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Stem cell derived interneuron transplants as a treatment for schizophrenia: preclinical validation in a rodent model

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Abstract

An increasing literature suggests that schizophrenia is associated with a reduction in hippocampal interneuron function. Thus, we posit that stem cell-derived interneuron transplants may be an effective therapeutic strategy to reduce hippocampal hyperactivity and attenuate behavioral deficits in schizophrenia. Here we used a dual-reporter embryonic stem cell line to generate enriched populations of parvalbumin (PV)- or somatostatin (SST)-positive interneurons, which were transplanted into the ventral hippocampus of the methylazoxymethanol (MAM) rodent model of schizophrenia. These interneuron transplants integrate within the existing circuitry, reduce hippocampal hyperactivity, and normalize aberrant dopamine neuron activity. Further, interneuron transplants alleviate behaviors that model negative and cognitive symptoms, including deficits in social interaction and cognitive inflexibility. Interestingly, PV- and SST-enriched transplants produced differential effects on behavior, with PV-enriched populations effectively normalizing all the behaviors examined. These data suggest that stem cell-derived interneuron transplants may represent a novel therapeutic strategy for schizophrenia.

INTRODUCTION

Schizophrenia is a devastating psychiatric disorder characterized by positive, negative, and cognitive symptoms. One of the longest-standing hypotheses of schizophrenia posits that

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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excessive subcortical dopamine release is responsible for the positive symptoms of the disorder. Support for this hypothesis stems from the fact that all antipsychotics are D2 receptor antagonists [1] and can reduce positive symptoms [2]. Unfortunately, these drugs are not effective in all patients, possess debilitating side effects, and have little to no impact on the negative or cognitive symptom domains [2]. Currently, there are no FDA-approved medications that specifically target the negative and cognitive symptoms of schizophrenia, which are associated with a dramatically reduced quality of life [3]. Negative symptoms, such as blunted affect and social avoidance, and cognitive symptoms, like deficits in attention and executive function, can be severely debilitating[3, 4]. Even among schizophrenia patients that respond to antipsychotic medications, disability rates remain high and functional outcomes poor, highlighting the urgent need to treat these residual negative and cognitive symptoms of schizophrenia [3]. Thus, new approaches targeting all three symptom domains are urgently needed.

Although the dopamine hypothesis has received wide support, there has been little observable primary pathology identified in the dopamine system of schizophrenia patients. This has led to the suggestion that the pathology may lie upstream in brain regions that regulate the dopamine system, such as the ventral hippocampus (vHipp) [5]. The vHipp not only regulates the activity of the dopamine system [6], but also projects directly to the prefrontal cortex [7], making this a reasonable therapeutic target not only for the positive symptoms associated with increased dopamine system function, but also for the negative and cognitive symptoms that rely on prefrontal cortical function [8, 9]. Hippocampal pathology is routinely observed in schizophrenia patients with an increased activity at rest [10] being correlated with both psychosis [11] and cognitive dysfunction [12]. This hyperactivity has been attributed to a deficit of inhibitory interneuron function [13]. Indeed, some schizophrenia patients display reductions in hippocampal interneurons that express the Ca^{2+}-binding protein, parvalbumin (PV), and those that express the neuromodulator, somatostatin (SST) [14, 15]. Recently, we demonstrated that a loss of PV expression is sufficient, in and of itself, to increase vHipp activity and produce downstream alterations in dopamine system function and schizophrenia-like changes in behavior [16]. Therefore, we hypothesize that a loss of interneuron function leads to an increase in hippocampal activity and aberrant dopamine system function. Restoring inhibitory interneuron function in the vHipp may normalize both hippocampal and dopamine neuron activity, resulting in a resolution of the positive, negative and cognitive symptoms of schizophrenia.

We have recently tested this hypothesis using interneuron transplants derived from rat fetal tissue [17]. During development, the majority of PV- and SST-positive interneurons are derived from the telencephalic structure known as the medial ganglionic eminence (MGE)[18]. Interneuron precursors born in the MGE have the remarkable ability to migrate tangentially across cortical regions [19]. Therefore, we used fetal MGE tissue to restore interneuron function in the methylazoxymethanol (MAM) developmental model of schizophrenia [17]. We found that interneuron transplants into the vHipp reduced hippocampal hyperactivity and dopamine population activity, and normalized amphetamine-induced locomotor activity, a model for the positive symptoms of schizophrenia. A subsequent paper showed similar effects of interneuron transplants on hippocampal hyperactivity and psychosis-related behaviors in a transgenic mouse model with reduced PV-positive interneurons in the

hippocampus [20]. While these results are exciting, this approach is not feasible in patients due to the source of the interneurons (fetal brain). Further, these studies did not examine negative and cognitive symptoms of schizophrenia, which are poorly treated by antipsychotics, and did not differentiate between interneuron subclasses. This is important since the distinct axon targeting and firing properties of interneuron subclasses is likely to differentially affect local activity after transplantation[13]. Moreover, different interneuron subtypes display discrete neurophysiological characteristics that may alter their therapeutic utility[13]. Thus, to address these issues in the current studies, we used embryonic stem cells that were differentiated into enriched populations of distinct interneuron subgroups [21]. Here we explore the role of specific interneuron subclasses in treating neurophysiological and behavioral alterations in the MAM model of schizophrenia and expand our recent findings to determine if vHipp interneuron transplants can also alleviate correlates of the negative and cognitive symptoms associated with this disorder.

MATERIALS AND METHODS

Drugs and Reagents

DNQX, picrotoxin, MK-801, kynurenic acid, guanosine 5′ triphosphate sodium salt hydrate (GTP), adenosine 5′ triphosphate magnesium salt (ATP), EGTA, Pentobarbital, and chloryl hydrate were purchased from Sigma-Aldrich. K-methylsulfate was purchased from Acros Organics. Tetrodotoxin citrate (TTX) was purchased from AbCam. Neurobiotin tracer was purchased from Vector Laboratories. Cell culture media and Leukemia inhibitory factor were purchased from Life Technologies. LDN-193189, Y27632, and XAV939 were purchased from Stemgent. Fibroblast growth factor-2, Insulin-like growth factor-1, and sonic hedgehog were purchased from R&D Systems. N2 and B27 supplements were purchased from Gibco. Cyclosporin A was purchased from Fisher Scientific.

MAM Administration

Timed pregnant female Sprague-Dawley rats were administered methylazoxymethanol (MAM: 22 mg/kg i.p.) or saline on gestational day 17 as reported previously [22]. Male pups were weaned on postnatal day 21 and all experiments included pups from multiple litters.

