
Cpe	1.0e-10 ± 0.545	0.021 ± 0.33	0.772 ± 0.235	$F_{2,25}=1.045$, $P = 0.366$
Dlg1	-5.6e-18 ± 0.183	-0.412 ± 0.196	0.218 ± 0.154	$F_{2,25}=3.009$, $P = 0.067$
Dlg3	-3.1e-18 ± 0.187	0.051 ± 0.187	0.048 ± 0.311	$F_{2,25}=0.017$, $P = 0.983$
Dlg4	-1.9e-17 ± 0.214	0.23 ± 0.25	0.445 ± 0.264	$F_{2,25}=0.811$, $P = 0.456$
Gria1	1.0e-10 ± 0.261	0.051 ± 0.247	0.695 ± 0.289	$F_{2,25}=1.978$, $P = 0.159$
Gria2	4.163e-18 ± 0.251	-0.01 ± 0.157	0.506 ± 0.228	$F_{2,25}=1.725$, $P = 0.199$
Grin2a	-5.573e-18 ± 0.259	-0.183 ± 0.233	0.474 ± 0.196	$F_{2,25}=1.903$, $P = 0.17$
Grin2b	0.0 ± 0.491	0.739 ± 0.177	1.087 ± 0.211	$F_{2,25}=2.628$, $P = 0.092$
Gucy1a1	1.0e-10 ± 0.393	0.317 ± 0.144	0.63 ± 0.205	$F_{2,25}=1.219$, $P = 0.312$
Gucy1a2	-2.0e-10 ± 0.17	-0.463 ± 0.341	-0.204 ± 0.146	$F_{2,25}=0.539$, $P = 0.402$
Gucy1b1	-1.0e-10 ± 0.469	0.425 ± 0.179	0.947 ± 0.128	$F_{2,25}=2.115$, $P = 0.142$
Map2k3	1.0e-10 ± 0.39	0.56 ± 0.444	1.54 ± 0.241	$F_{2,25}=3.835$, $P = 0.035$
Mapk14	-2.2e-17 ± 0.501	0.311 ± 0.15	0.886 ± 0.182	$F_{2,25}=1.668$, $P = 0.209$
Nos1	-3.0e-10 ± 0.407	-0.368 ± 0.563	0.477 ± 0.358	$F_{2,25}=0.785$, $P = 0.467$
Nos1ap	**-1.0e-10 ± 0.191**	**8.199 ± 0.273**	**-0.168 ± 0.117**	**$F_{2,25}=512.807$, $P < 0.001$**
Rasd1	2.0e-10 ± 0.305	-0.253 ± 0.381	-0.645 ± 0.225	$F_{2,25}=0.956$, $P = 0.398$
Scrib	1.0e-10 ± 0.627	0.884 ± 0.213	1.574 ± 0.189	$F_{2,25}=3.384$, $P = 0.05$
Syn1	1.0e-10 ± 0.441	1.02 ± 0.295	1.044 ± 0.532	$F_{2,25}=2.076$, $P = 0.147$

[1] Nominal (i.e. uncorrected) P-values are shown. Bonferroni-corrected significance threshold: $P_{B-C}=0.00278$

We previously showed that virally-overexpressed Nos1ap and a short isoform of Nos1ap, similar to $Nos1ap_{396-503}$, interact with endogenous nNOS [14]. Given their overabundance, endogenous nNOS appeared to be predominantly bound by these virally-expressed proteins, strongly reducing the interaction with endogenous Nos1ap, as shown by co-immunoprecipitation (Fig. 1F,G; $P < 0.001$ for Nos1ap and $Nos1ap_{396-503}$). Importantly, levels of endogenous Nos1ap and nNOS were not significantly affected by viral overexpression, as suggested by semi-quantitative immunoblot analysis (Figure S2A,B).

In keeping with recent findings suggesting that NOS1AP at least transiently mediates the function of the nNOS/PSD-95 NMDA receptor complex (discussed in [34]), using co-immunoprecipitation, we found that viral overexpression of Nos1ap and $Nos1ap_{396-503}$ strongly increased the nNOS/PSD-95 interaction (Fig. 1F,G; $P = 0.002$ for both) while overall PSD-95 levels were not significantly affected (Figure S2C).

In addition, we wanted to investigate if Nos1ap or $Nos1ap_{396-503}$ overexpression affects the expression of Nos1ap-associated genes. We found that the expression of *Map2k3* and *Scrib* was nominally affected ($P = 0.035$ and $P = 0.05$ respectively), but not after Bonferroni correction ($P_{Bonf}=0.00278$). The expression of all other tested genes was not significantly affected ($P > 0.05$; Table 1 and Figure S3), suggesting that the treatment only had a limited effect on the regulation of these genes.

