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Downregulation of Synaptotagmin 1 in the Prelimbic Cortex Drives Alcohol-Associated Behaviors in Rats

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ABSTRACT

BACKGROUND: Alcohol addiction is characterized by persistent neuroadaptations in brain structures involved in motivation, emotion, and decision making, including the medial prefrontal cortex, the nucleus accumbens, and the amygdala. We previously reported that induction of alcohol dependence was associated with long-term changes in the expression of genes involved in neurotransmitter release. Specifically, *Syt1*, which plays a key role in neurotransmitter release and neuronal functions, was downregulated. Here, we therefore examined the role of *Syt1* in alcohol-associated behaviors in rats.

METHODS: We evaluated the effect of *Syt1* downregulation using an adeno-associated virus (AAV) containing a short hairpin RNA against *Syt1*. Cre-dependent *Syt1* was also used in combination with an rAAV2 retro-Cre virus to assess circuit-specific effects of *Syt1* knockdown (KD).

RESULTS: Alcohol-induced downregulation of *Syt1* is specific to the prelimbic cortex (PL), and KD of *Syt1* in the PL resulted in escalated alcohol consumption, increased motivation to consume alcohol, and increased alcohol drinking despite negative consequences ("compulsivity"). *Syt1* KD in the PL altered the excitation/inhibition balance in the basolateral amygdala, while the nucleus accumbens core was unaffected. Accordingly, a projection-specific *Syt1* KD in the PL–basolateral amygdala projection was sufficient to increase compulsive alcohol drinking, while a KD of *Syt1* restricted to PL–nucleus accumbens core projecting neurons had no effect on tested alcohol-related behaviors.

CONCLUSIONS: Together, these data suggest that dysregulation of *Syt1* is an important mechanism in long-term neuroadaptations observed after a history of alcohol dependence, and that *Syt1* regulates alcohol-related behaviors in part by affecting a PL–basolateral amygdala brain circuit.

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Alcohol addiction is characterized by persistent changes in motivation, emotion, and decision making. These are thought to reflect long-term neuroadaptations in brain structures that subserve these functions, including the prefrontal cortex (PFC) (1). The PFC exerts top-down regulation of subcortical structures, such as the nucleus accumbens and amygdala complex, that are thought to be involved in the control of addiction-related behaviors (2). PFC function is also particularly vulnerable to disruption by stress, an important risk factor for alcohol addiction (3,4). We and others (5–7) have previously demonstrated that the PFC is sensitive to long-lasting changes in gene expression during withdrawal and protracted abstinence from alcohol. Specifically, we found that chronic intermittent alcohol exposure induced long-term expression changes of genes involved in exocytosis and neurotransmitter release (7,8).

Among genes dysregulated by alcohol, *SYT1* is of particular interest, as it plays a crucial role in several phases of synaptic transmission and plasticity (9). SYT1 belongs to a family of membrane-trafficking proteins called synaptotagmins, which consists of 17 members. It acts as the main calcium sensor for fast presynaptic vesicle exocytosis as well as for endocytosis (10). Recently, Wu *et al.* (11) demonstrated that Ca^{2+}-dependent exocytosis of AMPA receptors during long-term potentiation was driven by both SYT1 and SYT7 in the hippocampal CA1 region. Together, these data suggest a crucial role of SYT1 in neurotransmission and synaptic plasticity. Preclinical studies have also shown that inhibition of *Syt1* expression in the prelimbic cortex (PL) alters fear memory processes (12), which are affected by alcohol addiction (13).

Several studies indicate that *SYT1* expression is sensitive to alcohol exposure, but its mechanistic role remains unclear. For instance, acute alcohol exposure resulted in increased *Syt1* expression in mouse cortical neurons (14). In contrast, *SYT1* expression was decreased in postmortem tissue from the nucleus accumbens of human alcohol-dependent patients (15). In rats, we found decreased *Syt1* expression in the PL following chronic intermittent alcohol exposure (8). Although originating from different model systems, these observations potentially indicate differential effects on *Syt1* expression

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depending on the duration of alcohol exposure. Consistent with our data, *Syt1* expression was also downregulated in the PFC of Alko alcohol-accepting rats compared with their non–alcohol-accepting counterparts (16), suggesting that *Syt1* downregulation may be associated with increased alcohol consumption.

