NITRIC OXIDE SYNTHASE 1 ADAPTOR PROTEIN AS A TARGET FOR TREATMENT OF SCHIZOPHRENIA

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ABSTRACT OF THE THESIS

NITRIC OXIDE SYNTHASE 1 ADAPTOR PROTEIN AS A TARGET FOR TREATMENT OF SCHIZOPHRENIA

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Schizophrenia (SCZ) is a severe mental illness that affects 1% of the U.S. population. Antipsychotic medications are ineffective for many patients with SCZ, and *N*-methyl D-aspartate receptor (NMDAR) agonists may be potential treatments. Nitric oxide synthase 1 adaptor protein (NOS1AP) is overexpressed in the dorsolateral prefrontal cortex of patients with SCZ. NOS1AP negatively regulates NMDAR signaling and reduces dendrite branching *in vitro*, reproducing the abnormalities in dendrite branching observed in patients with SCZ. Here, we investigate 1) the effects of antipsychotics and NMDAR agonists on function and expression of NOS1AP, dopamine receptor D2 (D2), and disrupted in SCZ 1 (DISC1), and 2) the potential of NOS1AP as a biomarker for SCZ using human buccal cells to screen for NOS1AP expression. First, we treated cortical neurons with antipsychotics or NMDAR agonists (GLYX-13 and D-serine) for 24 hours and used Western blot analysis to determine the effects of these drugs on NOS1AP expression. Using Sholl analysis, we analyzed effects on dendrite branching in cortical rat neurons overexpressing NOS1AP. Additionally, we administered

haloperidol or D-serine to male and female Sprague Dawley rats via intraperitoneal injection for 12 days. We used Western blot analysis to determine the effects of treatment on cortical expression of NOS1AP, D2 receptor, and DISC1. Antipsychotics did not affect NOS1AP expression or dendrite branching *in vitro*, while GLYX-13 and D-serine reduced expression and corrected NOS1AP-mediated reductions in dendrite branching. Haloperidol significantly reduced D2 receptor expression in male, but not female, rats. Importantly, D-serine reduced NOS1AP expression in male, but not female, rats and had no effect on D2 receptor expression. D-serine also showed sex-specific effects on expression of disrupted in SCZ 1 (DISC1). To investigate the potential of NOS1AP as a biomarker for SCZ, we first collected buccal swabs from healthy control subjects. Using Western blot analysis, we confirmed that NOS1AP is consistently expressed in buccal cells, which had never before been investigated. We then assessed buccal cell NOS1AP expression in patients with SCZ and genotyped patients and healthy control subjects for single nucleotide polymorphisms (SNPs) in *NOS1AP*. Our data indicate that NOS1AP expression may be elevated in patients with SCZ who show rare disease-associated alleles in *NOS1AP*. Taken together, our data show for the first time that 1) D-serine influences the function and expression of NOS1AP, D2 receptor, and DISC1 in a sex-specific manner, and 2) buccal cell NOS1AP may be a biomarker for SCZ.

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Introduction

Potential of NMDAR agonists as treatments for SCZ

Schizophrenia (SCZ) is a debilitating psychiatric disorder that affects 1% of U.S. population [1]. Symptoms are categorized as positive (hallucinations and delusions), negative (withdrawal, loss of motivation, flat affect), and cognitive (deficits in attention, working memory, executive functioning) [2]. The dopamine hypothesis proposes that individuals with SCZ exhibit elevated dopamine signaling along the mesolimbic pathway [2-4]. Thus, current antipsychotic medications act as D2 dopamine (D2) receptor antagonists [5-8]. However, antipsychotics only alleviate positive symptoms and have minimal effect on negative and cognitive symptoms, suggesting that other signaling pathways may be involved [2]. The *N*-methyl D-aspartate receptor (NMDAR) hypothesis proposes that individuals with SCZ have reduced NMDAR functioning, leading to all symptom domains [9]. Evidence for this hypothesis comes from studies demonstrating that NMDAR antagonists, such as phencyclidine (PCP) and ketamine, reproduce all symptoms of SCZ in healthy individuals [10-13]. Furthermore, NMDARs are critical for synaptic plasticity, learning, and memory, which are impaired in SCZ [14-17], and NMDARs regulate dopamine signaling [4, 18-22]. NMDAR activation requires glutamate binding to the NR2 subunit of the NMDAR [23] and binding of a co-agonist, glycine or D-serine, to the glycine modulatory site (GMS) on the NR1 subunit [24]. Importantly, the GMS is not saturated *in vivo*, making it an ideal target for pharmacological manipulation to elevate NMDAR signaling [25]. Indeed, NMDAR co-agonists, such as D-serine, are being investigated in clinical trials as drug candidates for SCZ for treating all symptom domains [26-32].

Role of NOS1AP in SCZ

Nitric oxide synthase 1 adaptor protein (NOS1AP) is encoded by a SCZ susceptibility gene and negatively regulates NMDAR signaling [33-35]. NOS1AP was first identified in rat brain as a binding partner to nitric oxide synthase 1 (NOS1) [35]. Upon NMDAR activation, NOS1 is recruited to NMDAR by postsynaptic density 95 (PSD-95) [36]. However, NOS1AP competes with PSD-95 for binding to NOS1 and subsequently sequesters NOS1 from NMDAR, disrupting nitric oxide (NO) signaling, and thereby, NMDAR signaling [35, 37]. Furthermore, NOS1AP is elevated in the dorsolateral prefrontal cortex (DLPFC) of patients with SCZ [38]. We reported that three isoforms of NOS1AP - NOS1AP long (NOS1AP-L), short (NOS1AP-S), and short' (NOS1AP-S') - are upregulated in Brodmann's area 46 in postmortem tissue from patients [38]. Additionally, overexpression of NOS1AP-L and NOS1AP-S reduces dendrite branching [39] and alters dendritic spines *in vitro* [40], reproducing abnormalities present in patients with SCZ [41-43]. Moreover, NOS1AP-L mRNA is decreased in DLPFC of patients chronically treated with antipsychotic medications, suggesting that there may be a correlation between NOS1AP expression and antipsychotics [44]. However, it is unknown how investigational drugs, such as NMDAR agonists, affect NOS1AP expression.

Human buccal cells as a tool for biomarker discovery for SCZ

As of now, there are no reported biomarkers for SCZ to facilitate early diagnosis of the disorder. Collection of buccal cells is a non-invasive method for obtaining data regarding an individual's genetic make-up and protein expression

[45, 46]. Therefore, they represent an ideal tool for evaluating potential biomarkers of a disorder and the underlying factors that may correlate with an individual's drug response. Buccal cells have been investigated as a source of biomarkers for diseases such as cancer, Alzheimer's disease, and autism [47-50]. However, their potential application to SCZ has not been studied. This research has the potential to uncover easily accessible biomarkers for SCZ that may aid in early diagnosis and intervention. In particular, it is possible that NOS1AP may serve as a viable biomarker, as its expression is elevated in the DLPFC of patients with SCZ, and there is a correlation between NOS1AP expression in the DLPFC and presence of SNPs in *NOS1AP* [51]. However, this correlation has never been assessed using buccal cells.

Here, we 1) compare the effects of treatment with traditional antipsychotic medications with those of the NMDAR agonist, D-serine, on NOS1AP expression and function and expression of other proteins associated with SCZ, and 2) investigate the potential of buccal cell NOS1AP to serve as a biomarker for SCZ. We show that D-serine reduces NOS1AP in cultured rat cortical neurons and rescues NOS1AP-mediated reductions in dendrite branching while traditional antipsychotic medications have no effect. Furthermore, we show that haloperidol does not affect NOS1AP expression in the cortex of adult rats; however, it exerts a sex-dependent effect on D2 receptor expression. Haloperidol treatment reduces D2 receptor expression in male rats but has no effect in female rats. Conversely, D-serine treatment has no effect on D2 receptor expression in rat cortex; however, it reduces NOS1AP expression in the cortex of male, but not female, rats, suggesting a sex-based mechanism behind D-serine action. D-serine also exhibits sex-specific effects on expression of disrupted in schizophrenia 1 (DISC1), a protein

encoded by a SCZ susceptibility gene affecting glutamate signaling [52, 53]. Moreover, we demonstrate that NOS1AP is consistently expressed in human buccal cells, which has never been before studied, and that expression may be elevated in patients who exhibit rare disease-associated alleles in *NOS1AP*, suggesting that buccal cell NOS1AP may be an easy-to-acquire biomarker for SCZ. Taken together, these data provide critical insight into the sex-specific action of D-serine on NOS1AP and the clinical application of buccal cell NOS1AP as a biomarker for SCZ.

Methods

Antibodies

Primary antibodies for Western blot analysis and immunostaining are shown in **Appendix Table 1.**

Plasmids

pCAG-GFP was subcloned from EGFP of pEGFP-C1 (Clontech; Mountain View, CA) into vector with CMV–actin–β-globin promoter (pCAG). Human NOS1AP-L and NOS1AP-S cDNAs were subcloned into pCAG-GFP [39].

Primary cortical neuron cultures

Cortical neurons were isolated from embryonic rats at gestation day 18 as we described [54-56]. Neurons were plated at 2×10^5/well on 12-mm glass coverslips (Thermo Fisher) (dendrite analysis) or 35 mm dishes (analysis of NOS1AP expression) coated with poly-D-lysine.