Hippocampal overexpression of Nos1ap does not affect synaptic electrophysiology but changes dendritic spine morphology

An increased nNOS/PSD-95 interaction may influence synaptic signaling dynamics. To understand the influence of the nNOS/Nos1ap interaction on synaptic properties, we injected mice with the mCherry, Nos1ap, or $Nos1ap_{396-503}$ expressing rAAVs and, following 4–5 week of expression, performed patch clamp recordings on CA1 pyramidal neurons.

Paired-pulse ratios in CA1 Schaffer collateral synapses at inter-stimulus intervals of 30 or 100 ms were not altered ($P = 0.13$ and $P = 0.719$ respectively), indicating that overexpression of Nos1ap or $Nos1ap_{396503}$ does not influence their vesicle release probability (Fig. 2A). Equally, mEPSC frequency ($P = 0.713$), amplitude ($P = 0.793$), 20–80% rise time ($P = 0.158$), and τ_{decay} ($P = 0.128$) were not affected (Fig. 2B-E), suggesting that the number of functional synapses and the number of AMPA receptors per synapse were not influenced. AMPA and NMDA receptor-mediated currents in CA1 neurons, evoked by Schaffer collateral/commissural fiber stimulation showed that AMPA/NMDA ratios were comparable in all groups ($P = 0.082$), suggesting marginal effects on the relative number of AMPA and NMDA receptors (Fig. 2F).

We previously showed that overexpression of murine Nos1ap in cultured primary neurons resulted in an increase in filopodia and a reduction in dendritic spines [14]. Thus, we quantified dendritic spines in Golgi impregnated dorsal hippocampal CA1 neurons of mice with dorsal hippocampus injections (Fig. 2G-J).

The total number of mature spines (i.e. excluding filopodia) was significantly reduced in brains overexpressing Nos1ap (P = 0.004), but not in those expressing $Nos1ap_{396-503}$ (P = 0.196) when compared to control brains (Fig. 2I).

Functional properties of dendritic spines correlate strongly with their morphology [19]. Thus, we further analyzed different spine classes (Fig. 2J). We found a significant reduction in thin spines in brains overexpressing Nos1ap (P = 0.003) or $Nos1ap_{396-503}$ (P = 0.029), but no changes in the number of stubby (P = 0.852) or mushroom (P = 0.107) spines, or filopodia (P = 0.615).

Overexpression of Nos1ap in dorsal hippocampus disrupts selective behaviors related to mental disorders

Given the substantial effects of viral Nos1ap and $Nos1ap_{396-503}$ expression on nNOS/PSD-95 interaction and dendritic spines, we further investigated their effect on behavior.

Overexpression of Nos1ap or $Nos1ap_{396-503}$ did not affect basic behavioral phenotypes (Fig. 3). Specifically, horizontal (distance traveled: Fig. 3A, P = 0.191) and vertical (number of rearings: Fig. 3B, P = 0.202) activity in the open field were comparable across all groups. Previous studies in mice of the ICR strain have suggested that Nos1ap overexpression in the dentate gyrus, targeting a region more posterior to the one used here, had anxiogenic effects [13]. However, in our study, anxiety-related behaviors in the open field (time in the center: Fig. 3C, P = 0.227), the light dark-box (light time: Fig. 3D, P = 0.164; number of transitions: Fig. 3E, P = 0.459), and the elevated zero maze (open arm time: Fig. 3F, P = 0.858) were not affected by Nos1ap/$Nos1ap_{396-503}$ overexpression.

Sensorimotor gating, operationalized by PPI of the ASR is impaired in schizophrenia and other mental disorders (reviewed in [52]) and *NOS1AP* variants affecting PPI and startle have been identified [8]. Startle reactivity (Fig. 4A) was comparable in all groups (P = 0.611) and startle habituation (Fig. 4B) was intact in all groups (P < 0.001), with no effect of treatment (P = 0.683) or the interaction (P = 0.787). PPI significantly increased with prepulse intensity (Fig. 4C, P < 0.001), but was unaffected by the treatment (P = 0.599) or the interaction (P = 0.288) indicating that hippocampal Nos1ap/$Nos1ap_{396-503}$ overexpression does not influence sensorimotor gating.

NOS1AP was previously linked to depression and depression phenotypes, but not specifically within the hippocampus [6, 10]. Therefore, we tested our mice for anhedonia, a common negative symptom caused by impairments in reward-related pathways including the hippocampus [53]. However, no differences in anhedonia, measured by sucrose preference, were observed between groups (P = 0.508; Fig. 4D).