Given the central role of SYT1 in neurotransmitter release and neuronal functions, we hypothesized that *Syt1* downregulation may be a mechanism that promotes alcohol addiction-like behaviors following development of dependence. To examine this hypothesis, we used a viral *Syt1* knockdown (KD) strategy in rats and assessed the effects on a range of behaviors characteristic of the clinical alcohol addiction syndrome (17). These behaviors included alcohol consumption, motivation to obtain alcohol, and continued alcohol intake despite negative consequences; the latter behavior was operationalized as insensitivity to quinine adulteration and is hereafter referred to as "compulsivity" (18).

PL exerts top-down regulation of subcortical regions including the basolateral amygdala (BLA) and the nucleus accumbens core (NAcC) (2,19). Moreover, PL projections to these regions have been implicated in control of drinking behaviors (20,21). To determinate whether *Syt1* KD in the PL regulates alcohol addiction-like behaviors through these circuits, we used a projection-specific strategy that utilized a Cre-dependent KD vector in combination with a retrogradely transported Cre vector and assessed the specific contribution of PL projections to the BLA and NAcC, respectively. Because SYT1 is important for vesicle trafficking and Ca^{2+}-dependent exocytosis, we also evaluated the effects of *Syt1* downregulation on neurotransmission in the PL and in its downstream target regions: BLA, NAcC, and dorsomedial striatum (DMS).

METHODS AND MATERIALS

Animals

Adult male Wistar rats (200–225 g; Charles River, Wilmington, MA) were housed in a temperature- and humidity-controlled environment under a reverse light cycle (lights off at 7:00 AM) with food and water ad libitum. Rats were habituated to the facility and handled prior to experiments. Behavioral experiments took place during the dark phase. Procedures were conducted in accordance with the National Committee for Animal Research in Sweden and approved by the Local Ethics Committee for Animal Care and Use at Linköping University.

Surgery

***Syt1* KD PL.** Rats received 2 injections bilaterally (0.25 μL per injection; rate: 0.1 μL/min) directly into the PL [coordinates relative to bregma: anteroposterior: +3 mm, mediolateral: ±0.6 mm, dorsoventral: −3.5 mm (22)] of an adeno-associated virus (AAV) containing a short hairpin RNA targeting *Syt1* (AAV9.HI.shR.Syt1.CMV.ZsGreen.SV40; AACTGGGAAAGCTCCAAGCTCCAATATT; UPenn Core Facility, Philadelphia, PA) and a scrambled control (AAV9.HI.shR.luc.CMV.ZsGreen.SV40).

***Syt1* KD PL-BLA and PL-NAcC.** Rats received 2 injections bilaterally (0.25 μL per injection; rate: 0.1 μL/min) directly into the PL [coordinates relative to bregma: anteroposterior: +3 mm, mediolateral: ±0.6 mm, dorsoventral: −3.5 mm (22)] of an AAV containing a Cre-dependent microRNA targeting *Syt1* (AAV9.hSyn.DIO.-mcherry.miR.Syt1.WPRE; AACTGGGAAAGCTCCAAGCTCCAATATT; UPenn Core Facility). Rats also received 2 injections bilaterally (0.5 μL per injection; rate: 0.1 μL/min) into the BLA [coordinates relative to bregma: anteroposterior: +2.4 mm, mediolateral: ±5 mm, dorsoventral: −8.4 mm (22)] or the NAcC [coordinates relative to bregma: anteroposterior: +1.8 mm, mediolateral: ±1.4 mm, dorsoventral: −6.7 mm (22)] of an AAV-retro2-hSyn1-EGFP_iCre-WPRE-hGHp(A) (Addgene, Watertown, MA).

Overview of Behavioral Testing

Rats underwent multiple alcohol-related behavioral tests including operant alcohol self-administration, progressive ratio (PR), and quinine adulteration. Rats were also tested for locomotor activity, quinine preference, and saccharin self-administration.

Alcohol Self-administration

Operant training and testing were performed in operant chambers (30.5 × 29.2 × 24.1 cm; Med Associates Inc., St. Albans, VT) housed in sound-attenuating cubicles. Rats were trained to self-administer 20% alcohol under a fixed ratio 1 (FR1) schedule during 30-minute sessions as described previously (23–25). Reinforcement schedule was switched to FR2 once stable responding was obtained on FR1. Once baseline stabilized under FR2, rats received viral vector microinjections and were tested for alcohol self-administration after 2 weeks of recovery.

