REGULAR RESEARCH ARTICLE

Behavioral Deficits Induced by Somatostatin-Positive GABA Neuron Silencing Are Rescued by Alpha 5 GABA-A Receptor Potentiation

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Abstract

Introduction: Deficits in somatostatin-positive gamma-aminobutyric acid interneurons (SST+ GABA cells) are commonly reported in human studies of mood and anxiety disorder patients. A causal link between SST+ cell dysfunction and symptom-related behaviors has been proposed based on rodent studies showing that chronic stress, a major risk factor for mood and anxiety disorders, induces a low SST+ GABA cellular phenotype across corticolimbic brain regions; that lowering *Sst*, SST+ cell, or GABA functions induces depressive-/anxiety-like behaviors (a rodent behavioral construct collectively defined as "behavioral emotionality"); and that disinhibiting SST+ cells has antidepressant-like effects. Recent studies found that compounds preferentially potentiating receptors mediating SST+ cell functions, α5-GABAA receptor positive allosteric modulators (α5-PAMs), achieved antidepressant-like effects. Together, the evidence suggests that SST+ cells regulate mood and cognitive functions that are disrupted in mood disorders and that rescuing SST+ cell function via α5-PAM may represent a targeted therapeutic strategy.
Methods: We developed a mouse model allowing chemogenetic manipulation of brain-wide SST+ cells and employed behavioral characterization 30 minutes after repeated acute silencing to identify contributions to symptom-related behaviors. We then assessed whether an α5-PAM, GL-II-73, could rescue behavioral deficits.
Results: Brain-wide SST+ cell silencing induced features of stress-related illnesses, including elevated neuronal activity and plasma corticosterone levels, increased anxiety- and anhedonia-like behaviors, and impaired short-term memory. GL-II-73 led to antidepressant- and anxiolytic-like improvements among behavioral deficits induced by brain-wide SST+ cell silencing.
Conclusion: Our data validate SST+ cells as regulators of mood and cognitive functions and demonstrate that bypassing low SST+ cell function via α5-PAM represents a targeted therapeutic strategy.

Key Words: Somatostatin, GABA, depression, Gabra5, antidepressant

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Significance Statement

Human studies demonstrate somatostatin-positive GABAergic interneuron (SST+ cell) deficits as pathological features of major depressive disorder and anxiety disorders. Studies indicate reduced SST and GABAergic markers across corticolimbic brain regions. Past animal studies identified that SST+ cells regulate mood and cognitive functions related to symptoms of mood disorders but employed SST+ cell ablation or region-specific silencing not representative of disease-related processes. We developed a chemogenetic mouse model of brain-wide low SST+ cell function and performed behavioral characterization to demonstrate a role for these cells in regulating anxiety- and anhedonia-like behaviors, behavioral emotionality, and impaired memory. We next found that the α5-GABAA receptor positive allosteric modulator (α5-PAM), GL-II-73, rescued deficits induced by low SST+ cell function. These findings support SST+ cells as central regulators of symptom-related behaviors and validate α5-GABAA receptors as a therapeutic target to reverse deficits related to low SST+ cell function.

Introduction

Major depressive disorder (MDD) is a severe psychiatric illness affecting 322 million people (Friedrich, 2017; Vos et al., 2017; World Health Organization, 2017; Rehm and Shield, 2019) and is more common among females (5.1%) than males (3.6%) (World Health Organization, 2017). The diagnosis and treatment of MDD is limited by heterogeneity in pathological and clinical presentation, with low mood, anhedonia, physiological, and cognitive impairments and high comorbidity (approximately 40%–70%) with anxiety disorders. First-line antidepressants target monoaminergic imbalances that occur downstream from primary mood disorder pathologies and remain ineffective in 50% of treated patients (Gaynes et al., 2009; Holtzheimer and Mayberg, 2011). Therefore, a need exists to characterize how cellular changes contribute to specific symptoms to develop targeted drug therapies. Four decades of human and animal studies demonstrate reduced level, synthesis, and function of neurons expressing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) among primary pathological changes across mood disorders (Luscher et al., 2011; Newton et al., 2019). Among these, GABA neurons coexpressing the neuropeptide somatostatin (SST+ GABA cells, or SST+ cells) appear to be selectively vulnerable, wherein deficits were identified downstream of causal factors such as chronic stress and reduced neurotrophic factor signaling (Guilloux et al., 2012; Tripp et al., 2012).

GABA neurons play a critical role in information processing by cortical microcircuits to regulate mood and cognitive functions. These microcircuits encode neuronal information through activity patterns of excitatory glutamatergic pyramidal neuron (PN) ensembles, whereas GABAergic neurons shape this activity differently depending on nonoverlapping cellular identities coexpressing SST, the calcium-binding protein parvalbumin (PV+), or the ionotropic serotonin receptor 5HT3aR with or without vasoactive intestinal peptide (Rudy et al., 2011). SST+ cells gate excitatory PN input via dendritic inhibition and modulate output via perisomatic PN disinhibition through SST-PV+ cell afferents (Fee et al., 2017; Hu et al., 2019). Dendritic SST+ cell inhibition is partially mediated by GABA acting on GABA-A and -B receptors (GABAA-R/GABAB-R) (Urban-Ciecko et al., 2015; Schulz et al., 2018), whereas the SST peptide is released under distinct firing conditions (Katona et al., 2014; Yavorska and Wehr, 2016). SST has shared roles alongside GABA in pre- and postsynaptic PN inhibition (Tallent and Siggins, 1997; Schweitzer et al., 1998), plus distinct roles in negative regulation of stress-related hypothalamic-pituitary adrenal axis activation (Stengel and Taché, 2017).

Histological postmortem MDD studies identified SST and GABA marker reductions at mRNA and protein levels in the prefrontal cortex (PFC) (Sibille et al., 2011), anterior cingulate cortex (Tripp et al., 2011), and amygdala (Guilloux et al., 2012) that were more severe in females (Seney et al., 2013, 2015). Follow-up studies indicated reduced SST mRNA per cell spanning cortical layers (Tripp et al., 2011) and reduced detectable SST+ cell density without overall cell density changes in the amygdala (Douillard-Guilloux et al., 2017). In vivo studies revealed MDD-related reductions in PFC (Hasler et al., 2007) and ACC GABA levels (Price et al., 2009), with reduced GABAA-R and GABAB-R neurotransmission (Bajbouj et al., 2006; Levinson et al., 2010; Radhu et al., 2013). The regional and functional differentiation of GABAA-Rs depends on heteropentameric subunit composition, commonly containing 2 α, 2 β, and 1 γ-subunit (Rudolph et al., 2001; Sieghart and Sperk, 2002). SST+ cell functions are partially mediated by α5 subunit-containing GABAA-Rs given their localization to PN dendrites and PV+ cells in the PFC, hippocampus (HPC), and ventral striatum (Ali and Thomson, 2008; Schulz et al., 2018; Hu et al., 2019). GABRA5 transcripts (encoding α5GABAA-Rs) were also reduced in the PFC of MDD and aged patients (Oh et al., 2019), together suggesting that a low SST+ GABA cellular phenotype, affecting pre- and postsynaptic functions and arising from intrinsic brain-wide cellular vulnerabilities, contributes to common symptoms of mood disorders and age-related processes. Indeed, low SST+ markers are also commonly found in schizophrenia and bipolar disorder (Lin and Sibille, 2013).