Deficits in sociability and social cognition are commonly found in autism spectrum disorders [54], schizophrenia [55] and other mental disorders including depression [56]. When exposed to a juvenile conspecific, Nos1ap overexpressing mice showed a trend for reduced social interaction (Fig. 4E; P = $_{503}$ expressing mice was significantly reduced (P = 0.043). No

effect on the number of social contacts was observed (Fig. 4F). Thirty min later mice were tested for social memory by exposure to the same ('familiar') and an unfamiliar ('novel') juvenile conspecific (Fig. 4G). We observed significantly longer interaction with the novel mouse in controls (P = 0.004), while Nos1ap and $Nos1ap_{396-503}$ overexpressing mice showed no preference for the novel mouse (P = 0.32 and P = 0.144). These findings show that viral overexpression of Nos1ap and $Nos1ap_{396-503}$ in hippocampus leads to deficits in sociability and social memory.

Deficits in working memory have been proposed as a transdiagnostic endophenotype for mental disorders [57]. Performance in the novel arm paradigm, a test requiring limited SWM capacity [49] was not affected (Fig. 4H, P = 0.6), suggesting that Nos1ap/$Nos1ap_{396-503}$ overexpression does not influence basic SWM. Confirming this observation, mice from all groups performed well above chance level in the rewarded alternation paradigm on the T-maze (Fig. 4I). Control mice improved SWM performance (P = 0.006) across training. In contrast, neither Nos1ap (P = 0.833) nor $Nos1ap_{396-503}$ (P = 0.363) expressing mice showed a significant increase in SWM performance indicating limited SWM capacity. This deficit cannot be attributed to an overall spatial memory deficit, as spatial reference memory was intact (Fig. 4J, P < 0.001) with no effect for the viral vector (P = 0.759) or the interaction (P = 0.552).

4. Discussion

The adaptor protein NOS1AP, which interacts with the nNOS/PSD-95/NMDA receptor complex and links nNOS to downstream pathways, thus mediating their activation [34], has been linked to multiple mental disorders including depression, schizophrenia, PTSD and related (endo)phenotypes [1, 2, 6–12, 58]. Here we created a targeted overexpression mouse model and revealed that hippocampal Nos1ap overexpression led to changes in dendritic spine morphology and selective behavioral abnormalities. Increased hippocampal NOS1AP might thus give rise to a specifically altered behavior that contributes to mental disorders in a transdiagnostic manner, pinpointing the network-specific molecular underpinnings of these cross-disorder symptoms.

Strong support for a functional role of NOS1AP to various mental disorder (endo)phenotypes was provided by the COGS family study [7–9]. Translating our data to the human situation, the behavioral changes observed upon Nos1ap overexpression partially overlap with endophenotypes implicated by the COGS study (e.g. social interaction/memory, SWM), while other domains remained unaffected (e.g. sensorimotor gating, anhedonia, spatial reference memory). As the human COGS study examined genotypes, but not brain regions, one may speculate that the latter domains might be a consequence of non-hippocampal NOS1AP expression changes. The fact that we did not detect changes in baseline anxiety and depression-like behavior in mice with targeted over-expression in the dorsal hippocampus suggests that NOS1AP is linked to various behaviors depending on the neural circuit it is involved in.

Critically, the selective deficits observed in our study are precisely in line with our expectations given (i) the reported NOS1AP overexpression in other brain regions

described in patients with schizophrenia [4, 5] and depression [6], (iii) the differential contribution of the hippocampus along its longitudinal axis (e.g. reviewed in [44]) (iv) the high variability of symptoms in patients, and (v) the vast number of other molecules/pathways associated with mental disorders [39]. In fact, using this selective type of manipulation we can more clearly discern the specific effect of elevated NOS1AP encompassing all subregions of the dorsal hippocampus (i.e. CA1, CA3, and dentate gyrus) than with other rather unphysiological manipulations such as global gene overexpression or knockout. Subtle variations that specifically manipulate gene expression in selected circuits may better reflect the human situation [59].

Dendritic spines increase in size and become more stable with persistent stimulation [60]. The relatively large and stable mushroom spines have been considered 'memory spines', while the more transient thin spines have been regarded as 'learning spines' [61]. Thus, our findings suggest that overexpression of Nos1ap and $Nos1ap_{396-503}$ results in a loss of spines required for plasticity and thus learning, while memory-related spines remain unaffected. These findings are in line with our findings indicating compromised social memory and SWM capacity, but persistent spatial reference memory, as well as with *in vitro* data showing reduced dendritic growth and aberrant morphology of dendritic spines following overexpression of Nos1ap [14, 15, 17]. The observed integrity of basic electrophysiological signaling properties also supports the notion that more permanent memory-related changes are not affected by our treatment.