Embryonic Stem Cells

Enriched populations of parvalbumin (PV)- or somatostatin (SST)-positive interneurons were generated as published previously using the J27 mouse embryonic stem cell line containing dual reporters (Lhx6::GFP and Nkx2.1::mCherry) [21]. Briefly, undifferentiated stem cells were cultured on mouse embryonic fibroblasts (GlobalStem) in embryonic stem cell medium (knock-out DMEM containing knockout serum replacement, nonessential amino acids, beta-mercaptoethanol, leukemia-inhibitory factor, and L-glutamine). To differentiate the stem cells into interneurons, cells were grown in suspension as embryoid bodies in embryonic stem cell medium containing 1% N2, the BMP inhibitor, LDN-193189 (250nM), the Rho-kinase inhibitor, Y27632 (10μM), and the WNT inhibitor, XAV939 (10μM). On differentiation day 3, embryoid bodies were dissociated and plated onto an adherent dish in the presence of Fibroblast growth factor-2 (10ng/ml), Insulin-like growth factor-1 (20ng/ml) and sonic hedgehog (50ng/ml for SST-enriched cells only). Media was

changed daily until cells were sorted by flow cytometry in the presence of the viability marker, Zombie Violet. SST-enriched cells were sorted for GFP+ cells on differentiation day 12 (Figure 1a) and PV-enriched populations were sorted for mCherry+ cells on differentiation day 17 (Figure 1b).

Cell Sorting

Cells were incubated in accutase containing DNAse at 37°C for 30 min, then gently triturated to form a single cell suspension. Cells were centrifuged (250 *x g*) and the pellet was resuspended in DMEM:F12 with 20% fetal bovine serum (FBS) and 10μM Y-27632. Cells were incubated with Zombie Violet (Biolegend) for 15 min at room temperature. After centrifugation, the pellet was resuspended in DMEM:F12 containing FBS and Y-27632, passed through a 70um filter and stored on ice until sorting began. Cells were sorted using a BD FACSAria III (70 micron nozzle, 50 psi) and analyzed using FACS Diva 6.1.3 software (See Supplementary Figure 1). After excluding dead cells and doublets, mCherry-positive or GFP-positive cells were collected in neurobasal medium containing B27, 2mM L-glutamine, and 10μM Y-27632.

Cell Transplants

On postnatal days 40–45, animals were anesthetized (sodium pentobarbital, 60mg/kg, i.p.) and placed in a stereotaxic apparatus. Bilateral cannula aimed at the vHipp (A/P +4.8, M/L ±4.8, D/V −7.0 mm from bregma) were used to inject approximately 40,000 cells into each hemisphere. Control animals received dead cell transplants, produced by repeated freezing and thawing and confirmed by trypan blue. Rats were treated with an immunosuppressant (cyclosporin A, 10mg/kg, s.c.) for 7 days to prevent rejection of mouse embryonic stem cells. After day 7, cyclosporin A was administered in the drinking water (100ug/ml) for the duration of the experiment. All electrophysiological and behavioral experiments were performed at least 30 days post-transplantation, a time point at which the cells have had sufficient time to migrate and integrate into the cortex [21].

Ex vivo electrophysiology

To confirm that the transplanted cells integrated into the existing hippocampal circuitry, we performed patch clamp recordings in brain slices from transplanted rats. Briefly, rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and transcardially perfused for 60 seconds with ice cold aCSF containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, 11 D-glucose, and 1.25 kynurenic acid. After extraction, brains were blocked and mounted on a vibrating microtome (Leica Microsystems). Horizontal brain slices (200 μm thick) containing the vHipp were collected and maintained at 34°C in aCSF plus the NMDA receptor antagonist MK-801 (20 μM), continuously bubbled with 95% O_2/5% CO_2. Recordings were performed at 34°C under continual perfusion of aCSF at a flow rate of 2 ml/min. Pyramidal neurons were identified visually and whole cell patch clamp recordings were performed using pipettes of 1.5–2.2 MΩ resistance filled with an intracellular solution containing (in mM): 57.5 K-methylsulfate, 57.5 KCl, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 ATP, and 0.4 GTP plus 0.2% neurobiotin, pH 7.35–7.40, 267–275 mOsm/L. To isolate spontaneous and miniature $GABA_A$ receptor-mediated inhibitory postsynaptic currents (sIPSCs and mIPSCs), whole cell voltage clamp recordings

were performed at a holding voltage of −70 mV in the presence of the AMPA receptor antagonist DNQX (10 μM). During these brief (< 5min) experiments, electrical access to the interior compartment of the cell was continuously monitored and recordings were discarded if series resistance exceeded 5 MΩ. In some experiments, TTX was added (1 μM) to the bath to isolate mIPSCs. Data were acquired at 50 kHz and filtered at 6 kHz using a Multiclamp 700B amplifier (Molecular Devices). Data were recorded and analyzed using Axograph X software (www.axograph.com). Spontaneous and miniature IPSCs were analyzed by an investigator that was blinded to the experimental group. For each cell, 50 sec of data were analyzed with the detection software set to the following minimum criteria: slope threshold −50 pA/ms, separation between events 5 ms, amplitude 15 pA, rise time 100 μs, event half-width 600 μs.

In vivo electrophysiology

In order to measure putative pyramidal cell activity in the vHipp and downstream changes in dopamine neuron activity, we performed *in vivo* extracellular recordings. Rats were anesthetized with 8% chloral hydrate (400 mg/kg^{-1}, i.p.) and placed in a stereotaxic apparatus. Supplemental anesthesia was administered as necessary and a core body temperature of 37°C was maintained. Extracellular glass microelectrodes (impedance 6–14MOhms) were lowered into the vHipp (A/P −5.0, M/L +/−4.5, D/V −4.0—8.0 from bregma) or ventral tegmental area (VTA; A/P −5.3, M/L +/−0.6, D/V −6.5—9.0 from bregma). Putative pyramidal neurons in the vHipp (identified by a firing rate <2 Hz [23]) were recorded. In the VTA, previously established electrophysiological criteria were used to identify spontaneously active dopamine neurons [24] (Figure 3c). The following parameters of dopamine neuron activity were measured: 1) population activity, 2) basal firing rate, and 3) the proportion of action potentials occurring in bursts.

Optogenetics

To inhibit the transplanted interneurons, sorted cells were resuspended in neurobasal medium containing B27, plated, and incubated overnight with adeno-associated 2 virus (AAV2) vectors driven by human the alpha-synuclein promoter expressing -HaloRhodopsin (AAV2-hSyn-eNpHR3.0-EYFP: 5×10^{10} vm/well, UNC Vector Core). Cells were then dissociated, washed and transplanted into the vHipp as described above. On the day of the experiment, a fiber-optic cannulae, connected to a 100mW 593nm DPSS Laser (OEM laser systems), was lowered into the vHipp and yellow light (~20mW) was delivered continuously. Simultaneously, spontaneously active dopamine cells were recorded in the VTA as described above.

Latent Inhibition

The latent inhibition test was used to model a positive symptom of schizophrenia. Briefly, rats were placed into a 30.5×25.4×30.5 cm^3 square conditioning chamber with metal walls and a stainless steel grid shock floor (Coulbourn Instruments) and randomly assigned to two groups: tone pre-exposed and non-exposed. Pre-exposed rats were presented with a 20 sec tone over 16 trials with a pseudorandom intertrial interval averaging 2 min. Immediately following tone exposure, rats underwent an established fear conditioning procedure in which the 20 sec tone co-terminated with a mild (0.8 mA, 0.5 sec) footshock. The tone-shock

pairing was presented 4 times with a pseudorandom intertrial interval averaging 2 min. Twenty-four hours after conditioning, rats were returned to the conditioning chamber and re-exposed to the 20 sec tone over 4 trials. Behavior was video-recorded at each stage and freezing behavior was analyzed off-line using FreezeFrame and FreezeView software (Coulbourn Instruments). Freezing was defined as behavior below a motion index threshold of 10 lasting at least 1 sec. The percentage of latent inhibition was determined using the following formula: % LI = 100 − NoTone/Tone x 100.