Preclinical findings support that disrupted SST+ cell function may contribute to deficits reflecting mood disorder symptoms. In mice, chronic stress (a major risk factor for MDD and anxiety disorders) induced reduced mRNA levels of *Sst* and the GABA-synthesizing enzyme *Gad67* in the cingulate cortex, plus altered SST+ cell but not PN transcripts (Lin and Sibille, 2015; Girgenti et al., 2019). Mice with genetic *Sst* ablation also recapitulated elevated corticosterone, reduced growth factor and *Gad67* expression, and elevated anxiety-/depressive-like behaviors (collectively defined as "behavioral emotionality"; Guilloux et al., 2011). Furthermore, acute chemogenetic or optogenetic PFC SST+ cell silencing elevated behavioral emotionality (Soumier and Sibille, 2014) and working memory impairment (Abbas et al., 2018), whereas genetic disinhibition had antidepressant-like effects (Fuchs et al., 2017). Additionally, corticolimbic infusion of exogenous SST peptide had anxiolytic- and antidepressant-like effects (Engin et al., 2008; Engin and Treit, 2009; Yeung et al., 2011; Prévôt et al., 2017). Therefore, SST+ cells appear to regulate mood-related behaviors, but it is important to mention that genetic ablation and region-specific silencing do not recapitulate the brain-wide low (but intact) SST+ cell phenotype observed in humans (Fee et al., 2017).

Neuroimaging studies demonstrated that GABA levels normalized after pharmacological, cognitive-behavioral, and

neuromodulatory antidepressant treatment (Fee et al., 2017). Although first-line antidepressants do not target GABA/SST deficits directly, mixed efficacy has been observed from monotherapy or combination therapy with benzodiazepines (BZDs) that act as nonselective positive allosteric modulators (PAMs) at GABAA-Rs between $\gamma 2$ and $\alpha 1$–3, 5 subunits (Benasi et al., 2018; Gomez et al., 2018; Ogawa et al., 2019). However, BZDs confer side effects and abuse liability attributed to pan-α-subunit selectivity (Vgontzas et al., 1995). Recent preclinical studies assessing BZD derivatives with selectivity for $\alpha 5$-GABAA-Rs ($\alpha 5$-PAMs), which have restricted corticolimbic distribution, found anxiolytic-, antidepressant-like, and procognitive effects in young, aged, and chronic stress–exposed rodents (Koh et al., 2013; Piantadosi et al., 2016; Prevot et al., 2019a). Further, an exogenous formulation of the neurosteroid allopregnanolone was recently approved for postpartum depression and is believed to act partially as a PAM at extrasynaptic GABAA-R compartments where $\alpha 5$-GABAA-Rs are preferentially located (Farrant and Nusser, 2005; Lüscher and Möhler, 2019). However, studies to date have not assessed the potential of $\alpha 5$-PAMs to reverse deficits induced by specific pathologies, and even minor differences in GABAA-R subunit selectivity can have divergent outcomes (Prevot et al., 2019a), thus warranting further investigation.

Given evidence that a brain-wide low SST+ cellular phenotype contributes to mood disorder symptoms, we aimed to identify how acute silencing of brain-wide SST+ cells in mice led to changes along symptom-related behavioral dimensions, including anxiety- and anhedonia-like behavior, behavioral emotionality, and impaired memory. To achieve this, we combined multiple behavioral tests with a novel chemogenetic SST+ cell-silencing model. We then assessed whether the $\alpha 5$-PAM GL-II-73 could rescue behavioral deficits. We predicted that brain-wide SST+ cell silencing induces symptom-related behavioral deficits that can be rescued by $\alpha 5$-PAM.

Materials and Methods

A complete description of methods/study designs can be found in supplemental Information.

Animals

Viral transduction was validated in C57BL/6J $Sst^{Gfp/+}$ mice (Taniguchi et al., 2011; Lin and Sibille, 2015). Behavioral and molecular experiments were performed using C57BL/6J $Sst^{Cre/+}$ mice ($Sst^{tm2.1(cre)Zjh}$/J, Jackson Laboratories, Bar Harbour, ME; #913944), postnatal day zero at surgery and 9-16 weeks at testing. Mice were maintained under a 12-hour-light/-dark cycle (7 ᴀᴍ-7 ᴘᴍ) with ad libitum food and water and were group housed (4/cage) except during behavioral testing (1/cage). Tests were performed according to Institutional and Canadian Council on Animal Care guidelines.

AAV Vectors and Neonatal Injection

Enhanced serotype inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) vectors used were AAV-PHP.eB-hSyn-DIO-hM4D(Gi)-mCherry (Addgene #44362, Orono, ME) (Chan et al., 2017). Control vectors used were AAV-PHP.eB-hSyn-DIO-mCherry (Addgene #50459).

Brain-wide SST+ cell-specific hM4Di expression was achieved by low-dose bilateral intracerebroventricular (i.c.v.) infusion of PHP.eB serotype Flip-Excision AAVs in postnatal day zero $Sst^{Cre/+}$ mice (Kim et al., 2013) (Supplementary Methods).

For validation experiments, $Sst^{Gfp/+}$ neonates received DREADD (hM4Di) vectors. For characterization experiments, $Sst^{Cre/+}$ neonates received hM4Di ($Sst^{hSyn-hM4Di-mCherry}$ mice) or control ($Sst^{hSyn-mCherry}$ mice) vectors.

Chemogenetic Inhibition of Brain-Wide SST+ cells and $\alpha 5$-PAM Coadministration

For the first characterization experiment, $Sst^{hSyn-hM4Di-mCherry}$ mice received clozapine-N-oxide (CNO = 3.5 mg/kg) or vehicle (Veh = 0.9% saline) i.p. 30 minutes before testing (n = 16/group; 50% female; Fig. 2a). We confirmed that locomotor activity and anxiety-like behavior were not affected by CNO in separate $Sst^{Cre/+}$ cohorts not expressing hM4Di (supplemental Fig. 1).

For the second characterization experiment, we assessed whether deficits induced by SST+ cell silencing could be rescued by coadministering CNO and GL-II-73 (Prevot et al., 2019a). $Sst^{hSyn-mCherry}$ and $Sst^{hSyn-hM4Di-mCherry}$ mice were generated, and all groups received 3.5 mg/kg CNO, achieving SST+ cell silencing only in hM4Di-expressing mice (SST-silenced = $Sst^{hSyn-hM4Di-mCherry}$, SST-control = $Sst^{hSyn-mCherry}$). Mice were randomly assigned to receive Veh (vehicle+CNO) or 10 mg/kg GL-II-73 (+vehicle+CNO) totaling 4 groups (n = 10–12/group; 50% female; Fig. 4a).

Behavioral Analyses

In adulthood, anxiety-like behavior was assessed with the PhenoTyper test (PT), elevated plus-maze (EPM), open field test (OFT), and novelty-suppressed feeding test (NSF); anhedonia-like behavior with sucrose consumption test (SCT); mixed anxiety-/anhedonia-like behavior with novelty-induced hypophagia test (NIH); antidepressant-predictive behavior by the forced-swim test (FST); and memory impairment by the Y-maze (YM) and novel object recognition test (NORT) following past study designs (Fee et al., 2020). CNO was administered 30 minutes prior to test initiation, except for the PT where CNO was administered 30 minutes prior to the dark cycle (90 minutes prior to light challenge) to allow habituation.

Z-score normalization captured changes along behavioral dimensions, directionally, relative to controls (i.e., increase = deficit, decrease = improvement) for anxiety- (PT, EPM, OFT, NSF, NIH) and anhedonia-like behavior (NIH, SCT), behavioral emotionality (all previous + FST), and working and short-term memory impairment (YM, NORT) (Guilloux et al., 2011) (supplemental Methods). Viral transduction efficiency and SST+ cell specificity were visually inspected via immunohistochemistry (IHC) in all mice following behavioral experiments.

IHC and Microscopy

The transduction efficiency and SST+ cell specificity of neonatally delivered i.c.v. AAV-PHP.eB-hSyn-DIO-hM4D(Gi)-mCherry was assessed via IHC in sections from paraformaldehyde-perfused $Sst^{Gfp/+}$ mice fluorescently labeled for GFP and mCherry (n = 8, 2 fields/region/mouse; Fig. 1a).