Transfection, treatment, and immunostaining for dendrite analysis

Cortical neurons grown on glass coverslips were transfected at day *in vitro* (DIV) 6 using Lipofectamine LTX and Plus reagent (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. On DIV7, neurons were treated with 3.0 μg/ml clozapine, 0.25 μg/ml haloperidol, 8.4 μg/ml fluphenazine, 1 μM GLYX-13, or 10 μM D-serine (all Sigma-Aldrich, St. Louis, Missouri), or 0.1% DMSO vehicle. Following 24

hour, cells were immunostained with anti-MAP2 and chicken anti-GFP (both 1:200) and incubated with Hoechst dye for nuclear staining. Coverslips were mounted onto microscope slides with Fluoromount G (Southern Biotechnology; Birmingham, AL).

Assessment of dendrite number using Sholl analysis

Neurons were imaged at 200x on an EVOS FL Cell Imaging system. Semi-automated Sholl analysis was used to analyze dendrite branching as we described [57, 58]. Images were traced using the NeuronJ plugin [59] for ImageJ (NIH, Bethesda, MD). Axons were determined by the experimenter, who was blinded to the condition. Traces were converted to SWC files using MATLAB (Mathworks) and verified using NeuronStudio. Data were exported to Excel using MATLAB. Sholl curves were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test (Prism; Graphpad), and dendrite numbers were analyzed by one-way ANOVA followed by Dunn's multiple comparisons test.

Treatment and lysis of cultured cortical neurons

To analyze NOS1AP protein expression, at DIV21, neurons were treated for 24 hours with antipsychotics, NMDAR agonists, or vehicle. On DIV22, cells were washed with phosphate buffer saline (PBS) and lysed in ice cold TEE buffer (25 mM TrisHCl, 1 mM EDTA, 1 mM EGTA) containing 1 mM phenylmethylsulfonylfluoride (PMSF). Lysates were passed through a 25 ½ G needle five times, and Triton X-100 was added to final concentration of 1%. The lysates were incubated on ice for 30 minutes, vortexed

every 5 minutes, clarified by centrifugation at 12,000 × *g*, 4°C for 15 min, and stored at -20°C until Western blot analysis.

Animals

Adult male and female Sprague Dawley rats (Taconic, Hudson, NY) were single-housed with access to food and water *ad libitum* and maintained under 12-h light/dark cycle (on at 7:00 A.M.) at constant 22°C and relative humidity of 50%. Rats were injected with haloperidol (Henry Schein, Mylan Institutional LLC, Rockford IL, serial #7513033) diluted in sterile water at 1 mg/kg or vehicle (sterile water). Additional rats were injected with D-serine (Sigma Aldrich, S4250) diluted in sterile water at 200 mg/kg or vehicle (sterile water). All drugs were administered via intraperitoneal injection once/ day for 12 days. Animals were sacrificed on day 13 by CO_2 inhalation. Cortices were isolated, stored at -80°C, homogenized, lysed in TEE containing 1 mM PMSF and protease inhibitor cocktail (Sigma), and analyzed using Western blot analysis. All studies were reviewed and approved by Rutgers University Institutional Animal Care and Use Committee in accordance with the guidelines the National Institute of Health Laboratory Animals Resources Commission on Life Sciences' 1996 *Guide for the Care and Use of Laboratory Animals*.

Western blot analysis

Lysates from cultures and drug-treated rats were analyzed using Western blot analysis as we described [38-40]. Briefly, proteins were resolved on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to polyvinylidene difluoride membrane

in transfer buffer without SDS. Blots were incubated with NOS1AP primary antibody (1:500 in 3% bovine serine albumin (BSA) in buffer), and incubated overnight at 4°C. D2, DISC1, and GAPDH primary antibodies were used at 1:1000 in TBST (500mM tris-base, 60mM KCl and 2.8M NaCl, pH 7.4 with 0.1% tween) containing 3% BSA for 1 hour at room temperature. Membranes were developed using the enhanced chemiluminescence system (GE Healthcare; Piscataway, NJ) and Syngene G:BOX iChemi XR system and GeneSnap software (Version 7.09.a, Syngene, Frederick, MD). ImageJ was used for quantitation, and NOS1AP, D2, and DISC1 expression was normalized to GAPDH expression.

Analysis of NOS1AP mRNA using qRT-PCR

mRNA from cortex of rats injected with D-serine or vehicle-was extracted by homogenization in Trizol reagent (Life Technologies) following the manufacturer's instructions and stored at -80°C. mRNA (0.2 μg) from each sample was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Carlsbad, CA) following the manufacturer's protocol. The 2720 Thermal Cycler (Applied Biosystems) was set at the following: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, 4°C hold. cDNA was stored at -20°C until amplification by qPCR.

Each qPCR reaction was prepared according to the manufacturer's instructions for Taqman Fast Universal PCR Master Mix (Applied Biosystems). Briefly, each reaction included 10 μl Taqman Fast Universal PCR Master Mix (Applied Biosystems), 4 μl cDNA template, 5 μl RNase free water, and 1 μl either NOS1AP (FAM 20X

RN01490325, Thermo Fisher, catalog # 4351372) or β-actin (FAM 20X RN00667869, Thermo Fisher, catalog # 4331182) primer. All cDNA from the previous step was used to allow multiple technical replicates per sample. qPCR was performed using 7900HT real-time PCR system (Applied Biosystems) at the Department of Genetics, Rutgers University. DataAssist V3.01 was used to generate RQ (Fold Change), and p-values were computed using two-tailed Student's t-test comparing the $2^{(-\Delta CT)}$ values of the two groups. β-actin was internal control, and vehicle was a reference to calculate fold changes. The p-value was adjusted using Benjamini-Hochberg False Discovery Rate [60].

Subject Recruitment for buccal cell analysis:

Healthy control subjects for buccal cell studies were recruited from Rutgers University according to the following inclusion and exclusion criteria:

Inclusion Criteria:

1) Between the ages of 18-60,

2) Capable of not eating or drinking for a period of two hours,

3) Capable of providing consent.

Exclusion Criteria:

1) Diagnosis with a neurological or psychiatric disorder,

2) Unable to read or speak English,

3) History of seizures or epilepsy,

4) Currently taking antipsychotics or antidepressants.

Patients with SCZ were recruited from the Rutgers University Behavioral Health Care (UBHC) acute and extended partial hospitalization programs with the assistance of Dr. Steven Silverstein and UBHC staff members. As SCZ is more common in males, and the UBHC population is mostly male, we recruited only male patients for this study.

Patients were selected according to the following inclusion and exclusion criteria.

Inclusion Criteria:

1) SCZ diagnosis confirmed with the Structured Clinical Interview for the DSM-5 (SCID-5),

2) Between 18-60 years of age,

3) Male patients as defined by XY chromosome pair,

4) Capable of not eating or drinking for a period of two hours before buccal swab,

5) Capable of providing consent.

Exclusion Criteria:

1) Diagnosis with another psychiatric or neurological disorder, such as bipolar disorder or Tourette's syndrome,

2) Unable to read or speak English,

3) Currently inpatient,

4) History of seizures or epilepsy

Collection and lysis of buccal cells for NOS1AP protein analysis

Before buccal swabbing, all subjects were instructed to not eat or drink for a period of two hours to prevent contamination of the samples with food particles.

Buccal cells for protein analysis were collected using the Cytobrush Plus GT ® swab (Cooper Surgical), which was moved up and down on the inside of the subject's buccal mucosa for 60 seconds. The tip of the brush was placed in an eppendorf tube, containing 350 µL of lysis buffer (cold 25 mM TEE and 100 mM (DTT) with protease inhibitor cocktail and phosphatase inhibitors). The sample was incubated on ice for 30 minutes and then centrifuged at 14,000 x *g* at 4°C, for 10 minutes. The pellet was discarded, and the supernatant was stored at -80°C until Western blot analysis (see "Western Blot Analysis"). This protocol is adapted from Qian et al., 2015 [46].

Buccal swabbing of patients for DNA analysis

For DNA analysis, buccal cells were collected using the Whatman Omniswab ® (GE Healthcare). The tip of the swab brush was moved up and down on the inside of the patient's buccal mucosa for 15 seconds (as specified in the instructions provided by GE healthcare), and the tip of the brush was placed in 600 µL ice cold phosphate-buffered saline. All samples were stored at -80°C.

Detection of *NOS1AP* SNPs in DNA from patients with SCZ

All DNA samples collected were sent to EpigenDx for DNA extraction and genotyping. The SNPs and corresponding alleles specifically associated with SCZ are shown in **Table 1** and are based on previous genetic studies linking *NOS1AP* SNPs to SCZ.

Results

D-serine, but not antipsychotics, reduces NOS1AP expression and rescues NOS1AP-mediated decreases to dendrite branching *in vitro*

To investigate if traditional antipsychotics affect NOS1AP expression, we treated neurons (DIV21) with clozapine, haloperidol, fluphenazine, or DMSO (vehicle), all of which are commonly prescribed antipsychotics for treating SCZ. Using Western blot analysis, we detected NOS1AP-L, NOS1AP-S, and NOS1AP-S' (**Figure 1A-E**). Treatment with antipsychotics had no effect on NOS1AP isoform expression. Therefore, antipsychotics do not acutely affect NOS1AP protein levels *in vitro*, which are elevated in SCZ.

We then examined whether treatment with NMDAR agonists affects NOS1AP expression. We treated cortical neurons with GLYX-13, D-serine, or vehicle. We found that treatment with D-serine, a full agonist at the GMS on the NMDAR, but not GLYX-13, a partial agonist at the GMS, reduced NOS1AP expression (**Figure 1F-J**). Thus, antipsychotic treatment has no effect on NOS1AP expression *in vitro* while the full NMDAR agonist D-serine significantly reduces NOS1AP expression.