Social Interaction

To model a negative symptom of schizophrenia, the Social Interaction Test was performed as described previously [25]. Briefly, rats were placed in a test arena (100×100×40cm) alone for 15 min per day for 2 days prior to testing. On the test day, experimental rats were placed in the arena with a weight-matched “stimulus” rat. The 10 min test was recorded by video camera for offline analysis by a blind experimenter. The dependent measure was the time that the test animal spent actively engaged in social interaction, including sniffing, climbing on, following, grooming, or wrestling with the stimulus rat.

Attentional Set-shifting Test

To measure cognitive function, we performed the AST as described [26]. Briefly, rats were restricted to 12g food/day for 7 days prior to testing. Using a cheerio reward, rats were trained to dig in pots defined by cues along two stimulus dimensions: the digging medium filling the pot, and an odor applied to the inner rim of the pot. During testing, the rat was taken through a series of stages, each requiring a different discrimination, with a criterion of 6 consecutive correct trials required to proceed to the next stage. The first stage was a simple discrimination (SD), with only one stimulus dimension (odor or medium) present. Odor was the initial relevant dimension (signaling the location of the reward) for half the animals, and medium for the other half. The second stage was a compound discrimination (CD) in which the same discrimination was required and the second irrelevant dimension was introduced. The third stage was a reversal (R1) in which the same odors and media were used, and the same dimension remained relevant, but the negative cue from the previous stage became positive and the positive cue from the previous stage is now negative. Stage 4 was an intradimensional shift (ID), in which all new odors and media were introduced but the same dimension remained relevant. After performing a second reversal (R2), the last stage was an extradimensional set shift (ED). During ED all new odors and media were presented and the previously irrelevant stimulus became relevant.

Immunohistochemistry

After behavioral or electrophysiological testing, dual-fluorescence immunohistochemistry was used to determine the percentage of interneuron transplants that expressed SST or PV. Briefly, rats were transcardially perfused with saline followed by 4% paraformaldehyde. Brains were postfixed and cryoprotected in 10% sucrose in phosphate-buffered saline (PBS). Lhx6 is expressed in all PV- and SST-positive interneurons from the time cells exit the cell cycle into adulthood, therefore we used GFP, which is expressed under the control of Lhx6, to label transplanted cells. Free-floating coronal sections from the vHipp (50 μm) were washed in PBS, blocked (2% normal goat serum and 0.3% Triton X-100), then incubated

with mouse anti-GFP antibody (AbCam; 1:500) at 4C overnight. AlexaFluor 488 goat anti-mouse secondary antibody (1:1000) was then used to label transplanted cells. After washing, sections were incubated with rabbit anti-PV (AbCam; 1:2000) or rabbit anti-SST (Millipore; 1:400) antibodies at 4C overnight. Sections were then incubated with AlexaFluor 594 goat anti-rabbit secondary antibody (1:1000) and mounted on slides and coverslipped using Prolong gold anti-fade reagent with DAPI. Sections were imaged using an AxioCam ICc 1 (Zeiss) camera attached to an Axio Lab.A1 microscope (Zeiss). The number GFP-positive cells that colocalized with SST- or PV-positive cells were counted on 4 GFP+ sections per animal. The representative images were acquired using an Olympus IX81 Motorized inverted confocal microscope and FV10-ASW software and enhanced using ImageJ.

Data Analysis

In all figures, data are shown as mean ± s.e.m and *n* is indicated in the figure legend. Data was analyzed by one- or two-way ANOVA and the Holm-Sidak post-hoc test was used when significant interactions were present. When comparing groups with unequal variances the nonparametric Kruskal-Wallis test was used followed by Dunn's multiple comparison test. All tests were two-tailed, and significance was determined at $p<0.05$.

RESULTS

Embryonic stem cell-derived interneuron transplants

We have demonstrated previously that enriched populations of parvalbumin (PV)- or somatostatin (SST)-positive interneuron precursors can be generated from embryonic stem cells by recapitulating developmental programs *in vitro*[21]. After using flow cytometry to isolate the GFP- or mCherry-positive cells (Supplementary Figure 1a), we performed qPCR to confirm SST mRNA expression. We found that the GFP+ cells, which had been prepared according to the SST-enriching protocol (Figure 1a), had a significant increase in SST mRNA expression compared to negative cells (Supplementary Figure 1b). The mCherry-positive cells that had been treated according to the PV-enriching protocol did not show a significant increase in SST expression compared to negative control cells (Supplementary Figure 1b; Fold Increase: GFP+ cells = 7.15 ± 1.07; mCherry+ cells = 3.31 ± 0.83; One-way ANOVA: $F = 15.80$, $p < 0.05$), suggesting that the embryonic stem cells began to differentiate into specific interneuron subtypes in culture. To confirm that the precursor cells continued to develop into mature interneurons once they were transplanted into the rat hippocampus, we used dual fluorescence immunohistochemistry to label GFP and PV or SST. We found that when we used the SST-enriching protocol, 91% of GFP-positive cells also expressed SST (Figure 1a). The PV-enriching protocol resulted in 75% GFP-positive cells that were also PV-positive (Figure 1b). Together, these results confirm that our *in vitro* cell differentiation protocol produces enriched populations of SST- or PV-positive interneurons.

To demonstrate that the transplanted interneurons integrate into the existing circuitry, we first used dual fluorescence immunohistochemistry to show that the transplanted interneurons form synapses on endogenous pyramidal cells in the vHipp (Supplementary Figure 2). Next, we used *ex vivo* patch clamp electrophysiology to measure spontaneous

inhibitory postsynaptic currents (sIPSCs) in endogenous pyramidal cells throughout the vHipp. We found that the MAM animals that received PV- and SST- transplants show significant higher sIPSC amplitudes (Figure 2a; sIPSC Amplitudes: MAM/Dead cells = 56.38 ± 1.05 pA, MAM/SST cells = 84.32 ± 0.91 pA, MAM/PV cells = 75.64 ± 0.63 pA; Kruskal-Wallis: K = 292.5, $p<0.0001$) and significant shorter inter-event intervals (Figure 2b; sIPSC Intervals: MAM/Dead cells = 303.9 ± 19.8 ms, MAM/SST cells = 160.9 ± 4.28 ms, MAM/PV cells = 118.1 ± 3.25 ms; n=13–34 cells from 2–3 rats/group; Kruskal-Wallis: K = 844.7, $p<0.0001$) than the dead cell group, suggesting that the transplanted interneurons indeed integrated into the hippocampal circuitry. In the presence of the sodium channel blocker TTX there was no longer a difference in amplitudes (Figure 2c; mIPSC Amplitude: MAM/Dead cells = 43.14 ± 0.55 pA, MAM/SST cells = 41.42 ± 0.69 pA, MAM/PV cells = 41.80 ± 0.50 pA) but the event intervals remained significantly shorter (Figure 2d; mIPSC Intervals: MAM/Dead cells = 884.6 ± 73.5 ms, MAM/SST cells = 644.0 ± 111 ms, MAM/PV cells = 601.3 ± 36.8 ms; n=13–17 cells from 2–3 rats/group; Kruskal-Wallis: K = 18.36, $p<0.0001$). In all animal groups, sIPSCs and mIPSCs were eliminated by the $GABA_A$ receptor antagonist picrotoxin (100 μM). Representative traces are depicted in Figure 2e and a representative pyramidal cell is shown in Figure 2f.