Forty-eight hours after the last test, $Sst^{hSyn-hM4Di-mCherry}$ mice from behavioral experiments were injected with Veh or CNO and perfused 110 minutes later, approximating CNO + c-Fos peaks, to quantify neuronal activation following SST+ cell silencing (indexed by c-Fos+ cell counts) (Dragunow and Faull, 1989; Ferguson et al., 2011) (Fig. 2a). Cell counts were collected for every third section (N = 4–5 sections/region/mouse) in medial PFC (mPFC), dorsal HPC (dHPC), and basolateral amygdala (Amy). Image processing and counting was performed under blinded
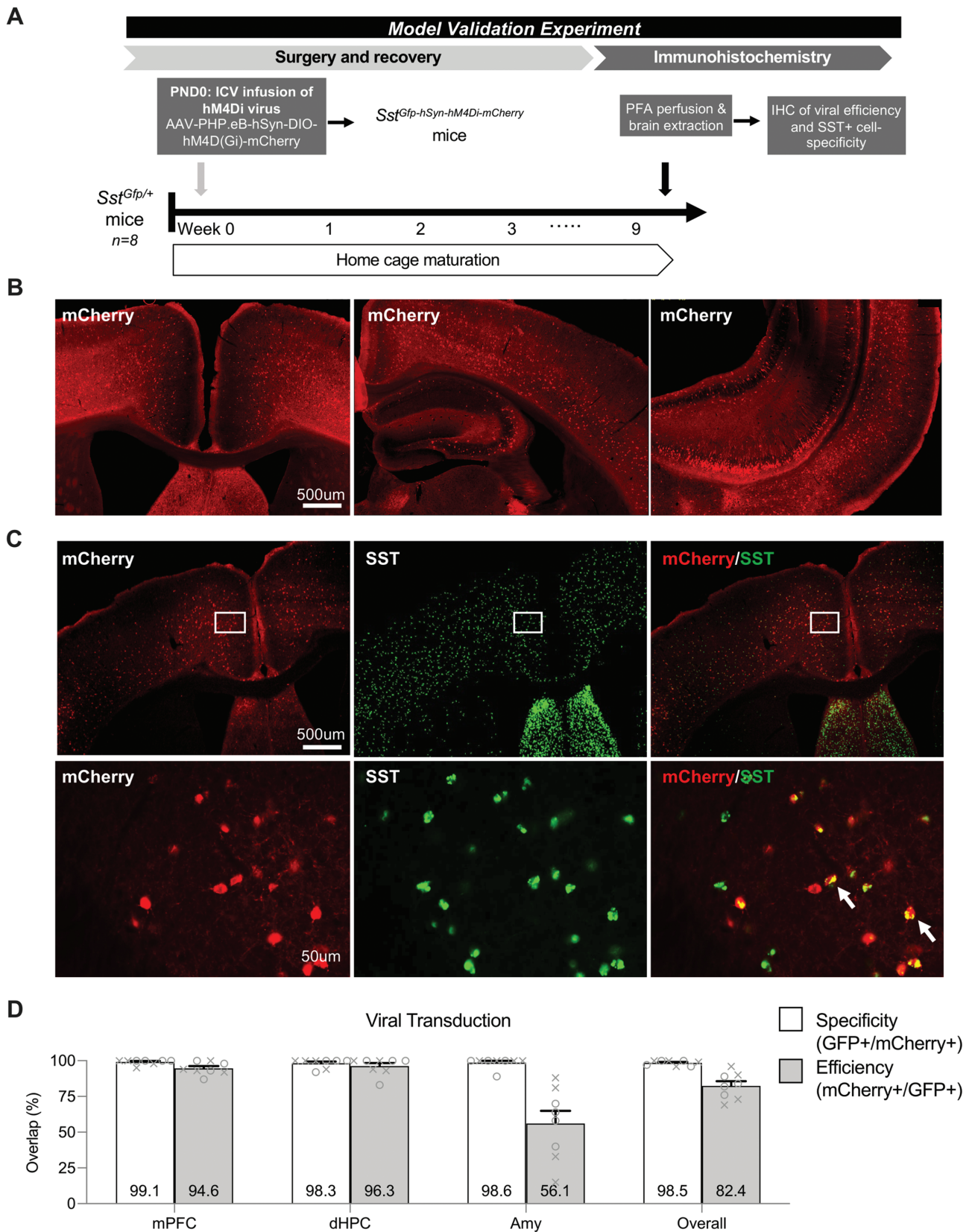
Figure 1. Validation of brain-wide somatostatin-positive (SST+) cell targeting. (A) Experimental design for validation of SST+ cell targeting by 2×10^{10}vg AAV-PHP.eB-DIO-hM4Di-mCherry i.c.v. in postnatal day zero *SstGfp/+* mice, validated by immunohistochemistry (IHC). (B) Representative images of brain-wide viral transduction in adult mice (red=conditional mCherry expression; scale=500 μm). (C) Representative images of hM4Di-mCherry (red) and SST-GFP (green) coexpression (white arrow) in the mPFC. (D) Efficiency (mCherry/GFP) and specificity (GFP/mCherry) of viral transduction in mPFC, dHPC, and Amy (n=8 mice; x=males, o=females).

conditions for Veh/CNO groups ($n = 12$/group; 50% female) using Fiji (Schindelin et al., 2012).

Corticosterone Measurement

In $Sst^{hSyn\text{-}hM4Di\text{-}mCherry}$ mice from behavior and c-Fos experiments, plasma corticosterone was assessed by ELISA (Arbor Assays, Ann Arbor, MI) 110 minutes after Veh/CNO administration from blood collected via cardiac puncture prior to perfusions ($n = 16$ mice/group; 50% female).

Data Analysis

Data were analyzed using SPSS (IBM, NY) and expressed as mean ± SEM. For Veh vs CNO, data were analyzed using 2-way ANOVA with treatment and sex as independent variables. For SST-control vs SST-silenced, data were analyzed using 3-way ANOVA, with group, treatment (Veh vs GL-II-73), and sex as independent variables, using Bonferroni-adjusted post hoc where appropriate. Timecourse parameters were assessed by repeated-measures ANOVA using Greenhouse-Geisser correction. Object recall in the NORT was assessed using paired-samples t tests. Given limitations on sample size, sexes were pooled when significant main effects or interactions with sex were not observed.

Results

Intracerebroventricular AAV-PHP.eB DREADD Infusion in Neonatal $SstCre$ Mice Enables Brain-Wide Manipulation of SST+ Cells in Adulthood

Validation of SST+ cell viral targeting was performed in $Sst^{Gfp/+}$ mice administered low-dose (2×10^{10}vg) AAV-PHP.eB-DIO-hM4Di-mCherry via neonatal i.c.v. infusion (Soumier and Sibille, 2014; Lin and Sibille, 2015) (Fig. 1a–d). In adults, IHC quantification revealed high viral transduction efficiency (mPFC = 95 ± 2%, dHPC = 96 ± 2%, Amy = 56 ± 9%, overall = 82 ± 5%) and SST+ cell specificity (mPFC = 99 ± 1%, dHPC = 98 ± 1%, Amy = 99 ± 1%, overall = 99 ± 1%) that was consistent with Sst expression patterns (NCBI, 2020) (Fig. 1b–d).

Based on established roles for GABA and SST in inhibitory regulation of local neuronal activity and endocrine signaling (Yavorska and Wehr, 2016), chemogenetic SST+ cell silencing was next validated in a separate cohort of $Sst^{hSyn\text{-}hM4Di\text{-}mCherry}$ mice by quantifying neuronal activity via c-Fos+ cell counts (Kovács, 1998) and plasma corticosterone levels 110 minutes following SST+ cell inactivation by CNO (Fig. 2a). Compared with Veh, CNO increased the number of c-Fos+ cells in the mPFC ($F_{1,23} = 30.62$; $P < .001$), HPC ($F_{1,23} = 11.33$; $P = .003$), and Amy ($F_{1,23} = 21.97$; $P < .001$; Fig. 2b). CNO induced a trend-level elevation in corticosterone as assessed by ELISA ($F_{1,28} = 3.51$; $P = .071$; Fig. 2c). No main effect or interactions with sex were found ($P > .3$).