In addition to Nos1ap, we overexpressed its carboxyterminus (i.e. $Nos1ap_{396-503}$), containing the nNOS interacting region [21]. While full-length Nos1ap can interact with downstream effectors (e.g. RasD1, MKK3) linking them to nNOS, $Nos1ap_{396-503}$, similar to the short form of NOS1AP [4], lacks the phosphotyrosine-binding domain and is therefore unable to interact with these proteins. Notably, most deficits in Nos1ap overexpressing mice were also observed in $Nos1ap_{396-503}$ mice. The dramatic increase in nNOS/PSD95 interaction observed in both groups is, most likely, the underlying reason for this. Importantly, PSD-95 has been suggested as a critical synaptic mediator of schizophrenia-related molecular consequences [62] and our previous study has shown that integrity of the Nos1ap/nNOS/PSD-95 complex is critical for the effect of Nos1ap on dendritic growth and spine plasticity [14]. Thus, downstream effectors such as RasD1or MKK3 might have a less direct role in the regulation of the (endo)phenotypes assessed here, as also supported by the lack of expression changes in Nos1ap interactions partners. Moreover, similar to what has been proposed for MAPK scaffolds [63, 64] it is possible that high levels of Nos1ap may negatively impact adequate formation of a trimolecular complex consisting of nNOS, Nos1ap and downstream effectors. In fact, interactions through the phosphotyrosine-binding domain and other binding regions (e.g. that for Cpe) have been shown to contribute to some of the effects mediated by NOS1AP [15, 16].

We and others have previously emphasized the potential for targeting the nNOS/PSD-95 interaction for the pharmacological treatment of schizophrenia, depression, and other mental disorders [1, 14, 65, 66]. The findings from the present study support this assertion and indicate a potential for directly targeting [illegible] using small molecules that are able to pass the blood brain

barrier. Such treatments will likely not adhere to diagnostic boundaries such as “schizophrenia” or “depression” but target defined neuropsychological deficits (here, compromised working memory and social interaction) in conjunction with evidence for abnormalities in NOS1AP interactions, in the sense of precision medicine approaches.

Taken together, we demonstrate a potential importance of hippocampal NOS1AP in the pathophysiology of different mental disorders. Through virus-mediated Nos1ap overexpression in the dorsal hippocampus of mice we recapitulated alterations in dendritic spine morphology also observed e.g. in patients with schizophrenia and mood disorders. Moreover, we reveal a role of Nos1ap overexpression in select (endo)phenotypes of psychiatric conditions including social memory and SWM capacity, without impacting on other aspects, such as PPI or anhedonia. Thus, this study enabled us to distinctly identify the contribution of hippocampal Nos1ap to phenotypes related to mental disorders. NOS1AP may thus provide an attractive target for disease stratification and targeting of NOS1AP protein interactions may be a potential target for novel pharmacological interventions at least in a subpopulation of patients.

Declarations

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Author contributions:

FF and EC cloned, purified, and validated the viral constructs and performed stereotaxic surgeries. FF, performed mouse behavioral experiments and analyzed

behavioral data. FF, LG, and MK performed and analyzed qPCR experiments. LLL, SB, and MJC performed Western blot and co-IP analyses and LLL analyzed data. XC performed electrophysiological experiments and XC and JvE analyzed electrophysiological data. DES, NS, VF, and NF performed Golgi stainings and quantified dendritic spines on Golgi-stained brain slices. EC, JM, SB, and AK sliced brains and microscopically analyzed brain slices. FF and AR conceived and supervised all experiments. FF and AR wrote the first draft of the manuscript. All authors contributed and proofread the manuscript and approved the final version.