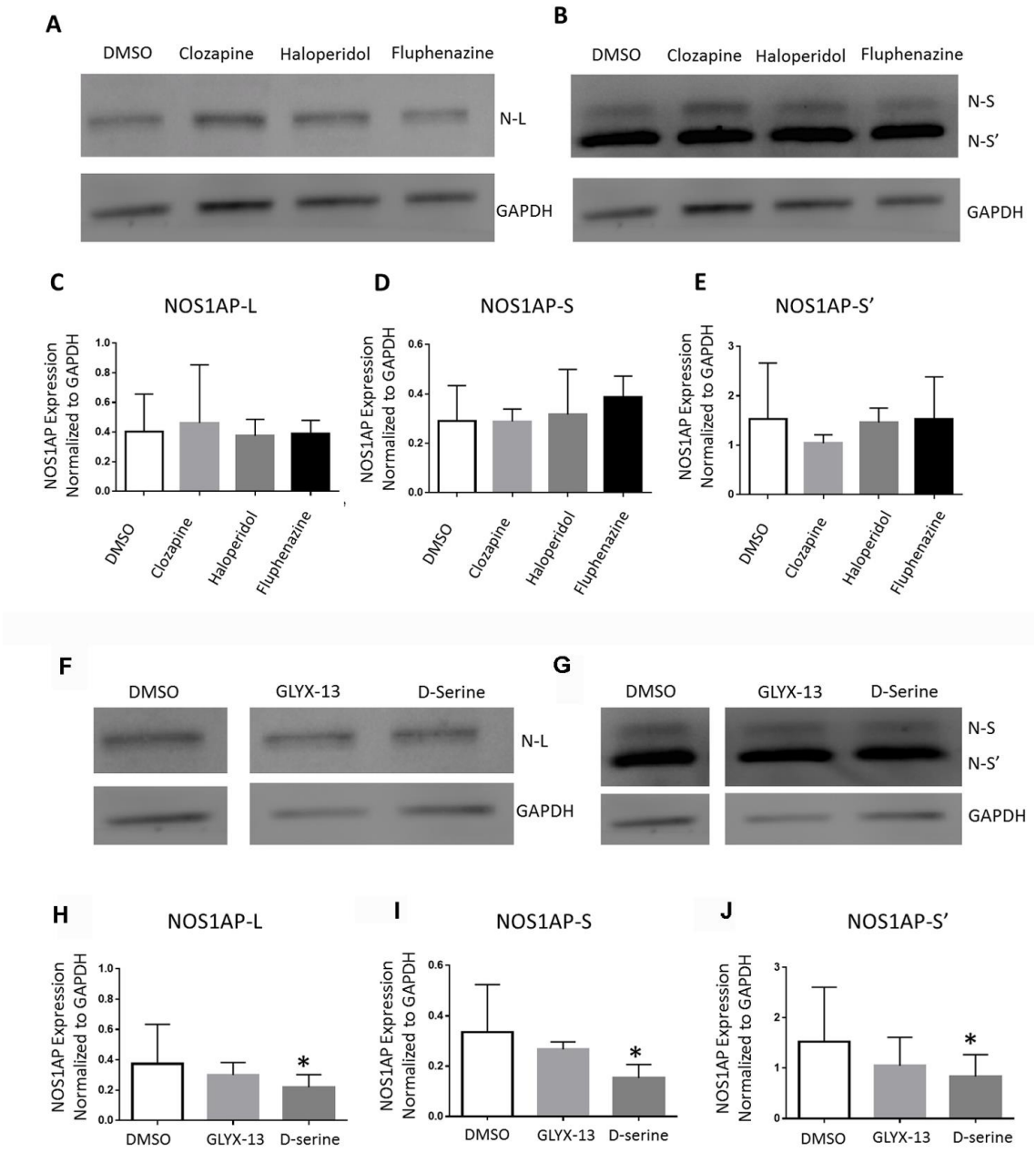

Figure 1. Treatment with D-serine, but not antipsychotics, reduces expression of NOS1AP isoforms in cortical neurons. A. Representative blots for NOS1AP-L (N-L) in cultured neurons treated with vehicle (DMSO), clozapine (3.0 μg/mL), haloperidol (0.25 μg/mL), and fluphenazine (8.4 μg/mL). **B**. Representative blots for NOS1AP-S (N-S) and NOS1AP-S' (N-S'). **C-E**, Quantitation of NOS1AP isoforms normalized to GAPDH expression. n=8 (N-L), n=8 (N-S), n=11 (N-S') independent cultures. No significant

differences detected. **F**. Representative blots showing detection of NOS1AP-L (N-L) in neurons treated with vehicle (DMSO), GLYX-13 (1 μM), and D-serine (10 μM). **G**. Representative blots for NOS1AP-S (N-S) and NOS1AP-S (N-S'). **H-J**, Quantitation of NOS1AP isoforms normalized to GAPDH expression following drug treatment. n=7 (N-L), n=9 (N-S), n=12 (N-S') independent cultures. *$p<0.05$ by one-way ANOVA followed by Tukey multiple comparisons test. Error bars represent SEM. Outliers excluded using Grubb's outlier test.

We next investigated whether treatment with these drugs functionally affects neuronal development. As NOS1AP overexpression reduces dendrites [39], we asked whether treatment with antipsychotics or NMDAR agonists rescues NOS1AP-induced reductions in dendrites. Unlike previously [39], we chose cortical, rather than hippocampal, neurons because D-serine reduces NOS1AP expression in cortical neurons, and NOS1AP is overexpressed in the DLPFC of patients with SCZ. Neurons were transfected at DIV6 with plasmids encoding GFP (control), NOS1AP-L, or NOS1AP-S. On DIV7, neurons were treated with antipsychotics or vehicle for 24 hours (**Figure 2**). Cells were fixed and immunostained for GFP and MAP2 on DIV8, a time point chosen within the period of active dendrite branching in our cultures [61]. Neurons overexpressing NOS1AP-L and treated with antipsychotics undergo significant amounts of cell death and could not be analyzed.

Treatment with haloperidol, but not clozapine or fluphenazine, decreased overall branching proximal to the soma (**Figure 2B, E, H**). No drug greatly affected the overall arbor with NOS1AP-S overexpression. However, clozapine decreased primary and secondary dendrites (**Figure 2C, D**), and fluphenazine reduced primary dendrites (**Figure 2I**) when NOS1AP-S was overexpressed. Haloperidol had no effect on primary or secondary dendrites (**Figure 2F, G**). Thus, antipsychotics do not rescue NOS1AP-mediated reductions in dendrite branching.

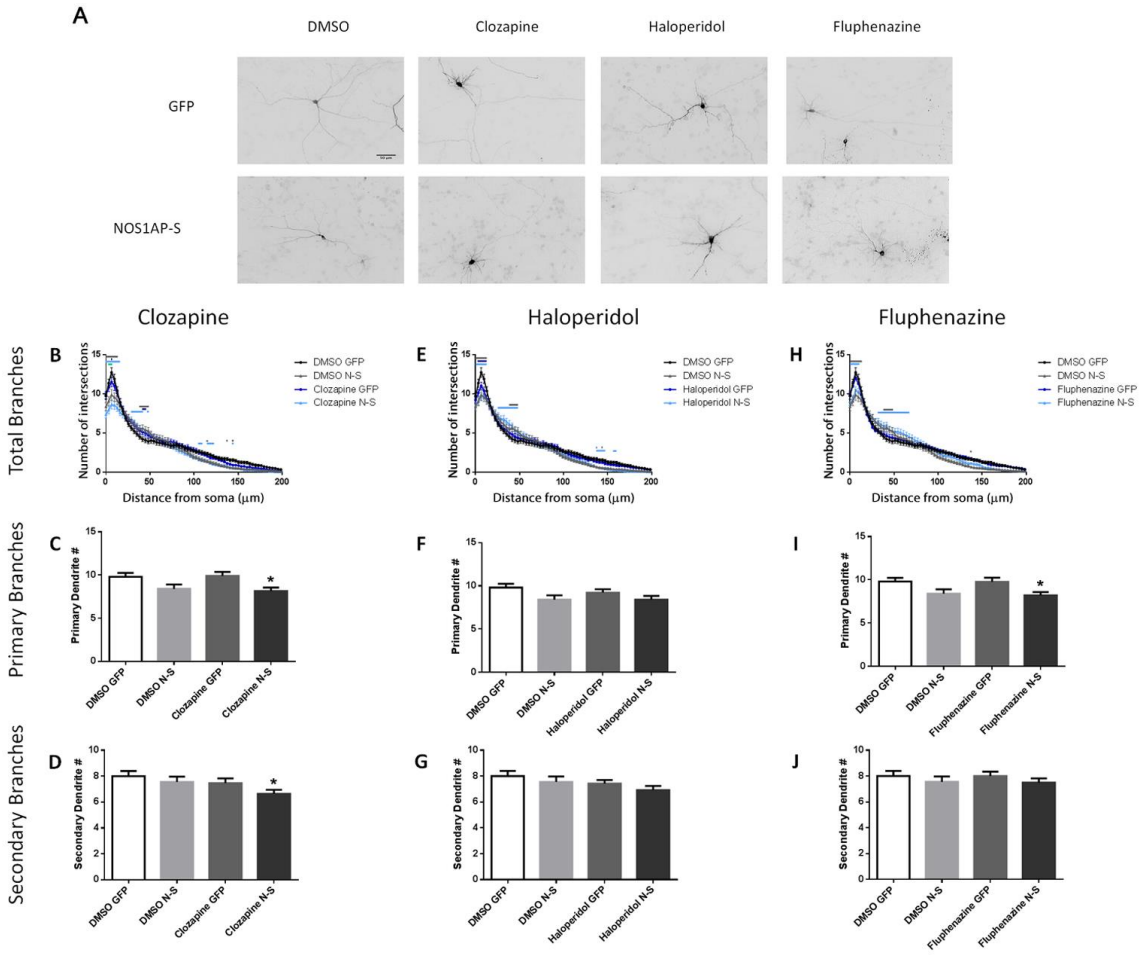

Figure 2. Effect of antipsychotic treatment on cortical neuron dendrites.