Brain-Wide SST+ Cell Silencing Increases Anxiety-Like Behaviors, Overall Behavioral Emotionality, and Memory Impairment

In the characterization experiment 1 cohort, we sought to determine whether SST+ cell silencing induced anxiety-like behavior (PT, EPM, OFT, NSF), anhedonia-like behavior (NIH, SCT), antidepressant-predictive behavior (FST), and memory impairment (YM, NORT).

Analysis of PT shelter zone activity before, during, and after a 1-hour light challenge in the dark cycle revealed significant

main effects of time ($F_{7.51,217.82} = 2.42$; $P < .05$), group ($F_{1,29} = 6.75$; $P < .05$), and group*time interaction ($F_{7.51,217.82} = 2.18$; $P < .05$; Fig. 3a). CNO significantly increased shelter zone time after injection and persisting for 6 hours after light challenge ($P < .05$). Overall anxiogenic response, indexed by shelter zone time area under the curve (AUC) from light challenge initiation until test completion, confirmed that CNO significantly increased shelter zone time ($F_{1,29} = 11.97$; $P < .01$; Fig. 3b).

Anxiogenic CNO effects were also detected from decreased EPM open arm time ($F_{1,25} = 7.06$; $P < .05$; Fig. 3c) and OFT center zone time ($F_{1,29} = 5.86$; $P < .05$; Fig. 3d). Distance travelled was unchanged between groups in the OFT and EPM (supplemental Table 1). In the NSF, no group differences were detected for novel environment latency to feed (Fig. 3e). In the NIH, CNO induced a trend-level increase in novel environment latency to drink ($F_{1,27} = 3.26$; $P = .082$; Fig. 3f). Locomotor activity (PT, EPM, OFT) and home cage latencies to feed or drink (NSF/NIH) did not differ between groups (supplemental Table 1). No main effect or interaction with sex was found for PT, EPM, OFT, NSF, or NIH parameters ($P > .1$).

In the SCT, CNO did not influence sucrose consumed (Fig. 3g) or sucrose relative to total fluid consumed (supplemental Table 1). There was a main effect of sex as females consumed significantly less sucrose ($F_{2,28} = 7.7$; $P < .01$; supplemental Table 1).

FST analysis revealed a significant effect of time ($F_{3.46, 100.39} = 15.15$; $P < .001$) and trend-level time*treatment interaction ($F_{3.46,100.39} = 2.32$; $P = .071$) as CNO increased immobility in minute 2 ($P < .05$; Fig. 3h).

In the YM, group differences were not detected for pretrial (supplemental Table 1) or trial alternation rates (Fig. 3i). In the NORT pretrial, left/right familiar object times were equivalent for both groups (Fig. 3j). In the NORT trial, Veh-treated mice spent significantly more time with the novel vs familiar object ($t = 4.44$, df = 15; $P < .001$) while CNO-treated mice did not ($t = 1.56$; df = 15; $P = .14$), indicating impaired short-term recall. There was no main effect or interaction with sex for FST, YM, or NORT parameters ($P > .2$).
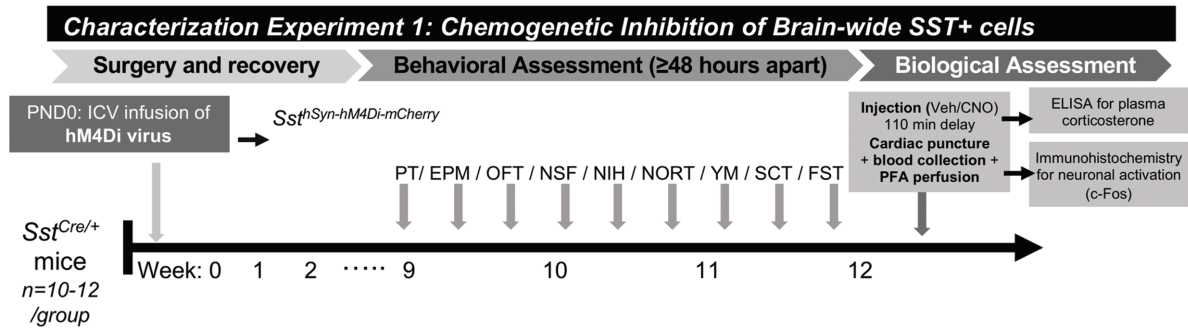
Given the well-characterized variability associated with preclinical behavioral tests (Willner, 2017; Prevot et al., 2019b), we next used Z-scores to assess the consistency of behavioral responses across tests assessing a priori-related dimensions (Guilloux et al., 2011). CNO significantly increased Z-anxiety scores, reflecting PT, EPM, OFT, NSF, and NIH parameters ($F_{1,29} = 17.53$; $P < .001$; Fig. 3k). Z-anhedonia scores did not differ between groups, reflecting SCT and NIH (Fig. 3l). CNO significantly elevated Z-emotionality scores, reflecting all previous tests plus FST ($F_{1,29} = 13.96$; $P < .01$; Fig. 3m). CNO induced a trend-level increase in Z-memory impairment scores, reflecting YM and NORT ($F_{1,29} = 3.49$; $P = .072$; Fig. 3n).

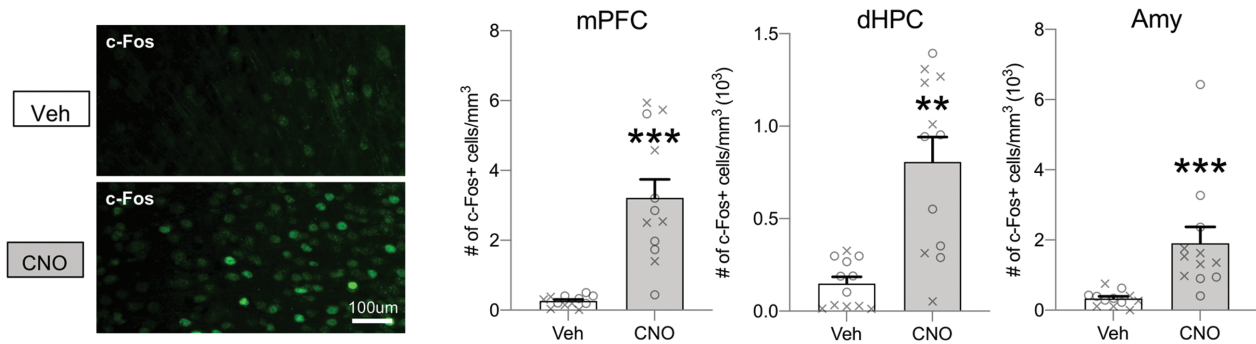## α5-PAM (GL-II-73) Rescues Behavioral Deficits Induced by Brain-Wide SST+ Cell Silencing

SST-control ($Sst^{hSyn\text{-}mCherry}$) and SST-silenced ($Sst^{hSyn\text{-}hM4Di\text{-}mCherry}$) mice were generated by neonatal infusion of control or DREADD viruses in $Sst^{Cre/+}$ mice, and in adulthood administered Veh (vehicle+CNO) or GL-II-73 (vehicle+CNO+GL-II-73) prior to behavioral testing (Fig. 4a).