Progressive Ratio

PR was performed as described previously (24). Briefly, rats were tested for PR after stable responding was reached on FR2. The progression of lever presses required to receive an alcohol reinforcer was 1, 2, 3, 4, 6, 8, 10, and 12, after which the ratio increased in steps of 4. The breakpoint was defined as the last ratio completed before 30 minutes passed without the completion of the next ratio and reflected how much the rat was willing to work to obtain 1 reinforcer.

Compulsivity/Quinine Adulteration

Rats were assessed for aversion-resistant alcohol intake using quinine adulteration. After stable alcohol self-administration under FR2, increasing concentrations of quinine (10, 25, 50, 75, 100, and 150 mg/L) were added to the ethanol (20%). Quinine concentration was increased every 3 sessions. Resistance to quinine adulteration was assessed by measuring the percentage of decreased reward after addition of quinine.

Locomotor Activity

Locomotor activity was tested for 30 minutes in sound-attenuated chambers (43 × 43 cm) equipped with an infrared beam detection system (Med Associates) and under ambient light level (190–210 lx).

Saccharin Self-administration

As a control of behavioral specificity, saccharin self-administration was performed under conditions similar to those of alcohol self-administration. Rats were trained to self-administer 0.2% saccharin in 30-minute sessions under FR1 and then an FR2/5 second time-out schedule of reinforcement. Once a stable self-administration baseline was reached, rats received viral vector microinjection into the PL. After 2 weeks, rats were tested for saccharin self-administration.

Quinine Preference

As a control for taste reactivity, quinine preference was assessed using a two-bottle choice paradigm, where increasing concentrations of quinine was added to one bottle (0, 5, 10, and 25 mg/L). Quinine concentration was increased every 4 days.

RNAscope: Fluorescent In Situ Hybridization

After euthanasia, brains were removed and flash frozen. Then 12-μm brain sections were collected at the PL level and kept at −80°C until use. In situ hybridization was performed according to the RNAscope Fluorescent Multiplex Kit User Manual (Advanced Cell Diagnostics, Newark, CA) and as previously described (6). *Syt1* (accession number NM_001033680.2) probe was purchased from Advanced Cell Diagnostics. Sections were incubated with a series of 4 amplifier probes at 40°C. During the last step, sections were incubated with fluorescently labeled probe Atto 550 (red) in C1 channel to visualize *Syt1*. Brains sections were examined with a confocal microscope (Zeiss LSM 700; Carl Zeiss AG, Jena, Germany) at 20× magnification to create images for quantification. *Syt1* messenger RNA levels were assessed as total pixels of the fluorescent signal (fluorescent "dots") using ImageJ software (National Institutes of Health, Bethesda, MD) (26).

Ex Vivo Electrophysiology

SYT1 is fundamental for synaptic exocytosis, and to assess the impact of *Syt1* KD on neurotransmission, ex vivo electrophysiology was performed on ethanol-naïve rats as previously described in detail (27) and in Figure S1. In brief, local field population spikes were evoked in acutely isolated coronal brain slices (300 μm) with a 20-second interpulse interval. Recording electrodes were positioned in the PL, NAcC, DMS, and BLA (see the Supplement). Stimulation electrodes were positioned locally, 0.2 to 0.3 mm from the recording electrode, and stimulus/response curves were created by stepwise increasing the stimulation strength in 7 steps.

To assess changes in release probability, responses were evoked with a paired pulse stimulation protocol (0.1 Hz, 50-ms interpulse interval), and the paired pulse ratio (PPR) was calculated by dividing the second pulse (PS2) with the first pulse (PS1). To monitor changes in GABAergic (gamma-aminobutyric acid) neurotransmission, changes in excitability were recorded during bath perfusion of the $GABA_A$ receptor ($GABA_AR$) antagonist bicuculline-methiodide (bicuculline) (20 μM). Drugs were purchased from Tocris Bioscience (Bristol, UK).

Statistical Analysis

Homogeneity of variance was assessed using the Levene test. When data violated the criteria, statistical analysis was performed using the Kruskal-Wallis nonparametric analysis of variance (ANOVA). When no violation was observed, parametric ANOVA was used, with factors for the respective analysis indicated in conjunction with its results. When appropriate, post hoc comparisons were performed using Newman-Keuls test. The accepted level of significance for all tests was $p < .05$. Electrophysiological data were analyzed using Clampfit version 10.2 (Molecular Devices, Sunnyvale, CA), Microsoft Excel (Microsoft Corp, Redmond, WA), and GraphPad Prism version 7 (GraphPad Software, San Diego, CA). Gaussian distribution was tested with D'Agostino-Pearson omnibus normality test. A 2-way ANOVA was used for comparisons over time and input/output function, while paired or unpaired Student's *t* tests were used for statistical analysis of PPR.