To further demonstrate that the interneuron transplants regulate vHipp activity, we performed *in vivo* electrophysiological recordings of putative pyramidal cells in chloral hydrate anesthetized rats (Fig 3a). As we have shown previously[17], MAM-treated rats show increased pyramidal cell firing in the vHipp compared to saline controls (Figure 3b; Saline/Dead cells = 0.60 ± 0.07 Hz, MAM/Dead cells = 0.82 ± 0.08 Hz; Two-way ANOVA: Interaction $F_{2,239}=3.995$, $p=0.02$; Holm-Sidak: t=2.034, p=0.043). This is consistent with our hypothesis that the increased vHipp activity is due to a loss of GABAergic interneuron function [5]. In line with this, we now demonstrate that both SST- and PV-enriched transplants can reverse aberrant pyramidal cell firing in MAM rats to control levels (Figure 4b; MAM/SST cells = 0.528 ± 0.9 Hz, MAM/PV cells = 0.58 ± 0.08; Holm-Sidak: MAM/Dead vs. MAM/SST t=2.564, p=0.033; MAM/Dead vs. MAM/PV t=2.269, p=0.048).

We have previously shown that the MAM model of schizophrenia has an increase in the number of spontaneously active dopamine cells in the ventral tegmental area (VTA)[27]. Further, we have also demonstrated that this increase in dopamine population activity is directly attributable to a pathologically enhanced drive from the vHipp [5, 28, 29]. In the current experiments, we now show that in MAM-treated animals, both SST- and PV-enriched transplants reduce the number of spontaneously active dopamine cells to control levels (Figure 3c, d; Saline/SST cells = 0.98 ± 0.128 cells/track, Saline/PV cells = 1.08 ± 0.14 cells/track, MAM/SST cells = 0.97 ± 0.06 cells/track, MAM/PV cells = 1.09 ± 0.08 cells/track; Holm-Sidak: MAM/Dead vs. MAM/SST t=4.229, p<0.001; MAM/Dead vs. MAM/PV t=3.618, p=0.003). Further, we used optogenetics in a subset of animals to demonstrate that this effect was dependent on the activity of the stem cell derived interneuron transplants (Figure 3). When HaloRhodopsin and a 593nm laser were used to inhibit the activity of the transplanted interneurons, the beneficial effect on dopamine population activity was completely abolished (Figure 3e; Dead cells/Halo = 1.84 ± 0.24 cells/track, SST cells/Halo = 1.92 ± 0.24 cells/track; PV cells/Halo = 2.04 ± .24 cells/track; Holm-Sidak: SST cells/Halo vs. MAM/Dead cells t=0.24, p=0.817; PV cells/Halo vs.

MAM/Dead cells t=0.60, p=0.91). Taken together, these data demonstrate that stem cell-derived interneuron transplants functionally integrate within the existing circuitry to normalize aberrant vHipp pyramidal cell activity and reverse downstream changes in dopamine system function.

Effect of cell transplants on behavioral correlates of positive, negative and cognitive symptoms

We have previously demonstrated that fetal-derived MGE transplants can reverse the augmented amphetamine-induced locomotor activity in MAM-treated rats[17]. To examine an additional behavioral correlate of positive symptoms we examined whether stem cell derived interneurons can reverse deficits in latent inhibition. Consistent with our previous observations, MAM-treated rats show a trend toward impaired latent inhibition (Supplementary Figure 3b, c; Saline/Dead cells = 29.81 ± 28.266% freezing, MAM/Dead cells = −14.64 ± 28.27% freezing). The Saline- and treated rats that received SST-positive interneuron transplants also show deficits in latent inhibition (Supplementary Figure 3b, c; Holm-Sidak, t = 2.52, p = 0.03; Saline/SST cells = −70.70 ± 28.27, −38.29 ± 30.53% freezing). The PV-positive interneuron transplants show a trend toward improvement in latent inhibition in the MAM-treated rats, although this effect did not reach significance (Supplementary Figure 3b, c; Saline/PV cells = 41.17 ± 30.53% freezing, MAM/PV cells = 9.32 ± 33.45% freezing).

Currently prescribed antipsychotic medications are poorly effective in treating negative symptoms of schizophrenia[2]. Therefore, we used the social interaction test to model the negative symptoms of schizophrenia to determine if interneuron transplants can improve performance in this symptom domain. Consistent with observations in schizophrenia patients, MAM rodents spend less time engaged in social interaction compared to saline controls (Figure 4; Two-way ANOVA, Interaction $F_{2,63}$=5.676, p=0.006; Holm-Sidak, t=2.396, p=0.02; Saline/Dead cells = 215.64 ± 12.291 sec, MAM/Dead cells = 182.86 ± 6.76 sec). The SST-enriched transplants had no effect on social interaction time in either the saline- or MAM-treated rats (Holm-Sidak, Saline/dead cells vs. Saline/SST cells, t=1.022, p=0.311; Holm-Sidak, MAM/dead vs. MAM/SST cells, t=0.395, p=0.694; Saline/SST cells = 230.8 ± 12.517 sec, MAM/SST cells = 177.3 ± 8.37 sec). In contrast, PV-enriched transplants abolished the schizophrenia-like deficit in social interaction without altering social interaction in control animals (Holm-Sidak, Saline/dead vs. Saline/PV cells, t=1.061, p=0.5; Holm-Sidak, MAM/dead vs. MAM/PV cells, t=2.443, p=0.035; Saline/PV cells = 199.44 ± 9.09 sec, MAM/PV cells = 217.2 ± 13.15 sec), suggesting that PV-positive interneuron transplants, derived from embryonic stem cells, may be effective in reducing negative symptoms of schizophrenia.