Clozapine, haloperidol, and fluphenazine do not reverse effects of NOS1AP-S (N-S) overexpression on dendrite branching. Colored significance bars (**Appendix Tables 2-4**) represent significance of at least $p<0.05$. **A.** Representative images from each treatment. **B**. Overexpression of N-S (DMSO) reduces proximal branching, but increases distal branching, and treatment with clozapine has no effect on overall dendritic arbor. **C.** Treatment of N-S overexpressing neurons with clozapine reduces primary dendrites and **D.** secondary dendrites. **E.** Treatment of N-S-overexpressing neurons with haloperidol does not affect dendrite branching, but haloperidol treatment of control (GFP) neurons

reduces proximal dendrites. **F, G.** Treatment of N-S-overexpressing neurons with haloperidol does not affect primary dendrites or secondary dendrites. **H.** Treatment of N-S-overexpressing neurons with fluphenazine does not affect dendrites versus neurons overexpressing N-S. **I, J.** Treatment of N-S-overexpressing neurons with fluphenazine reduces primary but not secondary dendrites. n = 54-79 total neurons analyzed from 4 independent cultures. Statistics by two-way ANOVA followed by Tukey multiple comparisons test. Error bars represent SEM. Scale bar = 50 μm.

We next determined the effects of NMDAR agonists in neurons overexpressing NOS1AP-S or NOS1AP-L (**Figure 3**). D-serine had no effect on branching in neurons overexpressing NOS1AP-S (**Figure 3H**), while treatment with GLYX-13 reduced proximal branching in neurons overexpressing NOS1AP-S (**Figure 3B**). However, treatment with either drug rescued dendrite branching with NOS1AP-L overexpression (**Figure 3E, K**). Specifically, GLYX-13 and D-serine rescued proximal dendrite branching in NOS1AP-L-overexpressing neurons. Therefore, unlike traditional antipsychotics, NMDAR agonists rescue NOS1AP-mediated reductions in dendrite branching.

We also analyzed the effects of NMDAR agonists on primary and secondary dendrites. Previously, we found that NOS1AP-L decreases both primary and secondary dendrites in hippocampal neurons [39]. Data here indicate that NOS1AP-L overexpression reduced only secondary dendrites in cortical neurons (**Figure 3F, L**). This could be due to different regulatory mechanisms and signaling pathways influencing neurite outgrowth in hippocampal and cortical neurons [62-64]. Importantly, GLYX-13 or D-serine partially restored secondary dendrites in NOS1AP-L-overexpressing neurons (**Figure 3G, M**). In sum, NMDAR agonists both reduce NOS1AP expression *in vitro* and have functional consequences on neuronal development.

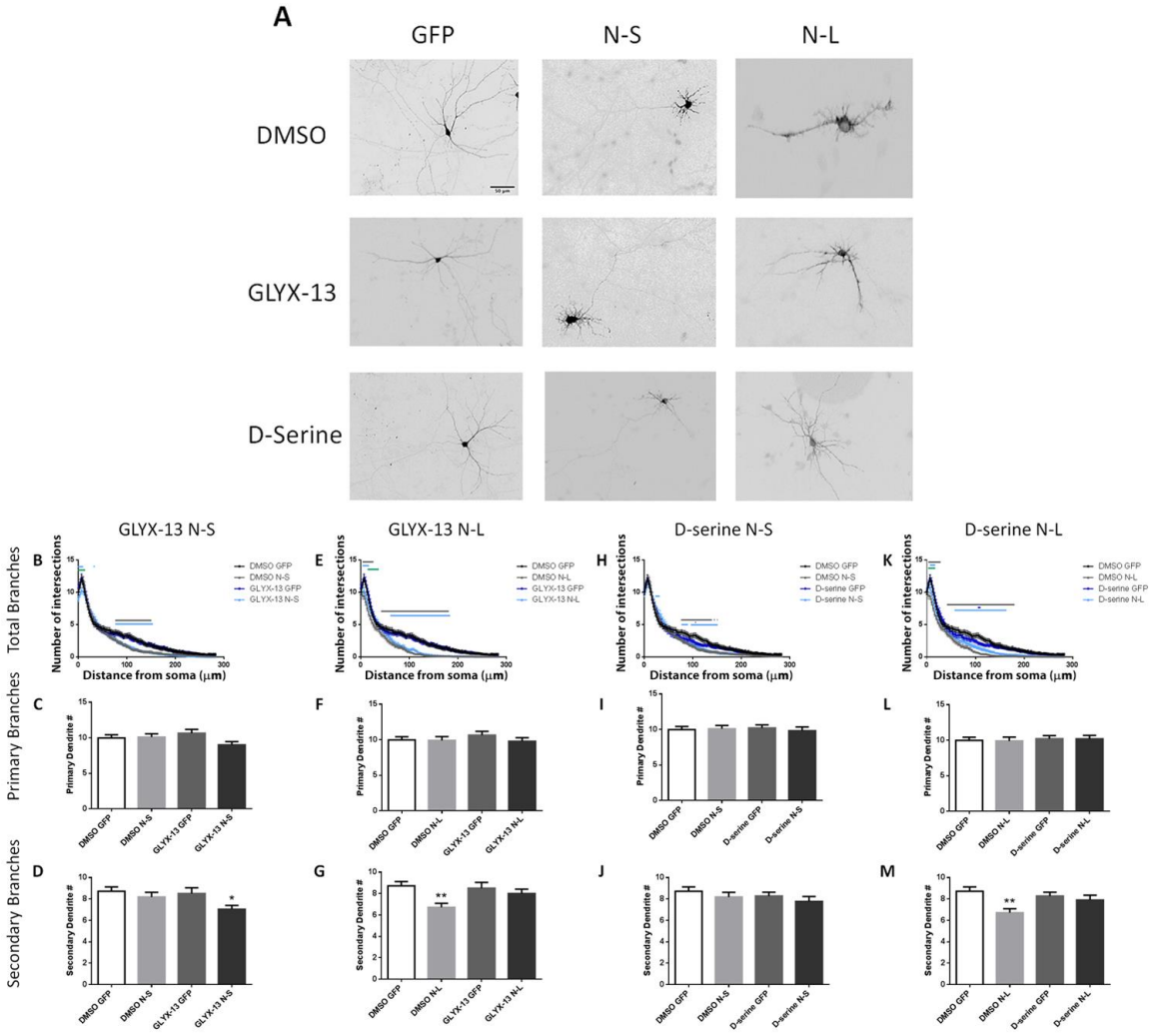

Figure 3. Treatment with D-Serine and GLYX-13 reverses NOS1AP-mediated reductions in dendrite branching. Neurons overexpressing NOS1AP-L (N-L) and NOS1AP-S (N-S) were treated with GLYX-13, D-serine, or DMSO vehicle. Colored significance bars (**Appendix Tables 5-8**) represent significance of at least $p<0.05$. **A.** Representative images from all treatments. **B.** Overexpression of N-S reduces distal branching, and treatment with GLYX-13 reduces proximal branching at five points (to 12.8 μm from soma). **C, D.** Treatment of N-S-overexpressing neurons with GLYX-13 does not affect primary but reduces secondary dendrites. **E.** Overexpression of N-L (DMSO) reduces dendrites, and treatment with GLYX-13 rescues proximal dendrites. **F.**

Treatment of N-L-overexpressing neurons with GLYX-13 does not affect primary dendrites. **G.** N-L overexpression reduces secondary dendrites, but treatment with GLYX-13 is not significantly different than control, indicating a partial effect of GLYX-13 on secondary dendrites with N-L overexpression. **H.** D-serine treatment of N-S-overexpressing neurons does not affect dendrite branching. **I, J.** Treatment of N-S-overexpressing-neurons with D-serine does not affect primary and secondary dendrites. **K.** Treatment of N-L-overexpressing neurons with D-serine rescues proximal dendrites. **L.** Treatment of N-L-overexpressing neurons with D-serine does not affect primary dendrites. **M.** Overexpression of N-L reduces secondary dendrites, but treatment of N-L-overexpressing neurons with D-serine is not different than control, indicating D-serine partially rescues N-L-mediated reductions in dendrite branching. n=56-64 total neurons per condition from 4 independent cultures. Statistics by two-way ANOVA followed by Tukey multiple comparisons test. Error bars represent SEM. Scale bar = 50 μm.

Haloperidol does not affect NOS1AP expression but has sex-specific effects on D2 receptor expression *in vivo*

Furthermore, we investigated whether antipsychotics and NMDAR agonists affect NOS1AP expression *in vivo*. We examined the effects of haloperidol treatment on NOS1AP-L expression in adult Sprague Dawley rats. We focused solely on expression of NOS1AP-L, as there is a correlation between antipsychotic medications and NOS1AP mRNA levels [44]. As *in vitro*, acute haloperidol treatment did not affect NOS1AP-L expression in male or female rats cortices (**Figure 4A, B**).