Analysis of PT shelter zone time revealed a significant main effect of sex ($F_{1,41} = 22.83$; $P < .001$; supplemental Table 2), time ($F_{9.5,389.4} = 6.971$; $P < .001$), and a time*group*treatment interaction ($F_{9.5,389.4} = 2.1$; $P < .05$). SST-silenced+Veh mice had significantly increased shelter zone time after the light challenge ($P < .05$ vs SST-control+Veh; Fig. 1b), and males overall spent more time
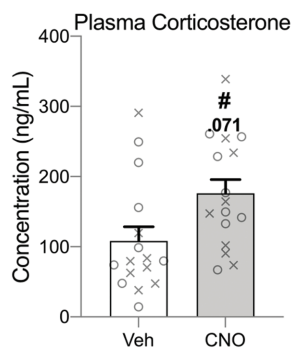
A

Characterization Experiment 1: Chemogenetic Inhibition of Brain-wide SST+ cells

Surgery and recovery ⟩ Behavioral Assessment (≥48 hours apart) ⟩ Biological Assessment

PND0: ICV infusion of **hM4Di virus** → $Sst^{thSyn-hM4Di-mCherry}$

Injection (Veh/CNO) 110 min delay
Cardiac puncture + blood collection + PFA perfusion

ELISA for plasma corticosterone

Immunohistochemistry for neuronal activation (c-Fos)

$Sst^{Cre/+}$ mice n=10-12 /group

Week: 0 1 2 ⋯⋯ 9 10 11 12

PT/ EPM / OFT / NSF / NIH / NORT / YM / SCT / FST

B

C

Figure 2. Validation of brain-wide somatostatin-positive (SST+) cell manipulation. (A) Experimental design for behavioral and biological characterization following chemogenetic SST+ cell silencing in *Sst*thSyn-hM4Di-*mCherry* mice. (B) Representative image of c-Fos+ cells 110 minutes after treatment with vehicle (Veh, top) or 3.5 mg/kg clozapine-N-oxide (CNO, bottom). CNO-hM4Di activation increased neuronal activity (c-Fos+ cell counts) in the mPFC, HPC, and amygdala (Amy) (n=12 mice/group, x=males, o=females). (C) From the same cohort, CNO induced a trend elevation in plasma corticosterone (n=16 mice/group, x=males, o=females). ***P<.001, **P<.01, #P<.1.

in the shelter (supplemental Table 2). This effect was reversed by GL-II-73 in SST-silenced mice (P<.05). For shelter zone AUC, ANOVA revealed a group*treatment interaction ($F_{1,39}$=8.45; P<.01), wherein SST-silenced+Veh mice had increased AUC (P<.01 vs SST-control+Veh) that was similarly reversed by GL-II-73 treatment (P<.05; Fig. 4c). Increased shelter zone AUC was also detected in SST-control+GL-II-73 vs SST-control+Veh mice (P<.05). Locomotor activity did not differ between groups ($F_{1,39}$=.65; P>.59; supplemental Table 2).

Analysis of EPM open arm time revealed a significant main effect of group ($F_{1,35}$=11.63; P=.002) and group*treatment interaction ($F_{1,35}$=8.83; P=.005) (Fig. 4d). GL-II-73 increased open arm time in SST-silenced mice (P<.05 vs SST-silenced+Veh). OFT center zone time was unchanged between groups (Fig. 4e). However, CNO+GL-II-73 significantly increased distance travelled in EPM (P=.044) and OFT (P<.001) (supplemental Table 2).

In the NSF, no group differences were detected for novel environment latency to feed (Fig. 4f). Analyses of NIH novel environment latency to drink revealed significant main effects of sex ($F_{1,39}$=6.57; P<.05), group ($F_{1,39}$=5.31; P<.05), treatment ($F_{1,40}$=16.66; P<.001), and a trend-level group*treatment interaction ($F_{1,40}$=2.93; P=.095), wherein SST-silenced+Veh mice had increased latency (P<.01 vs SST-control+Veh) that was rescued by GL-II-73 (P<.05) (Fig. 4g) and females overall had increased latency to drink (P<.05; supplemental Table 2). Home cage latency to feed (NSF) or drink (NIH) did not differ between groups (supplemental Table 2).