RESULTS

Selective *Syt1* KD Within the Prelimbic Medial PFC

We had previously reported a downregulation of *Syt1* following a history of alcohol dependence (8); using material from that study, we first established that this effect was selective for the PL and did not encompass the infralimbic (IL) medial PFC (mPFC) (Figure S1). We then proceeded to examine whether this *Syt1* downregulation within the PL is mechanistically involved in behavioral consequences of alcohol dependence. To this end, we downregulated *Syt1* expression in nondependent animals and assessed whether this would result in behavioral consequences similar to those observed following a history of alcohol dependence.

We targeted the PL and injected an AAV vector expressing a *Syt1* short hairpin RNA targeting *Syt1* in the PL, AAV9.-HI.shR.ratSyt1.CMV.ZsGreen.SV40 (*Syt1* KD). One week after the end of the behavioral testing, we measured *Syt1* expression using RNAscope. *Syt1* KD significantly downregulated *Syt1* expression in the PL compared with animals injected with a scrambled control vector (Kruskal-Wallis nonparametric ANOVA: main effect of group, $H_{1,12} = 8.1$; $p = .005$) (Figure 1C). This effect was highly selective for the PL; specifically, *Syt1* messenger RNA expression in the adjacent infralimbic cortex was unaffected (Figure 1D).

Syt1 KD in the PL Promotes Alcohol-Addiction-like Behaviors

***Syt1* KD Increases Alcohol Self-administration.** Decreasing the expression of *Syt1* in the PL resulted in significantly increased alcohol self-administration compared with injection of the control vector (repeated measures ANOVA: main effect of group $F_{1,72} = 21$; $p = .0006$) (Figure 1E). *Syt1* KD rats increased their alcohol self-administration rates compared with their own baseline (average rewards earned during the week prior to surgery). In contrast, rats injected with the scrambled control vector did not show any changes in self-administration rates compared to their presurgery baselines (2-way ANOVA: interaction phase (baseline vs. test) × treatment group (scrambled vs. *Syt1* KD): $F_{1,14} = 9.8$; $p = .007$; Newman-Keuls post hoc test:

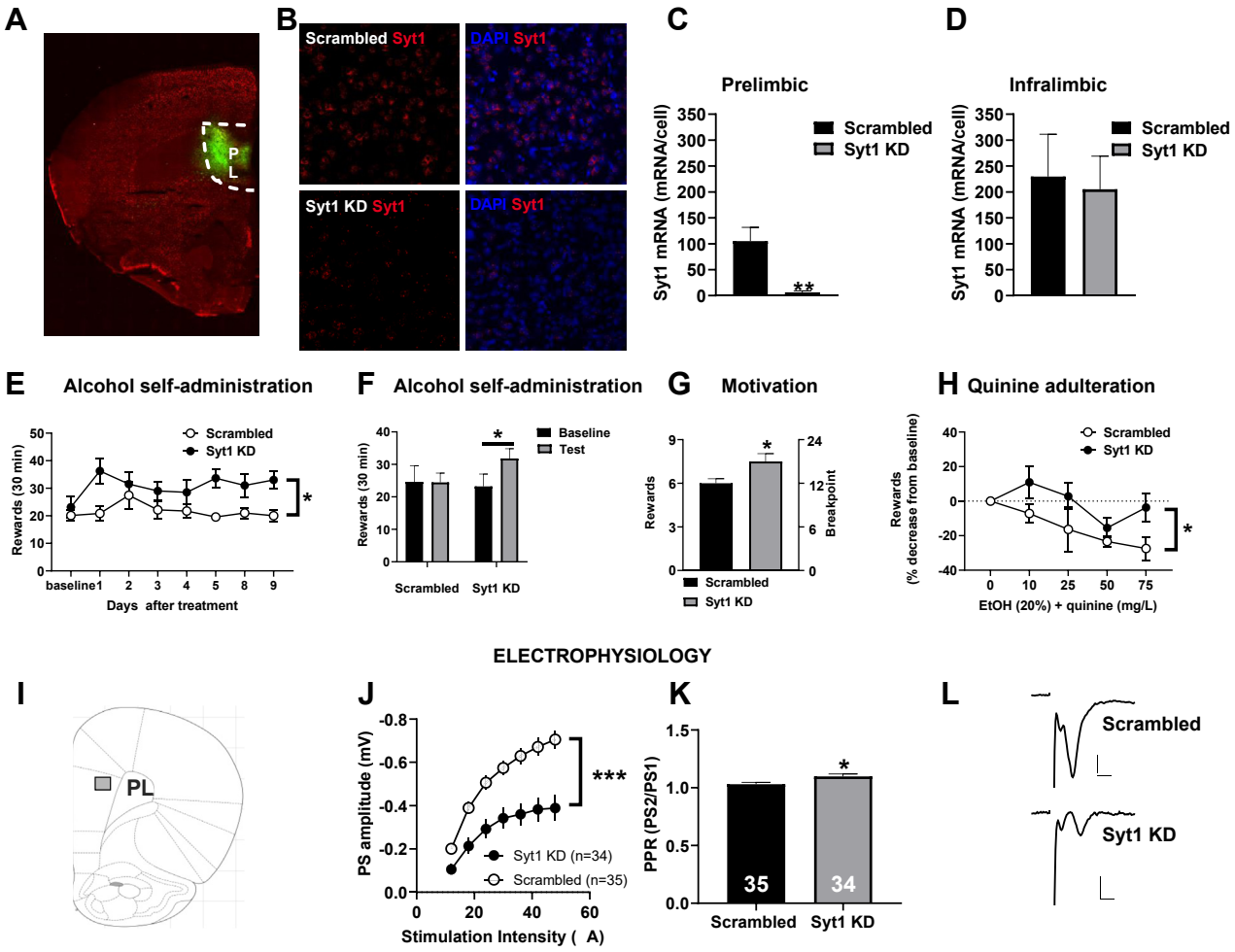

Figure 1. KD of *Syt1* in the PL promotes alcohol-addiction-like behaviors. **(A)** Representative image of virus injection site and spread is shown in panels **(A)** and **(B)**. RNAscope was used to quantify the KD in the PL (n = 5–7) **(C)** and the infralimbic cortex (n = 5–7) **(D)** to ensure viral efficiency and anatomical specificity. Representative micrographs are shown in panel **(B)**. KD of *Syt1* in the PL increased the alcohol consumption when compared with consumption in scrambled controls **(E)** and baseline **(F)**, an effect that lasted for 9 days (n = 7–8). It also increased the motivation to consume alcohol as measured by progressive ratio **(G)** and increased compulsivity as measured by insensitivity to quinine adulteration **(H)**. Field potential recordings in the PL **(I)** demonstrated significantly lower evoked potentials **(J)** and indicated a decreased release probability in *Syt1* KD rats compared with in controls **(K)**. Representative recordings are shown in panel **(L)**. *$p < .05$; ***$p < .001$. EtOH, ethanol; KD, knockdown; mRNA, messenger RNA; PL, prelimbic cortex; PPR, paired pulse ratio; PS, population spike.

Syt1 KD test vs. baseline $p = .003$; no other pairwise tests significant) (Figure 1F).

***Syt1* KD Increases Motivation to Consume Alcohol.** We then evaluated the effect of *Syt1* KD on the motivation to obtain alcohol using a PR schedule of reinforcement. One-way ANOVA showed a main effect of group (scrambled vs. *Syt1* KD; $F_{1,13} = 6.1$; $p = .02$) indicating that decreased expression of *Syt1* in the PL increased the motivation to consume alcohol, as shown by a higher breakpoint (Figure 1G).

***Syt1* KD Increases Compulsive Alcohol Intake.** Finally, we evaluated the role of *Syt1* KD on compulsive alcohol drinking, i.e., insensitivity to quinine adulteration. Repeated-measures ANOVA showed a significant main effect of group (scrambled vs. *Syt1* KD; $F_{1,13} = 0.3$; $p = .02$), indicating that downregulation of *Syt1* in the PL cortex reduced sensitivity to quinine adulteration (Figure 1H).