Since haloperidol acts as a D2 receptor antagonist, and patients with SCZ show altered dopamine receptor expression [7, 65], we examined the effect of haloperidol on D2 receptor expression. Interestingly, haloperidol administration resulted in sex-specific effect: haloperidol reduced D2 receptor expression in male, but not female, rat cortex (**Figure 4C, D**). Thus, acute treatment with haloperidol does not alter NOS1AP expression but has sex-specific effects on D2 receptor expression.

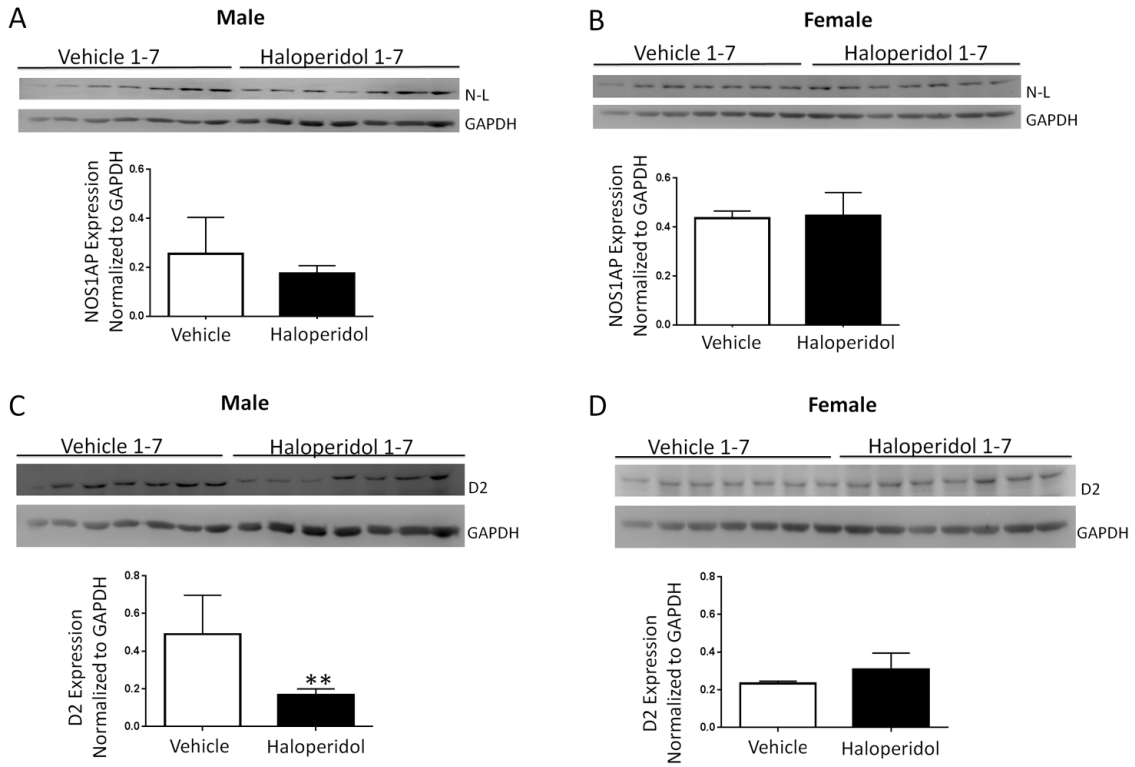

Figure 4. Haloperidol treatment affects D2 receptor expression in a sex-specific manner but does not affect NOS1AP-L expression in rat cortex. Haloperidol injection has no effect on NOS1AP-L (N-L) expression in cortices from male (**A**) and female (**B**) rats determined by two-tailed Student's t-test. Error bars represent SEM. n=7 animals per treatment group. Haloperidol injection significantly reduces cortical D2 receptor expression in male rats (**C**) but has no effect in female rats (**D**). **$p<0.01$ two-tailed Student's t test. Error bars represent SEM. n=7 animals per treatment group.

D-serine exerts sex-specific effects on NOS1AP and DISC1 but does not influence D2 receptor expression

We next investigated if D-serine affects NOS1AP expression *in vivo*. D-serine treatment significantly reduced NOS1AP-L expression in male, but not female, rat cortex (**Figure 5A, B**). This is relevant as SCZ occurs more frequently in males [66], and our data suggest that the effect of D-serine on NOS1AP expression is male-specific.

To investigate the mechanism by which D-serine affects NOS1AP expression, we performed qRT-PCR using cortices from rats. D-serine treatment did not affect NOS1AP-L mRNA levels in either male or female rat cortices (**Appendix Figure 1**), suggesting that D-serine regulates NOS1AP expression via transcriptional-independent mechanisms.

As haloperidol treatment significantly reduced expression of D2 receptor *in vivo*, we examined the effect of D-serine on D2 receptor expression. Unlike haloperidol, D-serine did not affect D2 receptor expression in male or female rats (**Figure 5C, D**), supporting the hypothesis that D-serine acts exclusively at the NMDAR [25].

Since SCZ is a heterogeneous disorder and linked to multiple risk genes, we investigated whether D-serine affects expression of DISC1, a protein encoded by a SCZ susceptibility gene [52]. DISC1 binds to and stabilizes serine racemase, the enzyme synthesizing D-serine, and mutant DISC1 leads to degradation of serine racemase and D-serine deficiency [67]. Moreover, DISC1 affects glutamatergic transmission [53, 68]. We observed that D-serine administration reduced DISC1 expression in male rats but increased DISC1 expression in female rats (**Figure 5E, F**). These data further underscore the sex-specificity of D-serine action.

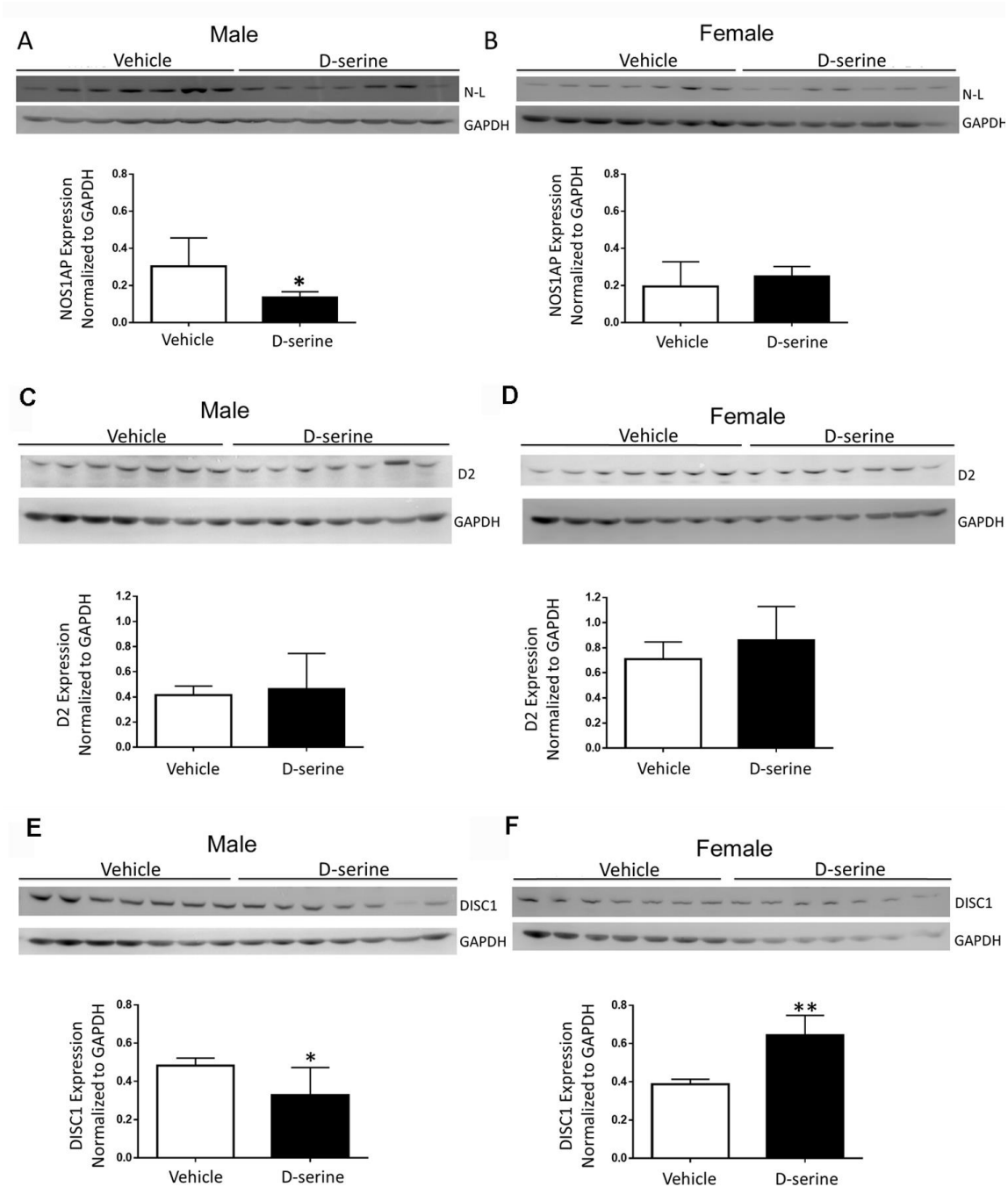

Figure 5. D-serine treatment reduces NOS1AP-L and DISC1, but not D2 receptor, expression in sex-specific manner in rat cortex. A. D-serine injection significantly reduces cortical NOS1AP-L (N-L) protein expression in male rats. **B.** D-serine injection has no effect on N-L expression in female rats. D-serine has no effect on expression of the dopamine D2 receptor in male (**C**) or female rats (**D**). D-serine reduces DISC1

expression in male (**E**) but increases DISC1 expression in female rat cortices (**F**). *p <0.05, **p<0.01 by two-tailed Student's t-test. Error bars represent SEM. n = 7 animals per treatment group.