In the SCT, a significant main effect of treatment was detected; GL-II-73 increased sucrose consumption ($F_{1,38}$=6.25; P<.05; Fig. 4h), but not sucrose ratio (supplemental Table 2), indicating increased overall fluid consumption rather than preference.
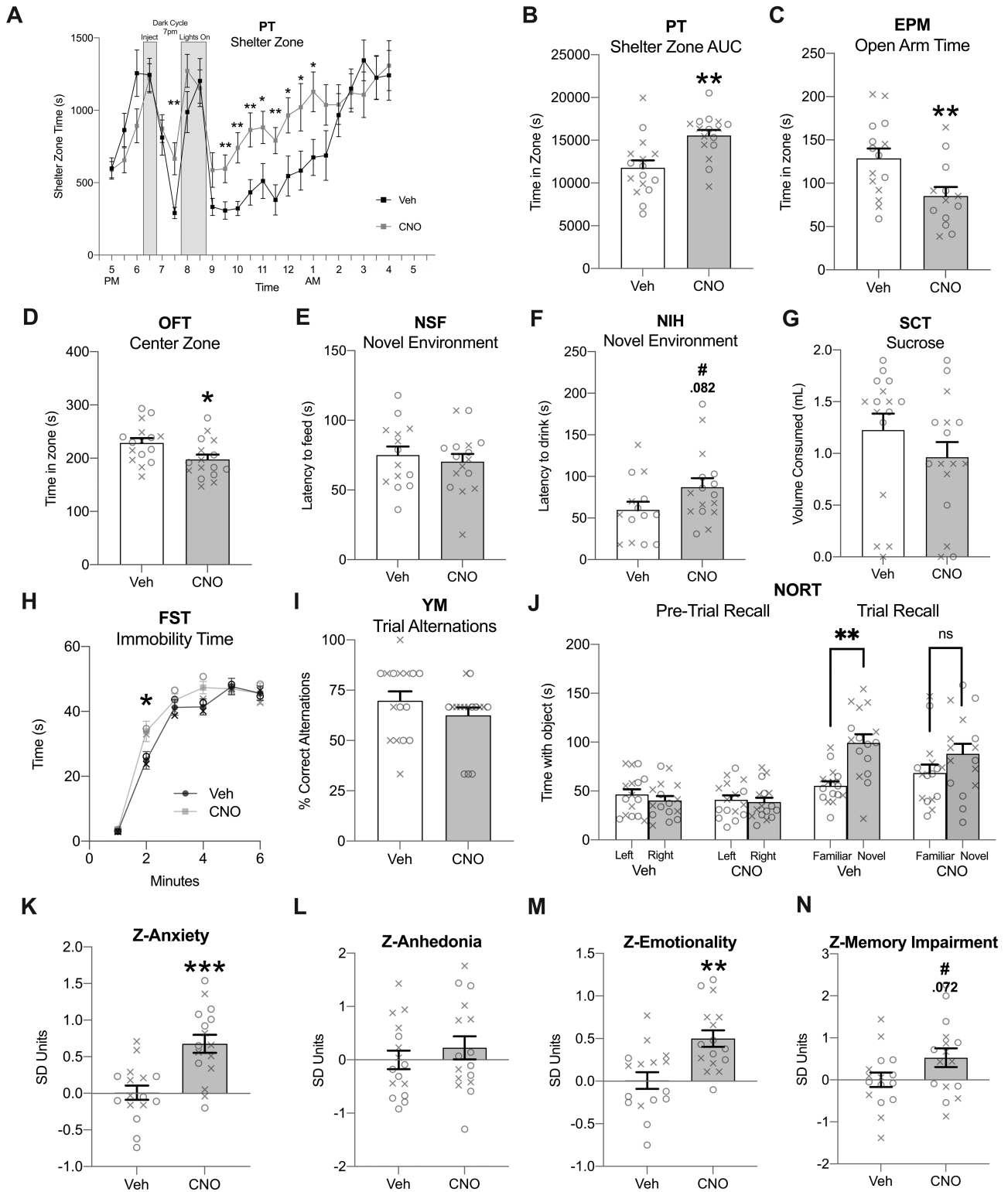
Figure 3. Brain-wide somatostatin-positive (SST+) cell silencing in *SsthSyn-hM4Di-mCherry* mice elevates anxiety-like behavior, overall behavioral emotionality, and memory impairment. (A–J) Behavioral tests administered 30 minutes after injection of vehicle (Veh) or 3.5 mg/kg clozapine-N-oxide (CNO) i.p. except for the PhenoTyper Test (PT) (administered 90 minutes prior to light challenge). (A) Shelter zone time before, during, and after light challenge in the PT. (B) Summary area under the curve (AUC) of shelter zone time from light challenge initiation until the test completion. (C) Time spent in open arms of the elevated plus maze (EPM). (D) Time spent in center zone of the open field test (OFT). (E) Novel environment latency to feed in the novelty suppressed feeding (NSF) test. (F) Novel environment latency to drink milk reward in the novelty-induced hypophagia test (NIH). (G) Sucrose consumed in the sucrose consumption test (SCT). (H) Immobility time in the forced swim test (FST). (I) Percent correct alternations in Y-maze (YM). (J) Pretrial left/right familiar object recall (left) and trial familiar/novel object recall (right). (K-N) Z-scores integrating test parameters assessing anxiety-like behavior (K), anhedonia-like behavior (L), behavioral emotionality (M), and memory impairment (N). n=16 mice/group; male=x/female=o. ***P<.001, **P<.01, *P<.05, #P<.01. Homecage measures in supplemental Table 1.
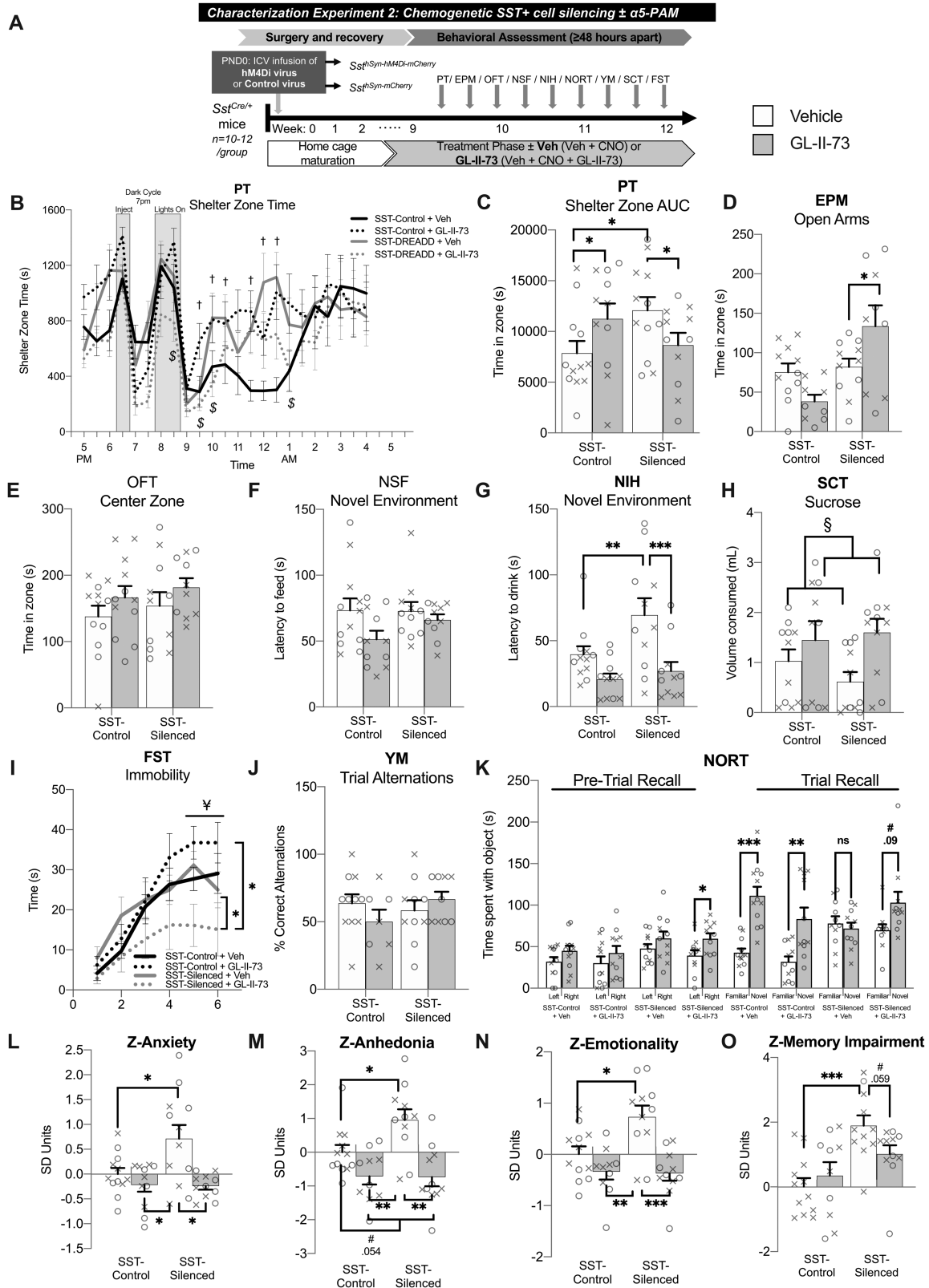
Figure 4. GL-II-73 rescues behavioral deficits induced by brain-wide somatostatin-positive (SST+) cell silencing. (A-J) Behavioral tests administered 30 minutes following i.p. injection of vehicle + CNO (Veh) or vehicle + CNO + GL-II-73 (GL-II-73) in SST-control (*SsthSyn-mCherry*) or SST-silenced (*SsthSyn-hM4Di-mCherry*) mice (except in PhenoTyper as indicated). (B) Shelter zone time before, during, and after light challenge and (C) derived shelter zone area under the curve (AUC) from light challenge initiation until PhenoTyper test (PT) completion. (D) Time spent in open arms of the elevated plus maze (EPM). (E) Time spent in center zone of the open field test (OFT). (F) Novel environment latency to feed for the novelty suppressed feeding (NSF) test. (G) Novel environment latency to drink milk reward for the novelty-induced hypophagia test (NIH). (H) Sucrose consumed in the sucrose consumption test (SCT). (I) Immobility time in the forced swim test (FST). (J) Percent correct alternations in Y-maze (YM). (K) Pretrial left/right familiar object recall (left) and trial familiar/novel object recall (right) in the novel object recognition test (NORT). (L-O) Z-scores integrating test parameters assessing anxiety-like behavior (L), anhedonia-like behavior (M), overall behavioral emotionality (N), and memory impairment (O). Abbreviations: n, 16 mice/group; x, males, o, females. ***$P<.001$, **$P<.01$, *$P<.05$, #$P<.01$, †=$P<.05$ SST-Control+Veh vs SST-Silenced+Veh; $=$P<.05$ SST-Silenced+GL-II-73 vs SST-Silenced+Veh; §= significant main effect of treatment; ¥= significant main effect of group. Homecage parameters in supplemental Table 2.

In the FST, a significant main effect of time ($F_{3,19,102}=6.74$; $P<.001$), time*group interaction ($F_{3,19,102}=3.2$; $P<.05$), and group*treatment interaction were detected ($F_{1,32}=6.87$; $P<.05$; Fig. 4i). GL-II-73 decreased immobility in SST-silenced mice ($P<.05$ vs SST-silenced+Veh, SST-control+GL-II-73). However, this may have been confounded by the previously identified hyperlocomotor effect of GL-II-73.

In the YM, pretrial (supplemental Table 2) and trial (Fig. 4j) alternation rates did not differ between groups. In the NORT pretrial, only SST-silenced+GL-II-73 mice spent significantly more time with the right-located object, indicating side preference that was balanced by rotating novel object position in the trial ($t=2.84$, df=10; $P=.018$; Fig. 4k). In the NORT trial, significant short-term recall was detected for SST-control+Veh ($t=1.62$, df=11; $P<.001$) and SST-control+GL-II-73 ($t=3.89$, df=10; $P=.003$). SST+ cell silencing impaired familiar object recall ($t=.695$, df=10; $P=.503$) that was partially rescued by GL-II-73 ($t=1.85$, df=10; $P=.09$).