Cognitive functions are also poorly treated by currently available antipsychotic medications[2]. Therefore, we used the attentional set-shifting test to measure two forms of cognitive flexibility that are impaired in schizophrenia patients [30]. Consistent with previous reports [31], MAM-treated rodents show deficits in reversal learning (Figure 5a; Two-way ANOVA, Interaction $F_{2,50}$=3.461, p=0.04; Holm-Sidak, t=3.641, p<0.001; Saline/Dead cells = 16.0 ± 2.28 trials, MAM/Dead cells = 25.22 ± 1.16 trials). Both SST- and PV-enriched

transplants completely abolish this deficit, returning trials to meet criterion to control levels (Holm-Sidak, MAM/dead vs. MAM/SST, t=3.054, p=0.011; Holm-Sidak, MAM/dead vs. MAM/PV, t=2.983, p=0.009; MAM/SST cells = 17.25 ± 1.84, MAM/PV cells = 17.67 ± 2.13). In contrast to the effects on reversal learning, PV and SST interneuron transplants produced disparate results on extradimensional set shifting (Figure 5b). Thus, while SST-positive interneuron transplants had no effect (Holm-Sidak, t=0.217, p=0.829; MAM/SST cells = 23.5 ± 3.21 trials), the PV-enriched transplants completely abolished the cognitive deficit observed in MAM rats (Holm-Sidak, t=3.244, p=0.005; MAM/PV cells = 12.0 ± 1.15 trials). There were no effects of prenatal treatment or cell transplants on any other stage of the test (Figure 5c).

DISCUSSION

In the current experiments we demonstrate that interneuron transplants, derived from embryonic stem cells, may be a viable treatment strategy to target negative and cognitive symptoms of schizophrenia, which are poorly treated by currently prescribed antipsychotic medications[2]. Previously, we have shown that interneuron transplants (derived from fetal tissue) can improve positive symptoms in the MAM model of schizophrenia[17]. This is consistent with data from a distinct model of schizophrenia, the cyclin D2 knock-out mouse, where Moore and colleagues also report that interneuron transplants, derived from fetal tissue, attenuate schizophrenia like deficits in amphetamine-induced locomotor activity[20], suggesting that the beneficial effect of interneuron transplants is not model-specific. In the current experiments, we have expanded on these findings to show that embryonic stem cells, differentiated into specific interneuron populations, can improve function in the MAM model of schizophrenia. Importantly, the interneuron transplants improve function in behavioral correlates of negative and cognitive symptoms, two symptom domains that are currently poorly treated[4].

The medial ganglionic eminence (MGE) gives rise to the majority of cortical PV- and SST-positive interneurons and the specification of MGE cortical interneurons has been well established (for review see[18]). The dorsal region of the MGE is enriched for SST-positive interneurons while more PV-positive interneurons are found in the ventral regions of MGE[32, 33]. This effect is thought to be due to sonic hedgehog (Shh) signaling, which occurs in a gradient in the MGE, with higher levels of Shh in the dorsal versus ventral areas of the MGE[32]. Sonic hedgehog (Shh) signaling is required for both the induction and maintenance of expression of the transcription factor, Nkx2.1[34]. We have shown previously that higher levels of Shh can promote differentiation of SST versus PV interneurons[21, 35]. Therefore, in the current experiments, we used high versus low Shh in culture to promote differentiation of SST or PV interneurons, respectively. In addition to Shh signaling, the fate determination of cortical interneurons also depends on the time at which the neurons are born[21, 33]. SST interneurons are generally born earlier and occupy deeper layers of the cortex while PV interneurons originate later in neurogenesis and are found in more superficial cortical layers[36]. Because interneurons in the MGE go through a stereotyped developmental program in which Nkx2.1 is expressed at high levels in dividing progenitor cells until the time of cell cycle exit, at which point Nkx2.1 decreases and Lhx6 expression begins to increase and remains high, we used Nkx2.1 and Lhx6 expression to identify early versus late born

interneurons[37]. Therefore, in the current experiments, we used a protocol to mimic the stereotyped pattern of SST- and PV-positive interneuron development to generate enriched populations of SST or PV interneurons.

Interestingly, we found that SST- and PV-enriched transplants had differential effects on behaviors related to schizophrenia. Given that both PV and SST interneuron transplants reversed vHipp hyperactivity, the differential behavioral effects of these transplants on extradimensional set shifting are likely associated with the differences in the unique anatomical, biochemical, and physiological characteristics of these two interneuron subtypes [38]. For example, PV- and SST- positive interneurons use laminar targeting to synapse on distinct subcellular compartments of post-synaptic cells[13]. SST-positive cells primarily target dendrites, allowing this interneuron subtype to regulate synaptic plasticity and integrate synaptic inputs[38]. Conversely, PV-positive cells can regulate the timing and generation of action potentials by targeting the cell soma or axon initial segment [38]. Further, PV- and SST-positive cells can be also distinguished by their firing patterns. PV-positive interneurons display a fast firing, non-adapting pattern [39] while SST cells show a non-fast firing, accommodating pattern [40]. Indeed, we have shown previously that transplanted PV- and SST-positive interneurons, derived from embryonic stem cells, display firing patterns that resemble endogenous PV- and SST-positive interneurons [21].

Another way in which SST- and PV-positive interneuron transplants may differentially influence schizophrenia-like behaviors is via the regulation of unique neural pathways from the vHipp. The classic dopamine hypothesis of schizophrenia posits that hyperactivity in the mesolimbic dopamine system underlies the positive symptoms of the disorder[41]. In the current experiments, we demonstrate that both SST- and PV-positive interneuron transplants into the vHipp can reduce dopamine population activity in MAM-treated rats, which is likely a result of normalization of the characterized polysynaptic vHipp-VTA pathway[5].

Reversal learning, a form of cognitive flexibility, is disrupted in schizophrenia patients [42]. Reversal learning is thought to involve the orbitofrontal cortex [43], a brain region that is not robustly targeted by the vHipp. However, an association between dopamine system function and reversal learning has been previously demonstrated. For example, reversal learning performance is correlated with striatal dopamine synthesis capacity [44] and striatal D2 receptor availability [45]. Although OFC-specific dopamine depletion has no effect [46], dopamine depletion at the level of the caudate nucleus impairs reversal learning [47]. Further, both pharmacological agonism [48] and antagonism [49] of D2/D3 receptors can disrupt reversal learning, suggesting that this type of cognitive flexibility requires tightly controlled dopamine signaling. Thus, similar to the effects of interneuron transplants on positive symptoms[17, 20], the beneficial effect of PV and SST transplants on reversal learning may be secondary to restoration of normal dopamine neuron activity.

Although both PV- and SST-positive transplants effectively normalized dopamine system function, latent inhibition, and reversal learning, only PV-positive transplants were also able to abolish deficits in social interaction and extradimensional set shifting. Interestingly, both social interaction and extradimensional set-shifting have been associated with the medial prefrontal cortex (mPFC), a brain region that receives direct projections from the vHipp [7]

and that is dysregulated in schizophrenia patients [50]. For example, elevating the balance between excitation and inhibition in the mPFC can impair social interaction, an effect that is normalized by restoring inhibitory signaling [9]. In the current experiments, restoration of parvalbumin interneuron function in vHipp was also able to increase social interaction in MAM rats, suggesting that this interneuron type may normalize signaling between the vHipp and mPFC.