NOS1AP is expressed in human buccal cells of healthy control subjects

We next investigated the potential of buccal cell NOS1AP expression as a biomarker for SCZ. We first confirmed that NOS1AP protein is expressed in buccal cells of healthy control subjects, as buccal cell NOS1AP expression had never been studied. Buccal cells were collected from 20 healthy individuals (males and females not diagnosed with a psychiatric or neurological disorder and not taking antipsychotics or antidepressants) using a buccal swab. The cells were lysed and analyzed using Western blot analysis. As shown in **Figure 6**, we detected expression of the NOS1AP-L isoform in all 20 individuals, comprised of 12 females and 8 males. Thus, our data show that NOS1AP protein is indeed expression in human buccal cells.

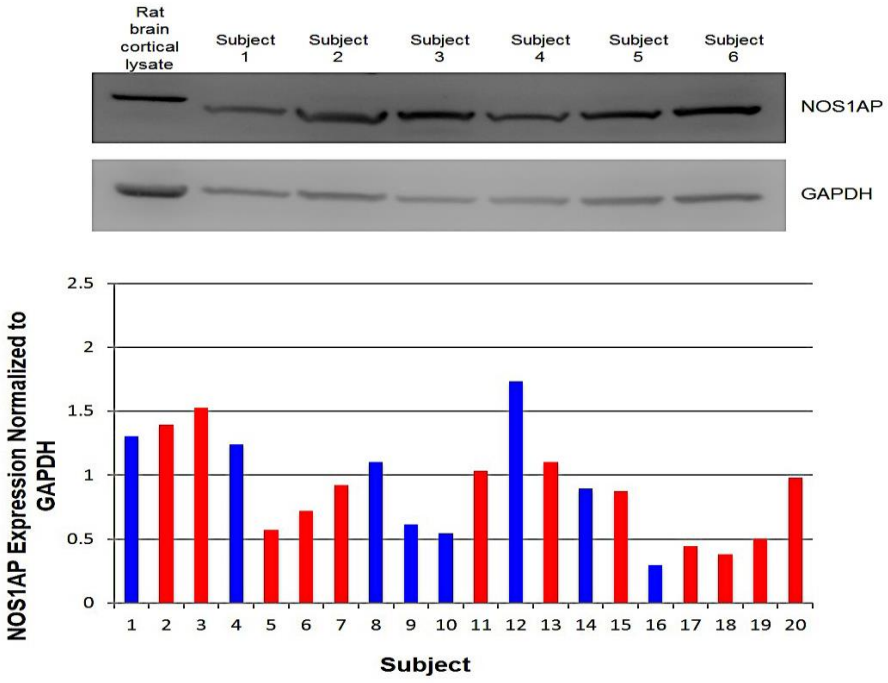

Figure 6. NOS1AP-L protein is expressed in buccal cells from healthy control subjects. Top panel. Representative Western blot of extracts from buccal cells from six control human subjects. Rat cortical lysate serves as a positive control. Immunoblot for

GAPDH serves as a protein loading control. Bottom panel. Quantitation of NOS1AP expression levels normalized to GAPDH levels for all 20 individuals (8 males, shown in blue, and 12 females, shown in red), demonstrating a normalized range of NOS1AP expression.

Buccal Cell NOS1AP expression may correlate to SNPs in *NOS1AP* in patients with SCZ

To determine the potential of buccal cell NOS1AP as a biomarker, we assessed buccal cell NOS1AP expression in 20 patients with SCZ to determine if NOS1AP is elevated in buccal cells of patients, as it is in the DLPFC, and furthermore, if NOS1AP protein expression correlates to SNPs in *NOS1AP*. All patients met SCID-5 criteria for diagnosis and were not formally diagnosed with another psychiatric or neurological disorder. We focused on male patients for this study, as SCZ is more common in men [66], and the majority of patients at UBHC are males. Quantitation from Western blot analysis revealed that one patient, identified as QF0, has elevated buccal cell NOS1AP expression (**Figure 7**). The range of NOS1AP expression values for patients with SCZ and control subjects is shown in **Figure 8**.

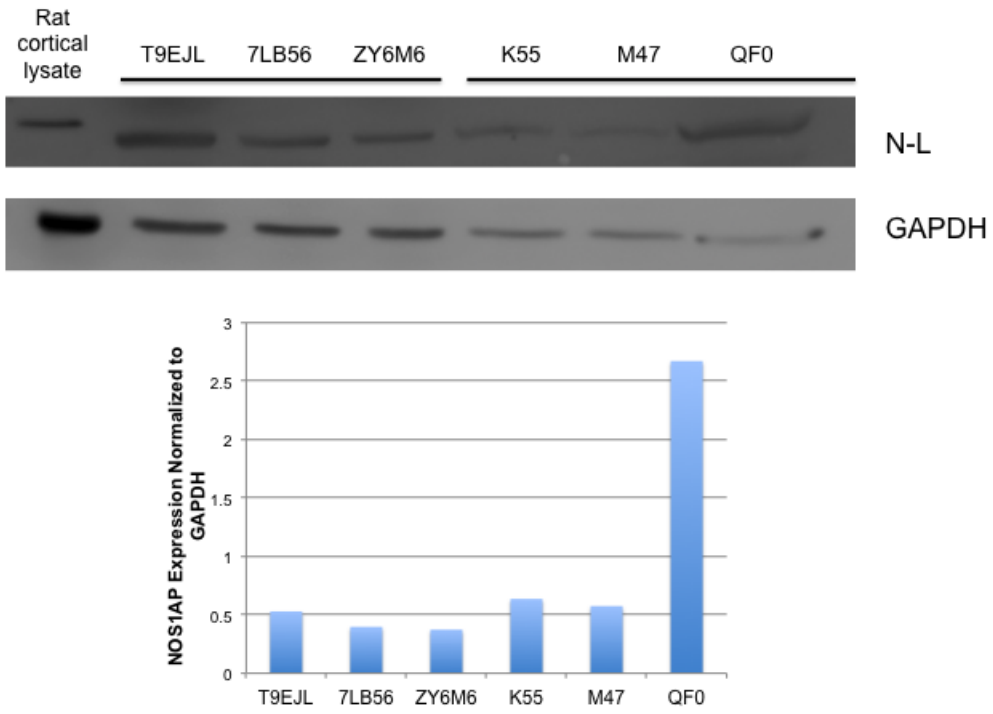

Figure 7. NOS1AP expression in buccal cells from patients with SCZ. Representative Western blot and quantification of NOS1AP levels in buccal cells from three each of control subjects (T9EJL, 7LB56, ZY6M6) and patients with SCZ (K55, M47, QF0). NOS1AP levels are normalized to GAPDH levels. As shown, buccal cells from patient QF0 have higher expression of NOS1AP than do control cells.

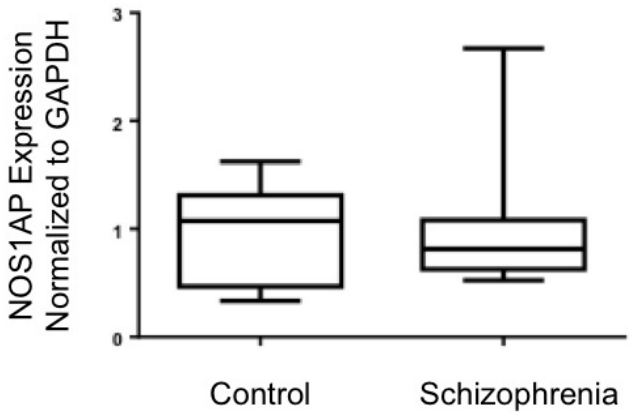

Figure 8. Range of NOS1AP-L expression in buccal cells from control subjects and patients with SCZ. Box and whisker plot of NOS1AP expression in buccal cell extracts from control subjects and patients with SCZ.

To determine if buccal cell NOS1AP protein expression correlates with *NOS1AP* SNPs, we genotyped patient QF0 and six patients with control levels of NOS1AP expression for SNPs in *NOS1AP* [33, 51, 69, 70], as shown in **Tables 1 and 2**. Data indicate that QF0 has rare disease-associated alleles in *NOS1AP* in rs1572495 and rs6680461 (**Table 2**), not observed in the other patients. To establish the frequency of these alleles in our population of healthy control subjects, we also collected DNA swabs from 7 healthy subjects and genotyped for the same 15 SNPs in *NOS1AP*. All control subjects were matched to races of patients as frequencies of alleles differs by race. Importantly, none of the healthy control subjects have rare variants for rs1572495 and rs6680461. Therefore, it is possible that rare disease-associated alleles in *NOS1AP* may correlate with elevated buccal cell NOS1AP expression, suggesting that buccal cell NOS1AP may serve as a viable marker for individuals with alterations in *NOS1AP*. However, future studies should be performed with a larger sample size to validate the consistency and reliability of this correlation.