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Figures

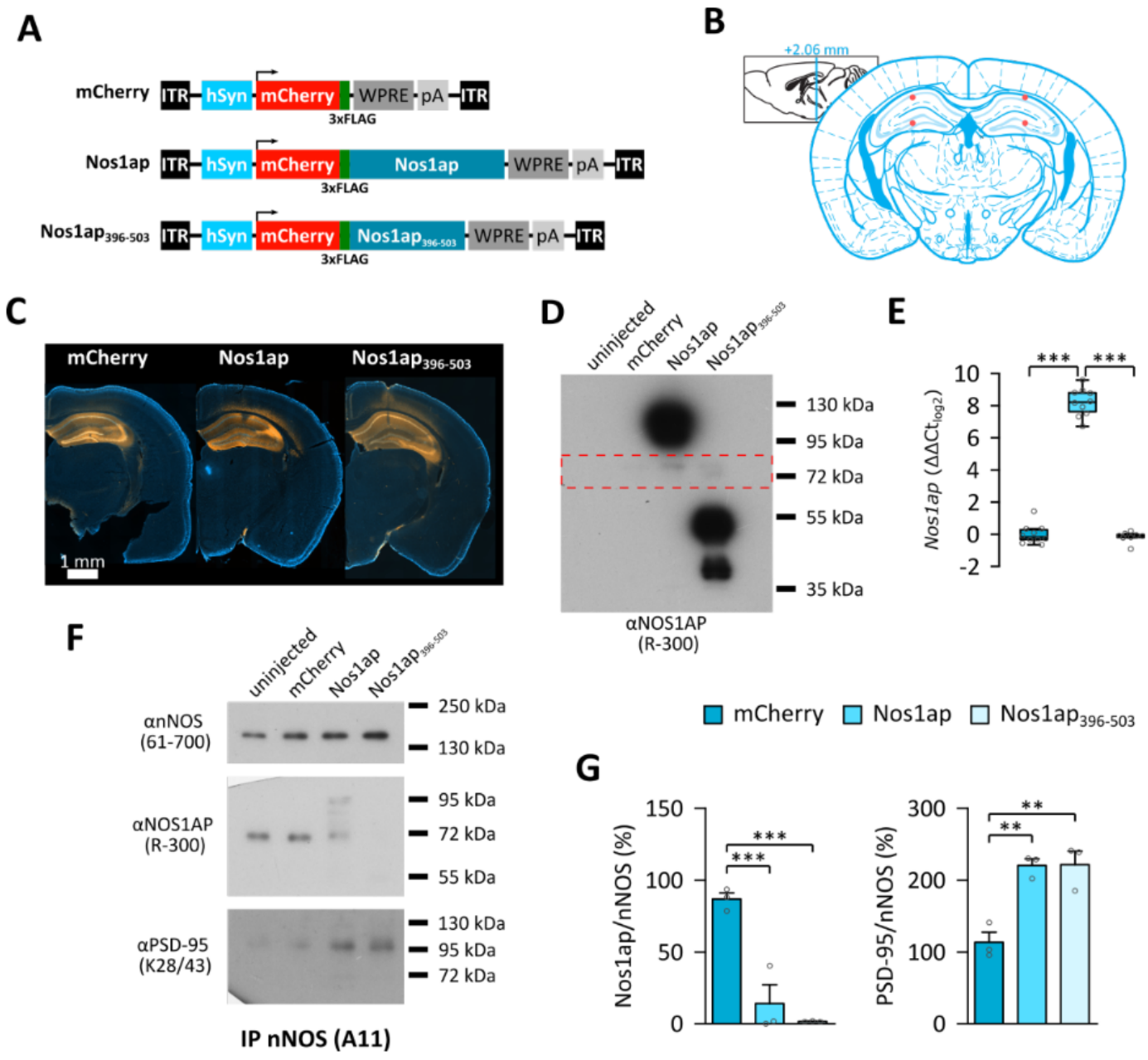

Figure 1

Hippocampus-specific overexpression of Nos1ap and Nos1ap396-503 enhances the interaction between PSD-95 and nNOS. (A) Schematic representation of the recombinant adeno-associated virus (rAAV) vectors used for expression of mCherry, the full-length long isoform of Nos1ap, and the carboxyterminal nNOS interaction domain of Nos1ap (i.e. Nos1ap396-503). (B) Schematic edited from [51] depicting the injection sites in dorsal hippocampus (red dots). (C) Exemplary images showing mCherry fluorescence in dorsal hippocampus of mice injected with the mCherry, Nos1ap, and Nos1ap396-503 expressing rAAVs. (D) Example immunoblot of protein lysates from dorsal hippocampus of C57BL/6JRj mice (uninjected or injected with the viral vectors) stained with an antibody raised against the carboxyterminal domain of Nos1ap. Endogenous Nos1ap is visible at ~72 kDa (indicated by the red box; for semi-quantitative

he virally encoded Nos1ap and Nos1ap396-503 can be seen

at ~95 kDa and ~50 kDa respectively but could not be quantified due to saturation of the signal. (E) Quantification of Nos1ap mRNA using quantitative PCR (qPCR). Data were normalized to the expression of Sdha and calculated relative to the average expression of the mCherry injected group. Data are displayed in log2. Nos1ap expression is significantly ($P<0.001$) increased ~294 fold (~8.2-fold in log2) in the hippocampi of Nos1ap injected mice ($F_{2,25}=512.807$, $P<0.001$). (F,G) Co-immunoprecipitation (IP) using nNOS as a bait (for input samples see Figure S4). (F) Exemplary immunoblots for nNOS, Nos1ap, and PSD-95 on the nNOS co-IP samples. (G) Quantification of co-IP data showing reduced interaction of endogenous Nos1ap with nNOS ($F_{2,6}=33.09$, $P<0.001$) and increased interaction of PSD-95 with nNOS ($F_{2,6}=18.89$, $P=0.003$), for both Nos1ap and Nos1ap396-503. (Abbreviations: hSyn = human Synapsin 1 gene promoter, ITR = inverted terminal repeat, pA = human growth hormone polyadenylation signal, WPRE = woodchuck hepatitis virus posttranscriptional regulatory element). Asterisks indicate significant differences of uncorrected post-hoc t-tests: **$P<0.01$, ***$P<0.001$.