For Z-score analysis, EPM, OFT (Z-anxiety), and FST parameters (Z-emotionality) were excluded due to potential locomotor bias, whereas this effect was not detected in other tests. Analysis of Z-anxiety scores (PT, NSF, NIH) revealed a significant main effect of treatment ($F_{1,41}=11.18$; $P<.01$), a trend-level group effect ($F_{1,41}=3.94$; $P=.054$), and a significant group*treatment interaction ($F_{1,41}=4.32$; $P<.05$) (Fig. 4l). SST-silenced+Veh mice had elevated anxiety-like behavior ($P<.05$ vs SST-control+Veh). GL-II-73 had anxiolytic effects in SST-control+GL-II-73 mice and reversed elevated anxiety-like behavior in SST-silenced+GL-II-73 mice ($Ps<.01$ relative to SST-silenced+Veh; Fig. 4l). For Z-anhedonia scores (NIH, SCT), ANOVA detected significant treatment effects ($F_{1,41}=20.13$; $P<.001$), and trend-level group ($F_{1,41}=3.34$; $P<.075$) and group*treatment effects ($F_{1,41}=3.8$; $P=.056$). Z-anhedonia was significantly elevated in SST-silenced+Veh mice ($P<.05$ vs SST-control+Veh) and reduced or rescued in SST-Control+GL-II-73 and SST-Silenced+GL-II-73 mice, respectively ($Ps<.01$ relative to SST-silenced + Veh; Fig. 4m). Z-emotionality scores (all previous tests) were significantly affected by treatment ($F_{1,41}=17.5$; $P<.001$), trend-level group effects ($F_{1,41}=4.1$; $P=.05$), and a significant group*treatment interaction ($F_{1,41}=4.77$; $P<.05$). Z-emotionality scores were increased in SST-silenced+Veh mice ($P<.05$ vs SST-control+Veh) and reduced or rescued in SST-Control+GL-II-73 and SST-Silenced+GL-II-73 mice ($P<.01$; Fig. 4n). Analysis of Z-memory impairment (YM, NORT) revealed a main effect of group ($F_{1,41}=15.18$; $P<.001$) and trend-level group*treatment interaction ($F_{1,41}=3.29$; $P=.07$), wherein SST+ cell silencing increased scores ($P<.05$ vs SST-control + Veh) and GL-II-73 partially rescued these ($P=.059$; Fig. 4o). There was no main effect or interaction with sex for Z-scores.

Discussion

In this report, we validated a novel approach to silence brain-wide SST+ cell function, finding increased neuronal activity in the PFC, HPC, and amygdala, elevated plasma corticosterone, and elevated anxiety- and anhedonia-like behaviors, behavioral emotionality, and memory impairment. Treatment with GL-II-73, an α5-GABAA-R-selective PAM, rescued behavioral changes. These results reveal that brain-wide low SST+ cell function contributes to behavioral changes related to symptoms of mood disorders, and that this low functioning cellular phenotype and its behavioral consequences can be rescued by α5-GABAA-R–selective PAM.

Silencing Brain-Wide SST+ Cell Function

To selectively silence brain-wide SST+ cell function, we employed neonatal i.c.v. infusion of Flip-Excision AAV-PhP.eB hM4Di vectors in $Sst^{Cre/+}$ mice. AAV-PHP.eB capsids have enhanced GABAergic interneuron transduction (Gholizadeh et al., 2013) but previously relied on high-dose systemic administration (Deverman et al., 2016; Chan et al., 2017). We achieved high SST+ cell transduction via low-dose i.c.v. infusion in neonates (Kim et al., 2013) as a cost-effective technique to overcome genetic leakage that is sometimes reported in lox-stop-lox models (Madisen et al., 2012). In $Sst^{Gfp/+}$ mice, cell specificity (approximately 98%–99%) and efficiency (56%–95%) were high, but not complete, consistent with systemic AAV-PHP.eB in $Sst^{Cre/+}$ mice (Allen et al., 2017). This may be due to lower IHC detectability in Sst^{Cre} mice that have reduced Sst levels and contain small numbers of non-SST Cre- or GFP-expressing cells (Ma et al., 2006; Taniguchi et al., 2011; Hu et al., 2013; Viollet et al., 2017).

Whereas past approaches used region-specific cell knockdown or Sst deletion (Soumier and Sibille, 2014; Lin and Sibille, 2015), we employed brain-wide inhibitory DREADDs that inhibit presynaptic firing and neurotransmitter release (Zhu and Roth, 2014), thus aiming to silence both GABA and SST functions. These changes may be more closely related to mood and neurodegenerative disorders where an intrinsic vulnerability causes low SST and GABA markers per cell across corticolimbic brain regions (Fee et al., 2017). Indeed, although not measured directly, putative suppression of SST/GABA functions was validated by corticolimbic hyperactivation in the PFC, HPC, and Amy, implying inhibitory neuron silencing and consistent with past approaches (Soumier and Sibille, 2014; Allen et al., 2017). Although we selected 3 regions for c-Fos analyses, SST+ cell targeting was confirmed via qualitative inspection throughout the neocortex in all mice. As a limitation, our approach did not distinguish SST vs GABA roles as both markers are reduced in MDD (Fee et al., 2017). However, corticolimbic hyperactivation is an expected outcome reflecting silencing both factors given their shared roles in PN inhibition (Tallent and Siggins, 1997; Schweitzer et al., 1998; Stengel and Taché, 2017) and consistent with past chemogenetic SST+ cell-silencing studies (Soumier and Sibille, 2014; Allen et al., 2017) and chronic stress studies wherein GABA and SST markers are selectively reduced (Lin and Sibille, 2015; Girgenti et al., 2019; Fee et al., 2020). Plasma corticosterone elevation also validated reduced SST function, given SST roles in inhibitory regulation of corticosteroid release (Engin and Treit, 2009; Prévôt et al., 2017; Prévôt et al., 2018). That corticosterone effects only reached trend level may reflect the long measurement window chosen (designed also for c-Fos half-life). Indeed, Sst-ablated mice had elevated corticosterone at baseline, but not during or after stress (Lin and Sibille, 2015). Behavioral findings were also consistent with low GABAA-R signaling (Ren et al., 2016) or SST knockdown mice (Lin and Sibille, 2015). Finally, given that GABA-AR–acting BZDs and SST receptor-acting analogs confer anxiolytic- and antidepressant-like effects in rodents (Sanders and Shekhar, 1995; Engin et al., 2008; Yeung et al., 2011; Prévôt et al., 2017), evidence suggests that both systems regulate mood and cognitive functions.

Behavioral deficits were induced by repeated acute CNO administration across 9 tests and were relatively lasting (i.e., persisting 6 hours after injection in PT), consistent with past chemogenetic studies (Guettier et al., 2009; Alexander et al., 2009). The amount of 3.5 mg/kg CNO was selected to achieve

brain penetrant levels >EC$_{50}$ for hM4Di for approximately 15–30+ minutes after administration and clearing from plasma within 2 hours (Jendryka et al., 2019). Therefore, consistent with past studies (Smith et al., 2016), we do not expect lingering subchronic effects from repeated hM4Di activation and consider behavioral deficits observed to reflect direct consequences of reduced upstream SST+ cell regulation of corticolimbic functions.