SNP ID (rs#)	Reference allele in Caucasians	Frequency	Reference Allele in Asians	Frequency	Reference Allele in African Americans	Frequency	Disease-associated allele	Reference
rs1572495	A	0.07627118	A	0.14444445	G	0.34999999	A	[33]
rs1538018	C	0.27876106	C	0.14634146	C	0.2300885	G	Internal Source
rs945713	C	0.38333333	T	0.31111112	T	0.05833333	A	[33, 69]
rs1415263	T	0.38938054	T	0.41860464	C	0.47345132	T	[33, 69]
rs12725553	G	0.38839287	G	0.41666666	A	0.38938054	A	Internal Source
rs4411117	A	0.4314	T	0.36399999	T	0.3691	A	Internal Source
rs3924139	G	0.32456142	A	0.40079999	A	0.375	T	[69]
rs4592244	G	0.39860001	A	0.40079999	A	0.3351	A	[69]
rs4145621	C	0.44067797	T	0.26666668	T	0.05	T	[69]
rs12742393	A	0.39823008	C	0.27906978	C	0.16371681	A	[51]
rs6680461	G	0.13559322	T	0.5	G	0.33050847	G	[69]
rs4657181	A	0.4761	T	0.29959998	T	0.0371	T	[69]
rs2661818	G	0.493	C	0.3211	C	0.0809	G	Internal Source
rs3751284	A	0.3549	A	0.48569998	A	0.28290001	T	[69]
rs348624	T	0.10267857	T	0.08536585	T	0.42857143	G	[70]

Table 1. Fifteen tested SNPs in *NOS1AP*. We assessed the presence of 15 SNPs in *NOS1AP* that have been linked to SCZ. Reference alleles are derived from HapMap database according to race: HapMap CEU (Western European/Caucasian), HapMap HCB (Asian), HapMap YRI (African) and the frequency of the rare allele by race

(frequency). Alleles associated with SCZ are indicated by "Disease-associated allele" and based on the literature referenced.

	Patient ID: QF0	Patient ID: HYX	Patient ID: WWS	Patient ID: YPB	Patient ID: XOM	Patient ID: GT1	Patient ID: UZN
	Caucasian	Caucasian	Caucasian	Asian	Asian	African American	African American
SNP ID	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype
rs1572495	G/A	G/G	G/G	G/G	G/G	A/A	G/G
rs1538018	G/G	G/C	G/C	G/G	G/C	G/G	G/C
rs945713	G/G	G/A	A/A	A/A	A/A	G/G	G/A
rs1415263	T/T	C/C	C/C	C/C	C/C	T/T	T/T
rs12725553	G/G	A/A	A/A	A/A	A/A	G/G	G/G
rs4411117	T/A	T/T	T/T	T/T	T/T	A/A	A/A
rs3924139	C/T	T/T	T/T	T/T	T/T	C/C	C/C
rs4592244	G/A	A/A	A/A	A/A	A/A	G/G	G/G
rs4145621	C/C	C/T	T/T	C/T	T/T	C/C	C/C
rs12742393	A/A	C/C	C/C	A/C	C/C	A/A	A/A
rs6680461	T/G	T/T	T/T	T/T	T/T	T/T	T/T
rs4657181	A/A	A/T	T/T	A/T	A/T	A/A	A/A
rs2661818	C/C	C/G	G/G	G/G	C/G	C/C	C/C
rs3751284	C/C	C/C	T/T	C/T	C/T	C/T	C/T
rs348624	C/C	C/C	C/C	C/C	C/C	C/T	C/C

Table 2. Genotypes of seven patients with SCZ. DNA extracted from buccal cells of patients was genotyped for 15 SNPs in *NOS1AP*. Blue text indicates the presence of at least one disease-associated allele (see **Table 1**) for that locus. Red text indicates presence of at least one disease-associated allele that is rare for the patient's race, defined here as frequency less than 0.15 (see **Table 1**). Patient QF0, who shows elevated NOS1AP protein in buccal cells (**Figure 7**), exhibits two rare disease-associated alleles (rs1572495 and rs6680461) not observed in the other patients.

	Subject ID: 7LB56	Subject ID: 00MBQ	Subject ID: 78295	Subject ID: T9EJL	Subject ID: 62678	Subject ID: 50928	Subject ID: 13543
	Caucasian	Caucasian	Caucasian	Asian	Asian	African American	African American
SNP ID (rs#)	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype	Genotype
rs1572495	G/G	G/G	G/G	G/G	G/G	G/A	G/A
rs1538018	G/C	G/C	G/G	G/C	G/G	C/C	G/G
rs945713	G/A	G/A	G/G	G/G	G/G	G/G	G/G
rs1415263	C/T	C/T	C/C	C/T	T/T	C/T	T/T
rs12725553	G/A	G/A	A/A	G/A	G/G	G/A	G/G
rs4411117	T/A	T/A	T/T	T/A	A/A	A/A	A/A
rs3924139	C/T	C/T	T/T	C/T	C/C	C/T	C/C
rs4592244	G/A	G/A	A/A	G/A	G/G	G/A	G/G
rs4145621	T/T	C/T	C/T	C/T	C/C	C/C	C/C
rs12742393	C/C	A/C	C/C	A/C	A/A	A/C	A/A
rs6680461	T/T	T/T	T/T	T/G	T/G	T/T	T/T
rs4657181	T/T	A/T	A/A	A/A	A/A	A/A	A/A
rs2661818	G/G	C/G	C/C	C/C	C/C	C/C	C/C
rs3751284	C/T	C/T	C/T	C/T	C/C	C/C	C/C
rs348624	C/C	C/T	C/C	C/C	C/C	C/T	C/T

Table 3. Genotypes of seven healthy control subjects. DNA extracted from buccal cells of seven healthy control subjects was genotyped for 15 SNPs. Blue text indicates presence of at least one disease-associated allele (see **Table 1**) for that locus. Red

text indicates presence of at least one disease-associated allele that is rare, defined here as frequency less than 0.15 (see **Table 1**). None of the seven healthy control subjects show rare disease-associated alleles for rs1572495 or rs6680461.

Discussion

Here, we provide evidence that 1) D-serine affects expression and function of NOS1AP, a protein encoded by a SCZ risk gene [33], and 2) buccal cell NOS1AP may serve as a biomarker for SCZ. We first investigated the acute effects of traditional antipsychotics and NMDAR agonists on NOS1AP in cortical rat neurons and cortices of treated rats. Our study links D-serine action to NOS1AP expression and function, providing evidence that D-serine reduces NOS1AP expression and reverses NOS1AP-mediated reductions in dendrite branching, while antipsychotics have no effect. Additionally, D-serine affects NOS1AP expression only in male rats, suggesting that this effect is sex-specific. Furthermore, we performed buccal cell studies to examine if NOS1AP protein is elevated in buccal cells of patients with SCZ compared to healthy control subjects, as in the DLPFC. Data indicate that buccal cell NOS1AP expression may be elevated in patients with particular SNPs in *NOS1AP*, suggesting buccal cell NOS1AP protein levels may be a biomarker for SCZ.

Effects of antipsychotics and NMDAR agonists on NOS1AP

Antipsychotic medications target dopamine receptors and are unable to treat all symptom domains of SCZ for the majority of patients [2]. As there is a correlation between chronic antipsychotic treatment and reduced NOS1AP mRNA levels in postmortem cortical tissue, we investigated if they reduce NOS1AP protein in primary cortical neurons and rat cortex. We found that antipsychotic medications do not affect NOS1AP expression *in vitro*, and acute treatment with haloperidol does not affect NOS1AP expression *in vivo*. Additionally, antipsychotic medications do not affect

dendrite branching *in vitro*. Thus, commonly prescribed antipsychotic medications do not affect NOS1AP protein levels or dendrite branching.

Due to the failure of antipsychotic medications to address all symptoms of SCZ, NMDAR agonists have been investigated as potential treatment options [71]. The NMDAR hypothesis of SCZ proposes that individuals with SCZ exhibit reduced NMDAR signaling [4, 9], which is important for perception, learning, memory, and synaptic plasticity [14-17, 72]. NOS1AP is proposed to play a role in NMDAR signaling by interacting with NOS1 [35]. Overexpression of NOS1AP, as in SCZ, interferes with NO signaling by sequestering NOS1, reducing NMDAR functioning [34, 37]. In this study, we find that D-serine, a full co-agonist at the NMDAR, reduces NOS1AP expression *in vitro* and in male rat cortex and corrects NOS1AP-induced reductions in dendrite branching. GLYX-13, partial agonist at the GMS [73, 74], does not affect NOS1AP expression *in vitro* but reverses NOS1AP-L-mediated alterations in dendrite branching. Importantly, both GLYX-13 [75] and D-serine [27-32] have been in clinical trials for depression and SCZ, respectively. Our data suggest the therapeutic benefit of D-serine may be due to two independent effects: increased NMDAR signaling and reduced NOS1AP expression. Taken together, these data shed light on the ineffectiveness of antipsychotics and further support the therapeutic potential of D-serine as a treatment option for SCZ.

Sex-specific actions of haloperidol and D-serine

Our results suggest that the effects of haloperidol and D-serine on expression of NOS1AP, D2 receptor, and DISC1 are sex-specific. Although treatment with haloperidol

does not affect NOS1AP expression, it reduces D2 receptor expression in the cortex of male, but not female, rats. Similarly, D-serine reduces NOS1AP expression only in male rats and shows opposing effects on DISC1 expression in male and female rats, suggesting its therapeutic effect may be restricted to males with SCZ. Thus, our data show, for the first time that haloperidol and D-serine influence proteins associated with SCZ through sex-specific pathways.