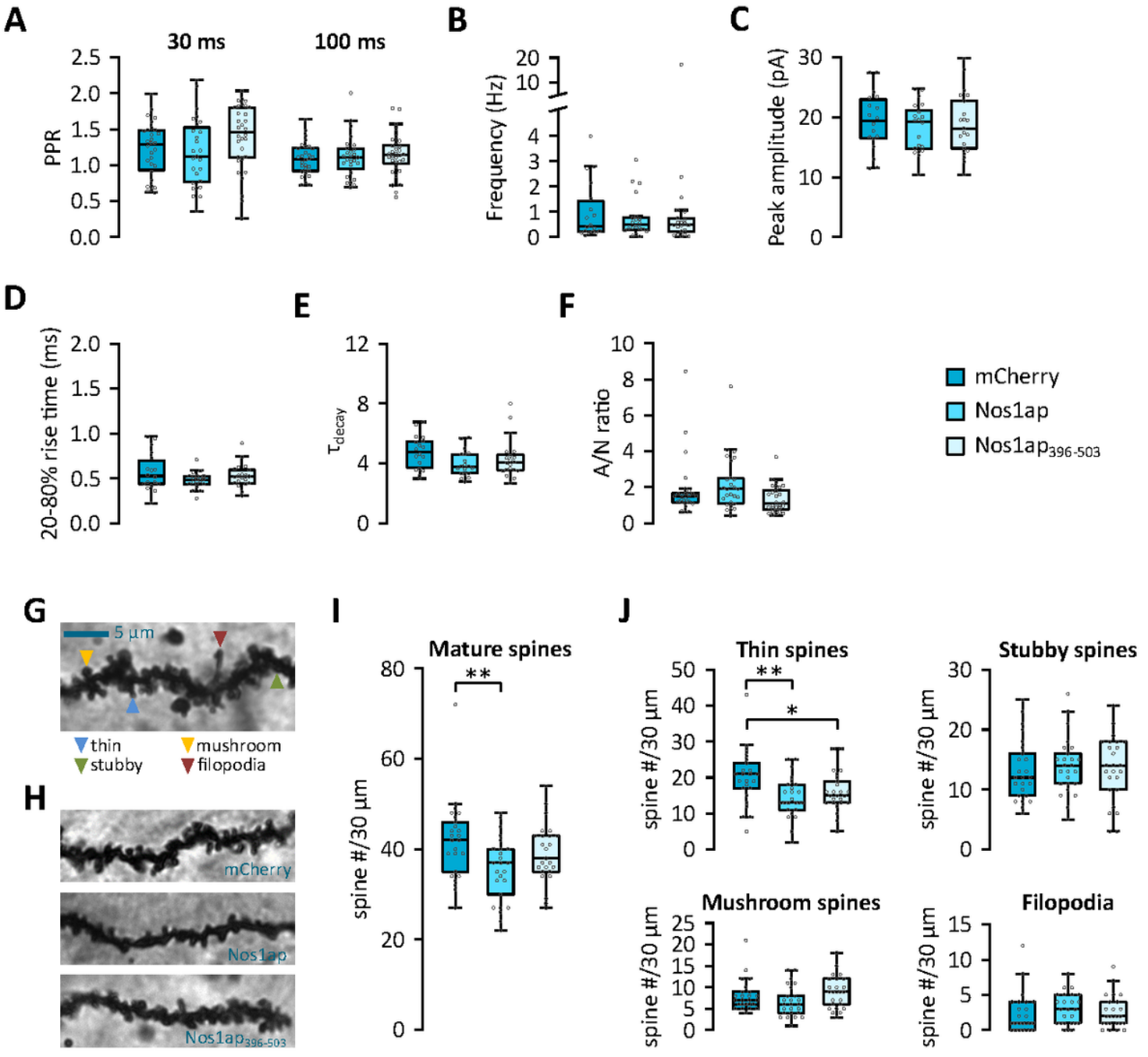

Figure 2

Overexpression of Nos1ap does not affect synaptic function but reduces spine number of CA1 neurons. (A-F) Electrophysiological properties of neurons from mice with viral overexpression of mCherry, Nos1ap, or Nos1ap396-503. (A) Paired-pulse ratio with 30 and 100 ms inter-stimulus interval was not affected (30 ms: $F_{2,86}=2.086$, $P=0.13$; 100 ms: $F_{2,85}=0.331$, $P=0.719$). (B-E) Miniature excitatory postsynaptic current (mEPSC) (B) frequency ($F_{2,56}=0.34$, $P=0.713$), (C) amplitude ($F_{2,56}=0.233$, $P=0.793$), (D) 20-80% rise time ($F_{2,54}=1.907$, $P=0.158$), and (E) τdecay ($F_{2,54}=2.133$, $P=0.128$) were comparable across all conditions. (F) Likewise, AMPA/NMDA (A/N) ratios were not affected by the treatment ($F_{2,76}=2.583$, $P=0.082$). (G-J) Brains of injected mice were Golgi-impregnated to quantify dendritic spines, including Example image highlighting the different spine types