Neuronal Excitability Is Reduced in the PL mPFC Following *Syt1* KD

We then assessed persistent effects of *Syt1* downregulation on PL neuronal excitability using field potential recordings (Figure 1I). Evoked field potentials in the PL were significantly depressed in brain slices from *Syt1* KD as compared to scrambled rats (main effect of group: $F_{1,67} = 16.7$; $p < .001$) (Figure 1J). Furthermore, the PPR was increased in slices from *Syt1* KD rats, indicative of a reduced probability for transmitter release ($t_{1,67} = 2.48$; $p = .016$) (Figure 1K). Together, these data

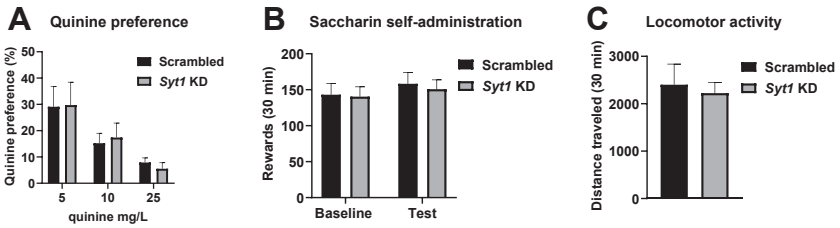

Figure 2. Observed effects of *Syt1* KD in the prelimbic cortex are specific to alcohol (n = 8/group). KD of *Syt1* in the prelimbic cortex did not affect quinine preference **(A)**, saccharin self-administration **(B)**, or locomotion **(C)**. KD, knockdown.

suggest a decreased neuronal excitability in the PL following *Syt1* KD.

Effects of *Syt1* KD in the PL on Alcohol-Addiction-like Behaviors Are Behaviorally Specific

To determine whether decreased PL *Syt1* expression specifically promotes alcohol-addiction-like behaviors, we also evaluated the effects of *Syt1* KD on operant saccharin self-administration, locomotor activity, and quinine preference. Repeated-measures ANOVA did not show any significant effect of group on any of these behaviors (Figure 2). This shows that *Syt1* KD–induced increases in alcohol self-administration, motivation, and compulsivity are not due to nonspecific effects of *Syt1*, such as generally increased valuation of appetitive rewards or behavioral disinhibition.

Syt1 KD in the PL-BLA But Not PL-NAcC Projection Increased Compulsivity

To identify the brain circuitry through which *Syt1* KD regulates alcohol-addiction-like behaviors, we selectively inhibited its expression in PL neurons projecting to the BLA or the NAcC, respectively. We found that *Syt1* downregulation in the PL-BLA projection increased compulsivity, as measured by insensitivity to quinine adulteration, without affecting other alcohol-related behaviors (Figure 3D–F). Repeated-measures ANOVA showed a significant main effect of group (scrambled vs. *Syt1* KD; $F_{1,125}$ = 6.65; p = .016) (Figure 3D) in the quinine adulteration test, whereas no significant effects were observed on alcohol self-administration or progressive ratio responding. *Syt1* KD in PL-NAcC did not affect any of the alcohol-related behaviors assessed (Figure 3J–L), demonstrating that the increase in compulsive alcohol taking following *Syt1* KD in the PL is mediated by the PL-BLA projection.

Syt1 KD in the PL Increased Neuronal Excitability in the BLA but Not in the NAcC

In the BLA, evoked potentials were significantly increased in slices from *Syt1* KD rats ($F_{1,65}$ = 10.1; p = .002), while the PPR was not significantly affected (t_{62} = 0.13; p = .90) (Figure 4B, D, respectively). Disinhibition induced by the $GABA_AR$ antagonist bicuculline (20 μM), however, was significantly less pronounced in brain slices from rats with a downregulation of *Syt1* in the PL ($F_{1,\ 28}$ = 11.8; p = .002) (Figure 4C). The PPR was reduced by bicuculline administration in both groups, suggesting that $GABA_AR$ antagonism facilitates transmitter release in both treatment groups (paired t test: *Syt1* KD: t_{11} = 9.35; $p <$ .001; and scrambled: t_{12} = 7.27; $p <$ .001) (Figure 4E). Bicuculline administration normalized stimulus/response curves ($F_{1,23}$ = 0.002; p = .96) (Figure 4G), indicating that a reduction in GABAergic neurotransmission underlies the increase in BLA excitability seen following *Syt1* KD.