Research regarding sex differences in SCZ pathophysiology and pharmacology is lacking. However, SCZ is more prevalent in men [66], and men have earlier age-at-onset than do females [76]. Additionally, there is evidence that estradiol affects dopamine and may be neuroprotective in females [77], and that antipsychotics may be more efficacious in women [78-82]. Our data show that haloperidol has sex-specific effects on expression of D2 receptor, the receptor to which most antipsychotics bind. Updated versions of the dopamine hypothesis propose that positive symptoms of SCZ are due to elevated dopamine release along the mesolimbic pathway, while negative and cognitive symptoms are the consequence of reduced dopamine release in the mesocortical pathway [2]. Therefore, a reduction in cortical D2 receptor expression, as observed in male rats, may further reduce dopamine release along the mesocortical pathway, and consequently, exacerbate negative and cognitive symptoms of SCZ. Our results underscore that sex is a critical factor to consider when evaluating treatment for a patient with SCZ. While D-serine may have a therapeutic effect in males by reducing NOS1AP expression, haloperidol may be more efficacious in females since cortical D2 receptor expression is not reduced.

Nonetheless, it would be beneficial to examine the effect of D-serine and haloperidol on NOS1AP, D2 receptor, and DISC1 expression throughout different phases of the estrous cycle, as the estrous cycles in the female rats used in the present study were not synchronized. Previous research suggests that estrogen fluctuations throughout the estrous cycle may influence expression of various synaptic proteins *in vivo* [83-85]. Therefore, it is possible that hormones, such as estrogen, may play a role in regulating response to treatment and resulting changes to expression of NOS1AP, DISC1, and D2 receptor in female rats

Buccal cell NOS1AP may be a biomarker for SCZ

There are currently no validated biomarkers for SCZ. Rather, diagnostic strategies rely on self-report and interview-based assessments, which can be inaccurate and lead to misdiagnosis [86]. Validated biomarkers would facilitate early diagnosis and ultimately support better clinical outcome [86]. Here, we investigated the potential of buccal cells as a source of biomarkers for SCZ. Previous studies have examined blood and cerebrospinal fluids as sources of biomarkers for SCZ [87, 88]; however, results have been inconsistent across studies, and collection of these fluids can be laborious, time consuming, and uncomfortable for patients [89]. Thus, we chose to investigate expression of NOS1AP in buccal cells, which are an easily accessible source for biomarker discovery. We show for the first time that NOS1AP protein is consistently expressed in buccal cells. Furthermore, we find that a patient with SCZ with elevated buccal cell NOS1AP exhibits rare disease-associated variants in *NOS1AP*. This suggests that there may be a correlation between particular disease-associated alleles in *NOS1AP* and buccal cell NOS1AP protein

expression. Therefore, buccal cell NOS1AP may serve as a viable biomarker for SCZ. However, a larger sample size is necessary to determine the validity of this correlation.

Importantly, D-serine has been investigated as a potential adjunctive treatment for SCZ, especially in terms of its ability to alleviate the cognitive symptoms of SCZ [29, 32]. However, previous clinical trials have yielded mixed results, and it is unknown why D-serine may improve symptoms in some individuals but not in others [26, 29, 32]. Patients with SCZ are heterogeneous in regards to many factors, including genetic susceptibility, premorbid functioning, speed of onset, symptom profile, cognitive impairments, treatment response, and long-term illness course [90-93]. It is possible that a patient's individual genetic profile and alterations in NOS1AP, which we show to be a target of D-serine, may be a factor in determining a patient's response to D-serine treatment. Therefore, future experiments should examine whether D-serine treatment in patients reduces NOS1AP in buccal cells, as it does *in vitro* and *in vivo*, and if changes to NOS1AP expression and/or SNPs in *NOS1AP* correlate to cognitive improvement. If so, this would indicate that buccal cell NOS1AP is a predictor of treatment response and that D-serine exerts its therapeutic effects specifically in individuals with alterations in NOS1AP, thus having the potential to accelerate development of a personalized medicine approach for treatment of SCZ.

Taken together, our results provide critical insight into the effects of haloperidol and D-serine on NOS1AP, D2 receptor, and DISC1. Our data emphasize that drug action is sex-dependent and provide a molecular basis for observed sex differences in drug efficacy in patients with SCZ. Furthermore, our results suggest that buccal cell NOS1AP

may be easy-to-acquire biomarker for SCZ, having the potential to significantly improve current diagnostic strategies.

APPENDIX

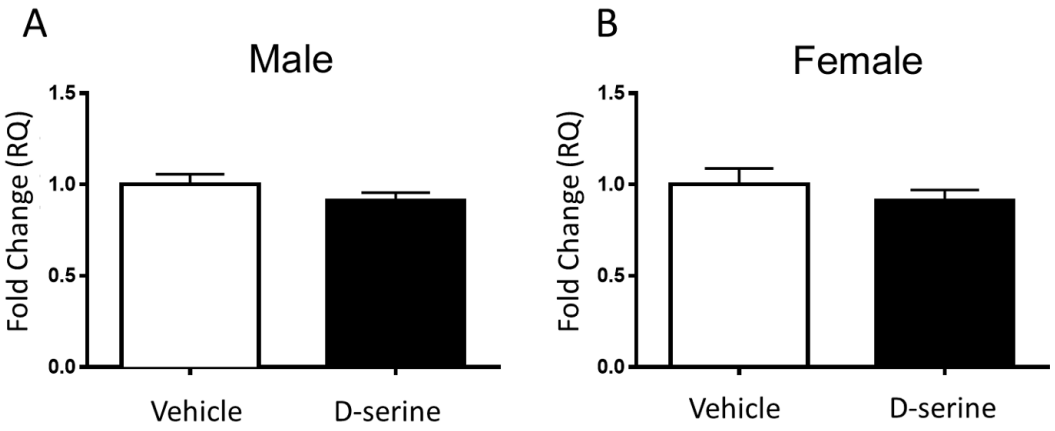

Figure 1. *In vivo* D-serine treatment does not affect NOS1AP-L mRNA expression. DataAssist V3.01 was used to generate RQ (Fold Change) and p-values were computed using two-tailed Student's t-test comparing the $2^{(-\Delta CT)}$ values of the two groups. The p-value was adjusted using Benjamini-Hochberg False Discovery Rate. n=7 animals per group and each had 3-4 technical replicates. Endogenous control = β-actin. Reference group = Vehicle. No significance detected. Error bars = SEM.

Table 1. Antibodies used in this study

Antibody	Catalogue number	Company	Use
rabbit polyclonal anti-NOS1AP	ab90854	Abcam (Cambridge, MA)	Western blotting
rabbit polyclonal anti-D2	ab5084P	EMD Millipore (Billerica, MA)	Western blotting
mouse monoclonal anti-GAPDH	MAB374	EMD Millipore	Western blotting
rabbit monoclonal anti-DISC1	ab192258	Abcam	Western blotting
monoclonal anti-rabbit IgG (γ-chain specific)-peroxidase	A1949	Sigma-Aldrich (St. Louis, MO)	Western blotting
polyclonal anti-mouse IgG (H&L) peroxidase	610-1319	Rockland Immunochemicals (Limerick, PA)	Western blotting
polyclonal anti-rabbit IgG (H&L) peroxidase	611-1102	Rockland Immunochemicals	Western blotting

chicken anti-Green Fluorescent Protein (GFP)	PA1-9533	Thermo Fisher Scientific	Immunostaining
mouse anti-microtubule-associated protein 2 (MAP2)	556320	BD Biosciences (San Jose, CA)	Immunostaining
anti-chicken IgG (H+L) conjugated to Alexa Fluor® 488	A11039	Life Technologies (Grand Island, NY)	Immunostaining
anti-mouse IgG (H+L) conjugated to Alexa Fluor® 555	A21424	Thermo Fisher Scientific	Immunostaining

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-S
Dark blue	DMSO GFP vs. Clozapine GFP
Light blue	DMSO GFP vs. Clozapine N-S
Green	DMSO N-S vs. Clozapine N-S

Table 2. Bar colors representing a significance of at least p<0.05 for Figure 2B.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-S
Dark blue	DMSO GFP vs. Haloperidol GFP
Light blue	DMSO GFP vs. Haloperidol N-S

Table 3. Bar colors representing a significance of at least p<0.05 for Figure 2E.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-S
Light blue	DMSO GFP vs. Fluphenazine N-S

Table 4. Bar colors representing a significance of at least p<0.05 for Figure 2H.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-S
Light blue	DMSO GFP vs. GLYX-13 N-S
Green	DMSO N-S vs. GLYX-13 N-S

Table 5. Bar colors representing a significance of at least p<0.05 for Figure 3B.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-L
Light blue	DMSO GFP vs. GLYX-13 N-L
Green	DMSO N-L vs. GLYX-13 N-L

Table 6. Bar colors representing a significance of at least p<0.05 for Figure 3E.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-S
Dark blue	DMSO GFP vs. D-serine GFP
Light blue	DMSO GFP vs. D-serine N-S

Table 7. Bar colors representing a significance of at least p<0.05 for Figure 3H.

Bar color	Comparison
Gray	DMSO GFP vs. DMSO N-L
Dark blue	DMSO GFP vs. D-serine GFP
Light blue	DMSO GFP vs. D-serine N-L
Green	DMSO N-L vs. D-serine N-L

Table 8. Bar colors representing a significance of at least p<0.05 for Figure 3K.

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