analyzed herein. (H) Example images of Golgi impregnated dendrites from mice injected with the mCherry, Nos1ap, or Nos1ap396-503 expressing virus. (I) The total number of mature spines was significantly reduced in neurons overexpression Nos1ap, but not in those expressing Nos1ap396-503 ($F_{2,72}=4.473$, $P=0.015$). (J) Analysis for spine types showed a significant reduction of thin spines in mice expressing Nos1ap or Nos1ap396-503 ($F_{2,72}=5.189$, $P=0.008$), but not stubby ($F_{2,72}=0.16$, $P=0.852$) or mushroom ($F_{2,72}=2.304$, $P=0.107$) spines. No changes filopodia-like protrusions were detected ($F_{2,72}=0.489$, $P=0.615$). Asterisks indicate significant differences in uncorrected post-hoc t-tests: *P<0.05, **P<0.01.

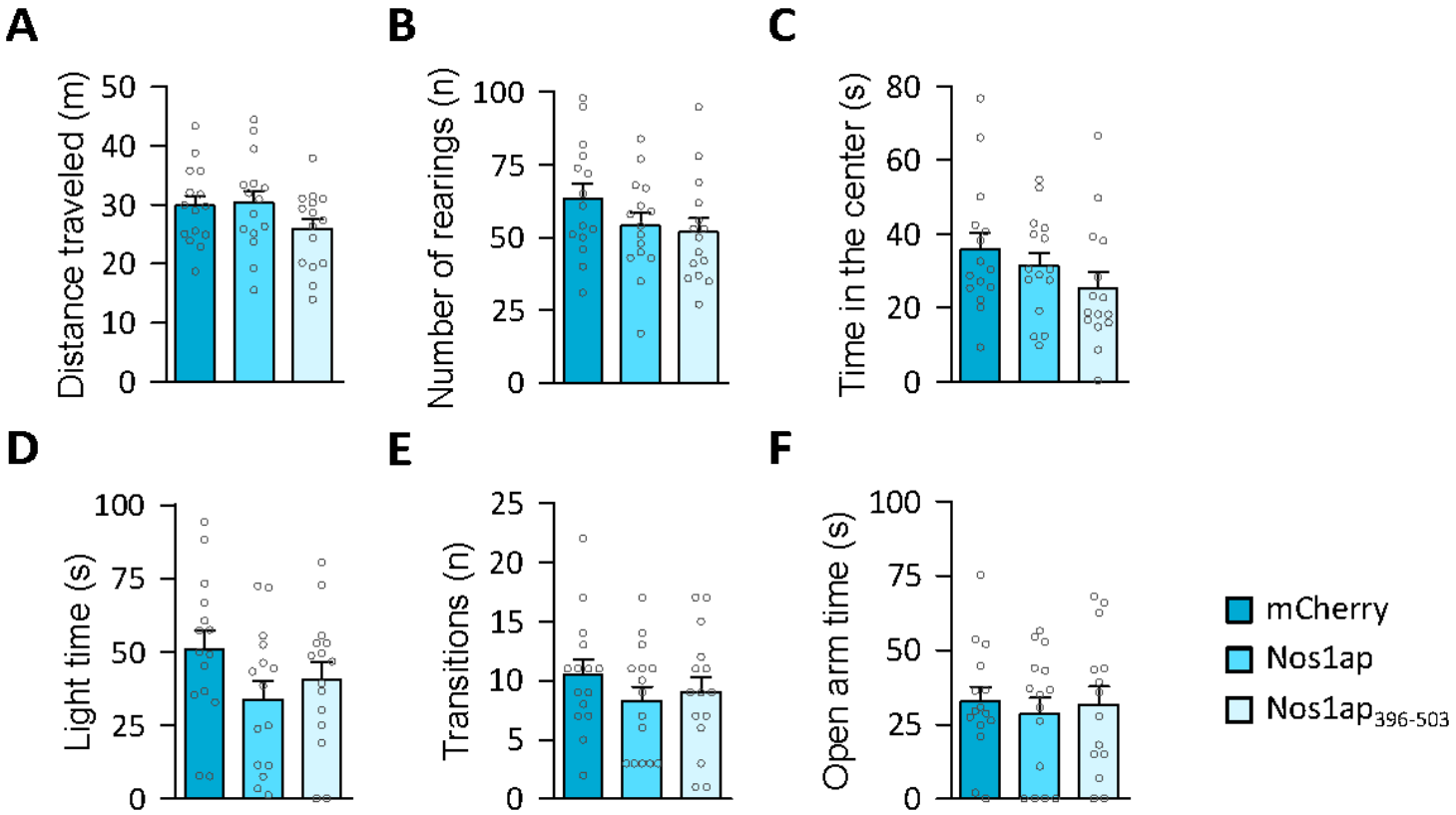

Figure 3

Basic behavioral phenotypes were not affected by overexpression of Nos1ap or Nos1ap396-503. (A) Distance traveled ($F_{2,42}=1.725$, $P=0.191$) and (B) number of rearings ($F_{2,42}=1.665$, $P=0.202$) in the open field were comparable in all groups. (C) The time spent in the center of the open field was also unaffected ($F_{2,42}=1.535$, $P=0.227$). (D) The time spent in the light compartment ($F_{2,42}=1.891$, $P=0.164$) and (E) the number of transitions ($F_{2,42}=0.793$, $P=0.459$) in the light-dark-box were comparable across groups. (F) Mice from all groups spent a comparable amount of time in the open segments of an elevated zero maze ($F_{2,41}=0.154$, $P=0.858$).