In contrast to the effects observed in BLA slices, downregulation of *Syt1* in the PL did not affect evoked stimulus/response curves in the NAcC ($F_{1,66}$ = 0.37; p = .54) (Figure 4I). There was also no effect by *Syt1* KD on bicuculline-induced disinhibition ($F_{1,25}$ = 0.24; p = .63) or stimulus/response curves following bicuculline-treatment ($F_{1,24}$ = 0.00; p = .99) (Figure S2). Because PL has previously been shown to project to the DMS and NAcC bilaterally via the anterior corpus callosum (28), we also performed additional experiments to assess putative effects on excitability in the DMS. These recordings did also not support an effect on prefrontostriatal excitability following *Syt1* KD in PL (Figure S2).

DISCUSSION

We previously reported that a history of alcohol dependence results in a persistent downregulation of *Syt1* expression in the PL (8). In the present study, we examined whether this effect contributes mechanistically to behaviors characteristic of alcohol addiction (29). In support of this overall hypothesis, we found that a *Syt1* KD within the PL increased alcohol self-administration and the motivation to consume alcohol in nondependent animals, mimicking what is observed following a history of alcohol dependence. Similar to alcohol postdependent rats, alcohol taking in *Syt1* KD rats was also resistant to quinine adulteration (30). Continued drug taking despite negative consequences is among the DSM-5 criteria for addiction and is thought to reflect compulsivity, a key behavioral characteristic of addictive disorders (31,32). *Syt1* KD did not influence saccharin self-administration, quinine preference, or locomotor activity, supporting the notion that effects of *Syt1* on alcohol-addiction-like behaviors are behaviorally specific. Together, these results suggest a role of SYT1 in the development of behaviors characteristic of alcohol addiction. However, additional experiments such as SYT1 manipulation in postdependent rats are needed to determine whether SYT1 is also involved in maintaining those behaviors.

In prior work, we identified DNA hypermethylation as the mechanism behind alcohol-induced *Syt1* downregulation in the mPFC; specifically, treatment with the DNA methyltransferase inhibitor RG108 restored levels of *Syt1* and partially rescued behavioral consequences of alcohol dependence (8). Together with our present data, this suggests that *Syt1*-dependent effects on neurotransmission, resulting from alcohol-induced DNA hypermethylation in the PL, contribute to alcohol-addiction-like behaviors. However, it is also clear that