Extradimensional set-shifting is another form of cognitive flexibility that is disrupted in schizophrenia [51, 52]. While reversal learning is associated with orbitofrontal activity and subcortical dopamine function, extradimensional set shifting, like social interaction, is more dependent on the mPFC [8]. Indeed, mPFC lesions cause specific deficits in extradimensional set-shifting without influencing other forms of cognitive flexibility, such as reversal learning [8]. In the current experiments, we demonstrate that only PV-positive interneuron transplants were able to reverse deficits in extradimensional set-shifting, which we posit is due to modulation of pyramidal cells that project directly to the mPFC [53]. Together, our results demonstrate that PV- and SST-positive cells in the vHipp differentially influence schizophrenia-like behaviors and suggest that specifically targeting PV-positive interneurons in the vHipp may be a more effective treatment strategy to alleviate multiple symptom domains of schizophrenia.

In the current experiments, we have demonstrated that interneuron transplants may be an effective treatment strategy for schizophrenia. However, before this strategy can move into the clinic, it will be important to consider the timing of the transplant surgeries. In the current studies, cells were transplanted between postnatal day (PND) 40–45, a time point that has been determined to be post-puberty based on balano-preputial separation and circulating androgen levels [54]. Using the MAM rodent model of schizophrenia, we have demonstrated previously that rats begin to show schizophrenia-like changes in behavior and physiology as early as PND 40[55]. Specifically, we found that PND 40 MAM rats show increased locomotor activity in response to amphetamine, a stable reduction of PV expression in the vHipp, and an increase in the number of spontaneously active dopamine cells in the VTA[55]. Studies in humans have demonstrated that poor prognosis is correlated with the time that schizophrenia is left untreated [56]; therefore, we believe that to be most effective, stem cells should be transplanted as early in the course of the disease as possible. However, this early intervention strategy raises the question of long-term outcomes. Although we demonstrated that interneuron transplants attenuated schizophrenia-like changes in behavior and physiology when animals were tested >30 days post-transplantation, it remains unclear whether the cells would continue to have beneficial effects throughout the animal's lifetime. A long-lasting therapeutic effect would depend first on the survival of the cells. In our experiments, we observed GFP+ interneurons up to 6 months after transplantation (data not shown). Further, in Parkinson's Disease patients, ventral midbrain grafts derived from fetal tissue have been shown to survive in the brain for up to 14 years[57]. Although these results suggest that transplanted cells can survive in the brain for extended periods of time, future experiments will be required to determine if the transplanted interneurons remain viable throughout the lifetime of the rat and whether these surviving cells continue to produce therapeutic changes.

While most pharmacological interventions only target symptoms, cell-based therapies hold considerable promise for the cure of neurological disorders such as Parkinson's Disease and Stroke [58]. To date, very few groups have explored the potential of cell transplants in schizophrenia[17, 20]. Those groups that have tested the therapeutic benefit of interneuron transplants in schizophrenia have used interneuron precursors derived from fetal tissue[17, 20]. Here we provide the first preclinical evidence that PV-positive interneuron transplants, derived from embryonic stem cells, may effectively reduce not only positive but also negative and cognitive symptoms in an animal model of schizophrenia. Further, most literature focuses on positive symptoms of schizophrenia, which are already the target of currently prescribed antipsychotic medication[2]. Here, we demonstrate that interneuron transplants into the vHipp alleviate not only positive, but also negative and cognitive symptoms in the MAM rodent model. Together, our results provide insight into the role of PV- and SST-positive interneurons in the pathology of schizophrenia and offer an exciting new treatment strategy to target all symptom domains of this debilitating disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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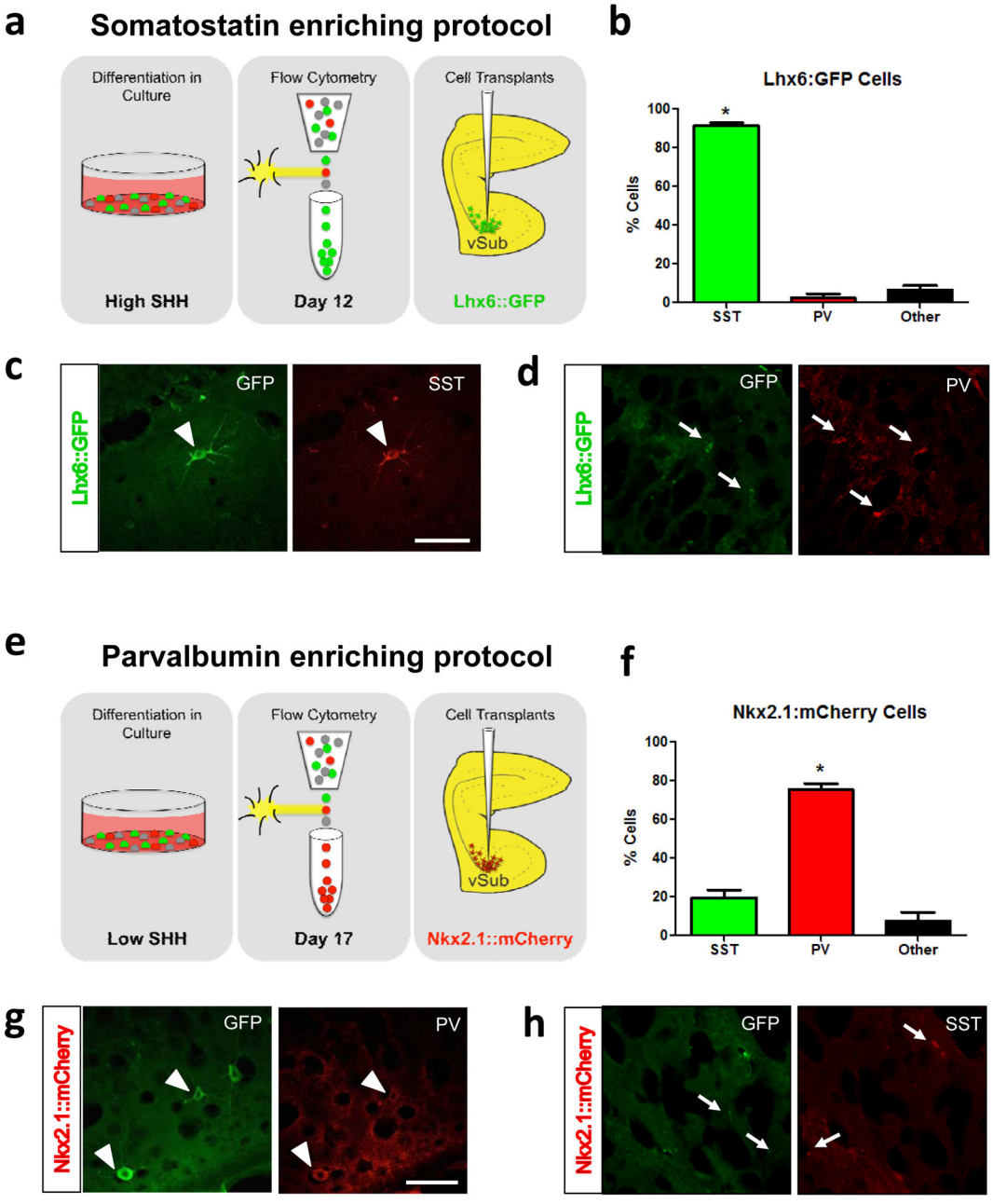

Figure 1.