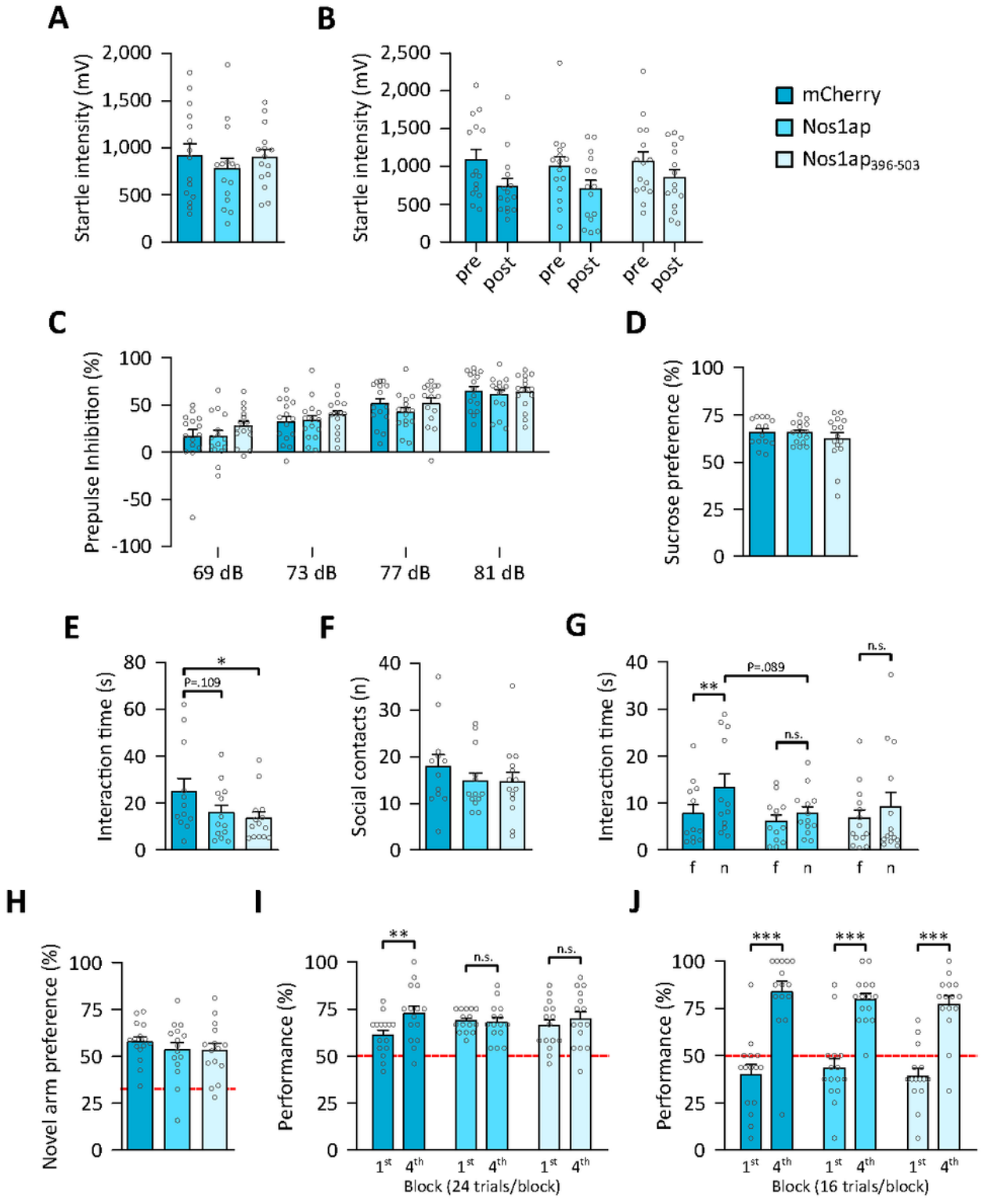

Figure 4

Sensorimotor gating, operationalized by PPI of the ASR is impaired in schizophrenia and other mental disorders (reviewed in [52]) and NOS1AP variants affecting PPI and startle have been identified [8].

Supplementary Files

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