The DREADD approach has several technical caveats, including CNO to clozapine back-metabolism that may confer off-target effects (Gomez et al., 2017; Jendryka et al., 2019). However, CNO-derived clozapine did not exceed hM4Di EC$_{50}$ at the selected dose (Gomez et al., 2017), and CNO alone did not alter behavior in *Sst*$^{Cre/+}$ control mice (supplemental Fig. 1) or locomotion in SST-control mice (Fig. 3). Furthermore, when all groups received CNO, behavioral changes were detected only in hM4Di-expressing mice (Fig. 4). As human pathologies reflect chronic conditions, we tested chronic SST+ cell silencing via frequent repeated i.p. injections or oral CNO delivery but found no behavioral changes (Unpublished data, Fee, 2020). Rather than this being a consequence of chronic SST+ cell silencing, we attributed these effects to technical limitations of DREADDs, including that CNO has a rapid half-life and is challenging to administer at levels exceeding hM4Di EC$_{50}$ orally over long time periods without back metabolism and accumulation of lipophilic clozapine (Gomez et al., 2017; Jendryka et al., 2019). Indeed, evidence of chronic chemogenetic hM4Di activation is sparse, including null or opposite effects compared with acute hM4Di activation (Soumier and Sibille, 2014; Poyraz et al., 2016; Urban et al., 2016), whereas chronic excitatory DREADD activation is more common due to the unique pharmacokinetic properties of hM3Dq (Jain et al., 2013). These challenges justify the ongoing development of improved DREADD actuators or delivery systems (Bonaventura et al., 2019).

SST+ Cells and Depressive-Like Behavior

Low SST and GABA markers in postmortem MDD patients and elevated behavioral emotionality in mice with altered SST+ cell function suggest a contributing role in mood disorders (Fee et al., 2017). We replicated past findings, including anxiety-like behaviors observed in *Sst* knockout (Lin and Sibille, 2015) and acute chemogenetic PFC SST+ cell-silenced mice (Soumier and Sibille, 2014). Our findings extended roles for SST+ cell regulation of behavioral deficits to antidepressant-predictive (e.g., FST) and short-term memory dimensions (e.g., NORT). However, we did not find working memory impairment as with optogenetic PFC SST+ cell silencing, possibly due to distinct inhibition timing, that is, initiated before vs during the test (Abbas et al., 2018).

Notably, past studies found that acute and chronic blockade of PFC SST+ cell function had opposite physiological and anxiogenic outcomes (Seybold et al., 2012; Soumier and Sibille, 2014). However, region-specific manipulations may confer compensatory neural circuit adaptations that are not reflective of human pathology, especially on regional cellular ablation rather than cell silencing (Fee et al., 2017). Indeed, we found behavioral changes closely paralleling rodent chronic stress studies (Nikolova et al., 2018; Prevot et al., 2019b; Fee et al., 2020) that demonstrated selective SST+ cell functional deficits via dysregulated cell integrity pathways (Lin and Sibille, 2015; Girgenti et al., 2019; Oh et al., 2019). These findings suggest that SST+ cell deficits are an intermediary causal factor between upstream risk factors (e.g., chronic stress, altered proteostasis and neurotrophic factor signaling) and symptom emergence (Fee et al., 2017; Prévot and Sibille, 2021).

In SST-control vs SST-silenced mice (using control vs hM4Di viruses), we replicated anxiogenic findings from the first characterization experiment (using Veh vs CNO) but found a stronger loading on anhedonia-like deficits. The lack of reproducibility on individual tests of anxiety-like behavior is a limitation of the current findings. However, the well-noted replicability challenges of individual behavioral tests (Willner, 2017) justified development of the PhenoTyper Test (a fully automated test with higher reproducibility than EPM and OFT; Prevot et al., 2019b) and Z-scoring methods that increase the power and reliability of preclinical phenotyping (Guilloux et al., 2011). To this extent, we found that SST+ cell silencing induced consistent anxiogenic changes detected by the PT and Z-scoring across experiments.

Given that SST+ cell deficits are more severe in females (Seney et al., 2013, 2015), sex was investigated as a main factor. However, differences were sparse as, for example, anhedonia-like behavior was increased in female mice but unrelated to SST+ cell silencing.

Antidepressant Potential of α5-PAM

α5-GABAA-R potentiation via GL-II-73 had antidepressant-, anxiolytic-like, and pro-memory effects in mice with brain-wide low SST+ cell function. GL-II-73 rescued deficits in the EPM and FST, consistent with effects in wild-type mice (Prevot et al., 2019a) and with phenotypes of mice having disinhibited SST+ cell function (Fuchs et al., 2017). However, CNO+GL-II-73 conferred hyperlocomotor effects in the EPM, OFT (but not the PT), and presumably the mobility-dependent FST (supplemental Table 2), so these results were excluded from Z-score analysis. These changes did not appear in past studies of GL-II-73 alone (Prevot et al., 2019b), so we cannot preclude potential drug-drug interactions. However, corrected Z-scoring revealed that GL-II-73 reduced anxiety- and anhedonia-like behavior, overall emotionality, and (trend-level) memory impairment in both SST-control and SST-silenced mice, consistent with past studies (Prevot et al., 2019a). Contrasting findings in chronic stress-exposed mice, GL-II-73 did not improve YM performance, potentially due to the reactive phenotype of *Sst*^{Cre} mice and the handling necessary for this test (Viollet et al., 2017). Indeed, SST-controls had lower YM alternation rates compared with wild-type mice in past studies (Prevot et al., 2019a). Finally, GL-II-73 induced trend-level rescue of NORT deficits (a low-handling test), consistent with HPC α5-GABA-AR mediation of short-term memory (Möhler and Rudolph, 2017).

Others reported antidepressant-like and pro-cognitive properties from α5-knockdown or negative allosteric modulators (NAMs) (Martin et al., 2010; Fischell et al., 2015; Zanos et al., 2017; Bugay et al., 2020). Although these findings appear to conflict with α5-PAMs, it is proposed that SST+ cell regulation of microcircuit activity benefits from "tightening" in contexts characterized by underinhibition (i.e., poor information encoding) and "loosening" in contexts characterized by overinhibition (i.e., poor information transfer), supporting the utility of α5-PAM/NAM for different contexts, behaviors, or disorders (Prévot and Sibille, 2021). Indeed, we found that α5-PAM had therapeutic effects in SST-silenced mice with corticolimbic hyperactivity. Another possibility is that α5-PAMs and NAMs could increase the signal-to-noise ratio of corticolimbic microcircuits through different mechanisms, for example, by promoting phasic vs tonic PN activity with α5-PAMs or by normalizing PN signaling through ketamine-like synaptic plasticity with α5-NAMs (Bugay et al., 2020).

Future Directions

Despite marked improvement in sedative and amnesic side-effect profiles of BZDs, α5-selective derivatives such as GL-II-73 have mild α1–3 GABAA-R potentiation (Prevot et al., 2019a) that may worsen or improve therapeutic efficacy. For example, more selective α5-PAMs (Stamenić et al., 2016) had weaker antidepressant-like and pro-cognitive effects only in chronic stress-exposed female (Piantadosi et al., 2016) and nonstressed male mice (Prevot et al., 2019a). Given that regional localization impacts α1-5-subunit functional differentiation (Sieghart and Sperk, 2002), characterization studies using gene profiling (Lin and Sibille, 2015) or RNA labeling (Hu et al., 2019) may inform therapeutic strategies.

In conclusion, we demonstrated that brain-wide SST+ cell function regulates mood and cognitive functions and found support that acute disruptions contributing to anxiety- and anhedonia-like behaviors, overall behavioral emotionality, and impaired memory may reflect similar processes in psychiatric diseases over a longer scale. Deficits arising from brain-wide low SST+ cell function were rescued by α5-PAM, representing a promising new avenue for the development of targeted antidepressants.

Interest Statement

D.K., G.L., P.M., J.C., E.S., M.B., and T.P. are co-inventors or listed on US patent applications that cover GABAergic ligands and/or their use in brain disorders. E.S. is co-founder of Alpha Cog, a biotech company developing ligands, including GL-II-73, as procognitive therapeutics. C.F. and K.M. have no conflicts of interest to disclose.

Author Contributions

C.F., M.B., and E.S. conceived of the study design. C.F. performed all experiments, data acquisition, and analysis with assistance from T.P., K.M., and M.B. C.F. wrote the manuscript with critical input from T.P., M.B., and E.S. D.K., G.L., P.M., and J.C. contributed to distinct steps for the synthesis and development of GL-II-73.

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