Schematic depicting the protocol used to generate SST-enriched cells is shown in **(a).** The majority of cells grown using the SST-enriching protocol express SST. * is significantly different than the percentage of cells that express PV **(b)**. Representative image of a transplanted Lhx6::GFP cell in which GFP and SST are co-localized **(c)**. Representative image of a transplanted Lhx6::GFP cell in which GFP and PV are not co-localized **(d)**. Schematic depicting the protocol used to generate PV-enriched cells is shown in **(e).** The majority of cells grown using the PV-enriching protocol express PV. * is significantly different than the percentage of cells that express SST **(f)**. Representative image of a

transplanted Nkx2.1::mCherry cell in which GFP and PV are co-localized **(g)**. Representative image of a transplanted Nkx2.1::mCherry cell in which GFP and SST are not co-localized **(h)**. Arrowheads indicate double-labeled cells. Arrows indicate cells that are not double-labeled. Scale bar is 100 microns. n = 11–31 cells per rat.

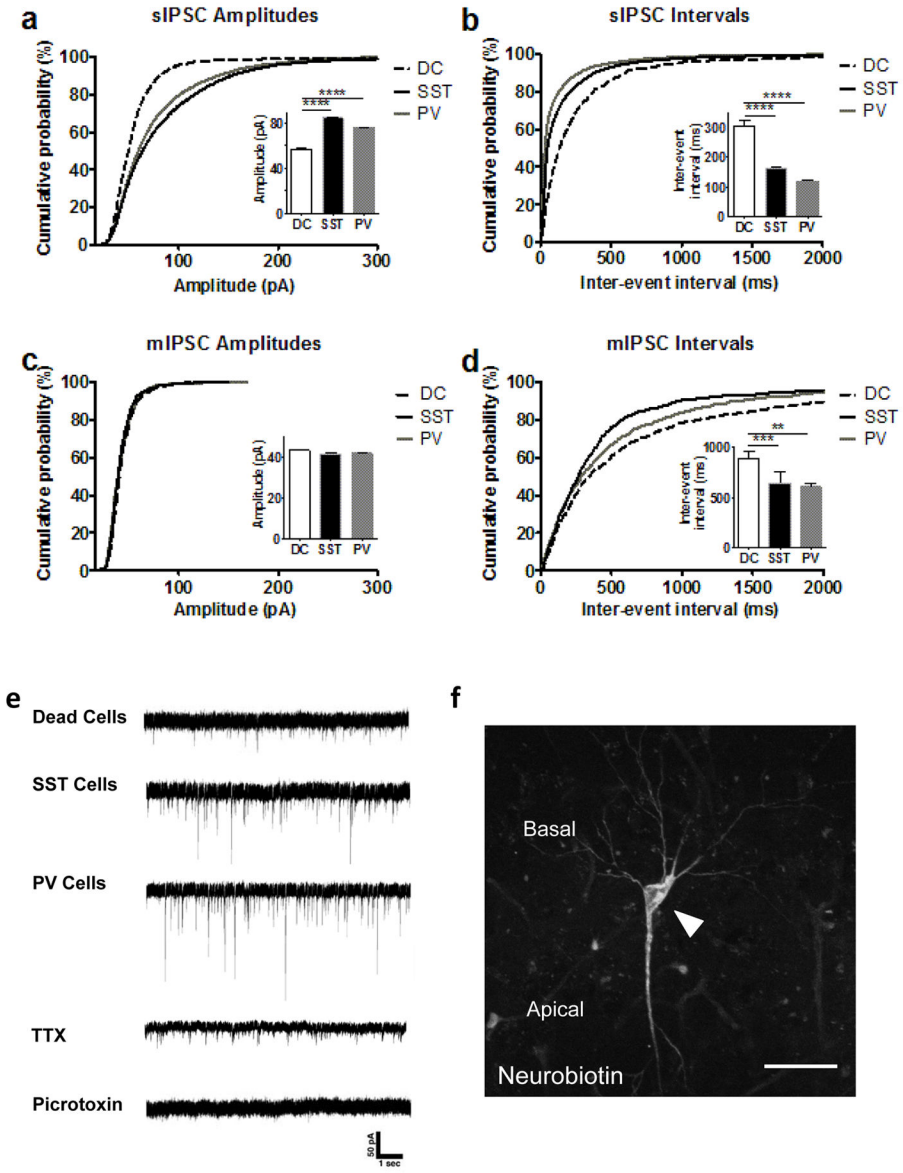

Figure 2.

Stem cell derived interneuron transplants functionally integrate within the existing circuitry. *Ex vivo* patch clamp electrophysiology (holding voltage: −70 mV) of endogenous pyramidal neurons demonstrated significant higher sIPSC amplitudes **(a)** and shorter inter-event intervals **(b)** compared to dead cell controls. When TTX was added and mIPSCs were analyzed, the difference in amplitudes between groups was eliminated **(c)**, but inter-event intervals remained shorter **(d)** compared to dead cell controls. Example traces are depicted in **(e)**. A representative pyramidal cell, labeled with neurobiotin is shown in **(f)**. Scale bar is 50 microns. n = 13–34 cells from 2–3 rats/group.

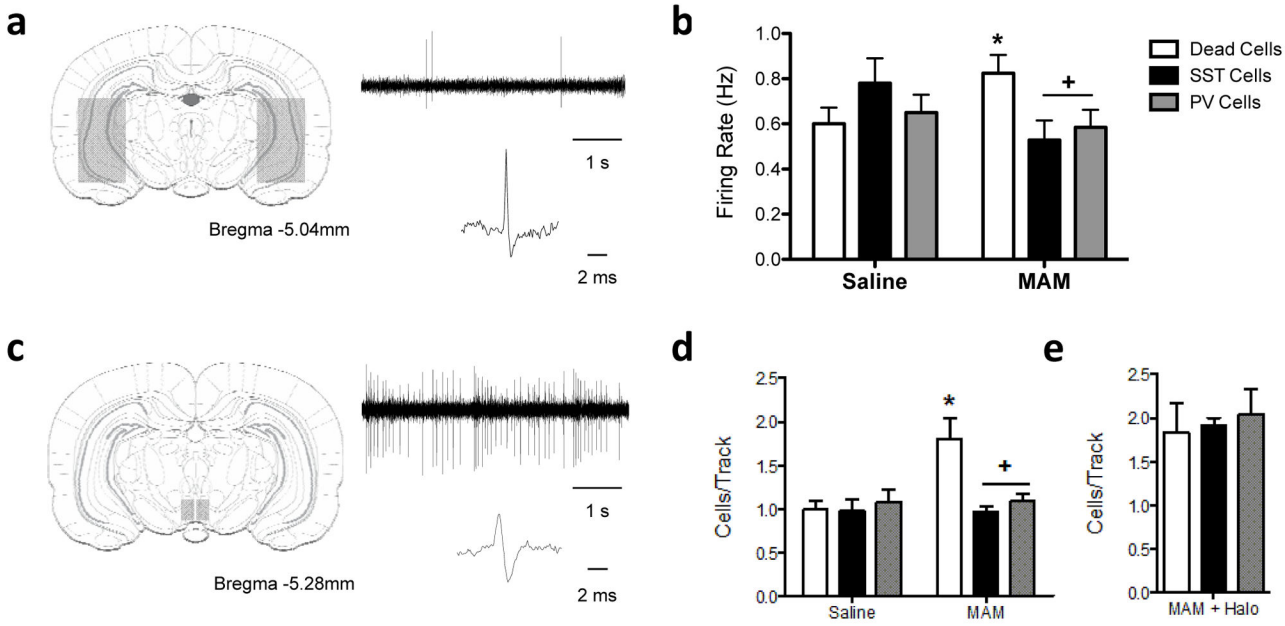

Figure 3.

Stem cell derived interneuron transplants reverse aberrant neuronal activity in the vHipp **(a)** and VTA **(c)**. MAM-treated rats display an increase in vHipp pyramidal neuron activity that was normalized by both PV- and SST-positive transplants (**b**: n=23–48 cells per group). Similarly, PV- and SST-positive vHipp transplants were also able to normalize downstream alterations in dopamine neuron population activity in the ventral tegmental area (**d:** n=5–7 rats/group). The effect on interneuron transplants on dopamine activity is completely reversed when transplanted cells are optogenetically silenced (**e:** MAM + Halo n=2–5 per group). + denotes significant difference from MAM-treated animals that received dead cell transplants while * represents significant difference from saline controls.

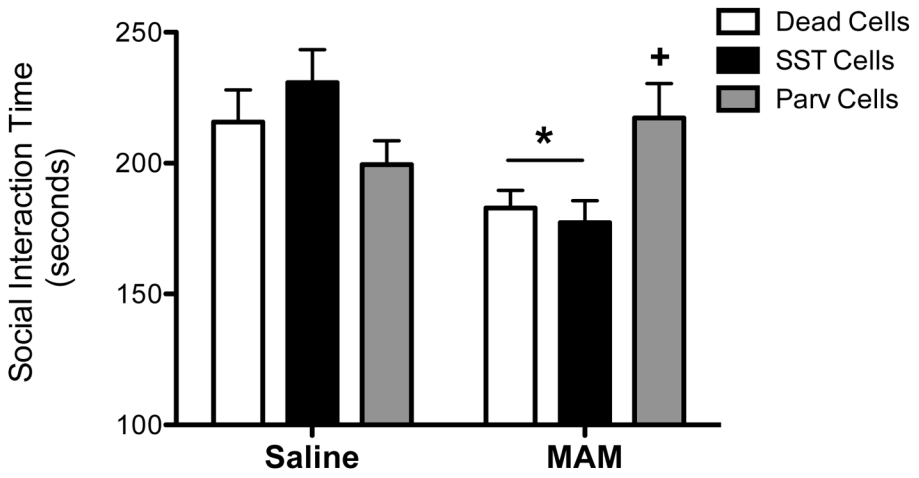

Figure 4.

PV-positive transplants, but not SST-positive transplants, into the ventral hippocampus reverse deficits in social interaction in MAM-treated rats. * demonstrates significant difference from saline-treated controls while $^{+}$ represents significant difference from MAM-treated animals that received dead cell transplants. n=9–14 per group.

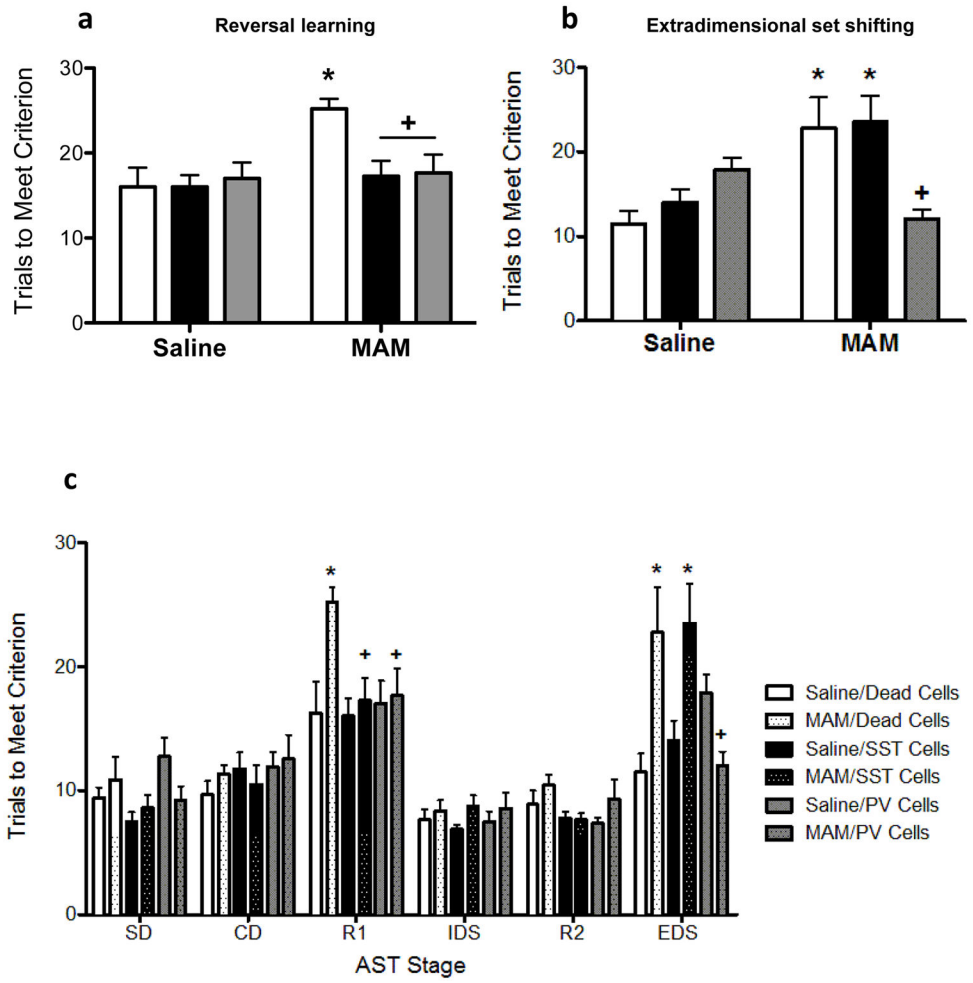

Figure 5.

PV- and SST- interneuron transplants differentially affect cognitive flexibility as determined by the attentional set shifting test. MAM-treated rats display deficits in reversal learning **(a)** and extradimensional set shifting **(b).** Both PV- and SST-positive vHipp transplants abolish reversal learning deficits in MAM-treated rats. However, only PV- enriched transplants also reverse extra-dimensional set shifting deficits. Neither prenatal treatment nor cell transplants influenced the other stages of the attentional set shifting test **(c)**. SD is simple discrimination. CD is compound discrimination. R1 is the first reversal. ID is intradimensional set shift. R2 is the second reversal. ED is extradimensional set shift. * demonstrates significant difference from saline-treated controls while $^{+}$ represents significant difference from MAM-treated animals that received dead cell transplants. n=8–9 per group.