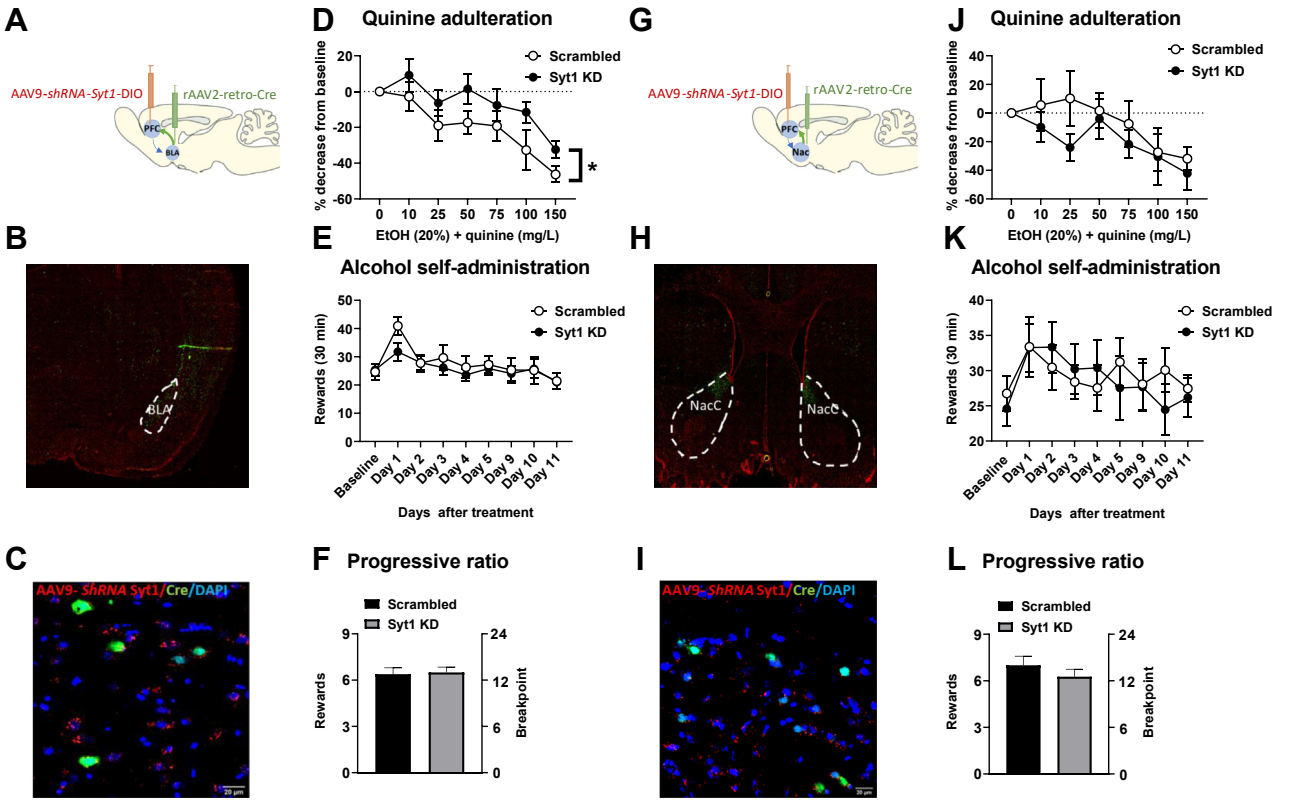

Figure 3. KD of *Syt1* specifically in neurons projecting to the BLA increase compulsivity, as measured by insensitivity to quinine adulteration. Panels **(A)** and **(G)** show the dual virus approach where an AAV9 with floxed short hairpin RNA against *Syt1* was injected in the prelimbic cortex and a rAAV2 retro-Cre was injected into the BLA (n = 13–14) **(A, B)** and NAcC (n = 7–9) **(G, H)**. **(C, I),** Microscopic pictures of the prelimbic cortex (20×) showing cells infected by the rAAV2retro-Cre (green) and by the AAV-ShRNA Syt1-DIO (red). KD of *Syt1* specifically in neurons projecting to the BLA increased insensitivity to quinine adulteration **(D)** but did not affect alcohol self-administration **(E)** or progressive ratio responding **(F)**. *Syt1* KD in neurons projecting to the NAcC did not influence any of the observed behaviors **(J–L)**. *$p < .05$. BLA, basolateral amygdala; EtOH, ethanol; KD, knockdown; NAcC, nucleus accumbens core; PFC, prefrontal cortex.

alcohol effects on PL *Syt1* expression can only partially account for behavioral consequences of alcohol dependence. We previously found that alcohol-induced DNA hypermethylation in the mPFC also results in a repression of another synaptotagmin, SYT2, an effect that similarly contributed to compulsive alcohol taking (8). Thus, a KD of either *Syt1* or *Syt2* in the PL promotes compulsive alcohol taking, but neither of them alone is sufficient to account for the full effect observed following a history of alcohol dependence. Collectively, these observations suggest that both *Syt1* and *Syt2* are repressed by a history of alcohol dependence and additively contribute to the resulting compulsive alcohol taking.

It is well established that exposure of the PFC to alcohol influences neurotransmission in this region and its projections (33). However, few studies have investigated the effects of alcohol on the synaptic machinery that mediates synaptic transmission, such as fusion proteins required for neurotransmitter release. A postmortem study found changes in the expression of genes involved in synaptic neurotransmission in the PFC of alcohol-dependent patients with cirrhosis (34). A majority of these genes were downregulated, suggesting an overall inhibition of neuronal neurotransmission in these patients. Our finding that *Syt1* KD resulted in a decreased neuronal excitability in the PL is broadly consistent with these findings.

Our *Syt1* KD resulted in depressed evoked potentials within the PL. Field potentials in the PL are primarily glutamatergic and mediated through AMPA receptor activation, as they are rapidly blocked by the AMPA/kainate-antagonist CNQX (35). Thus, our findings suggest that glutamatergic neurotransmission within the PL was reduced by the *Syt1* KD and, by extension, is reduced in this region by alcohol dependence. The suppression of the stimulus/response curve was paralleled by an increase in PPR, suggesting that the *Syt1* KD depressed evoked potentials by reducing the release probability of glutamate. This is in agreement with the postulated role of SYT1 as a calcium-sensing membrane protein that signals to the sodium *N*-ethylmaleimide-sensitive factor attachment protein receptor complex to elicit transmitter release. It is also in line with a previous study, in which transfection with *Syt1* short hairpin RNA suppressed NMDA receptor–mediated excitatory postsynaptic currents in the mPFC (12). Integrating these findings, a possible interpretation is that a history of alcohol dependence, through repression of *Syt1* in the PL,

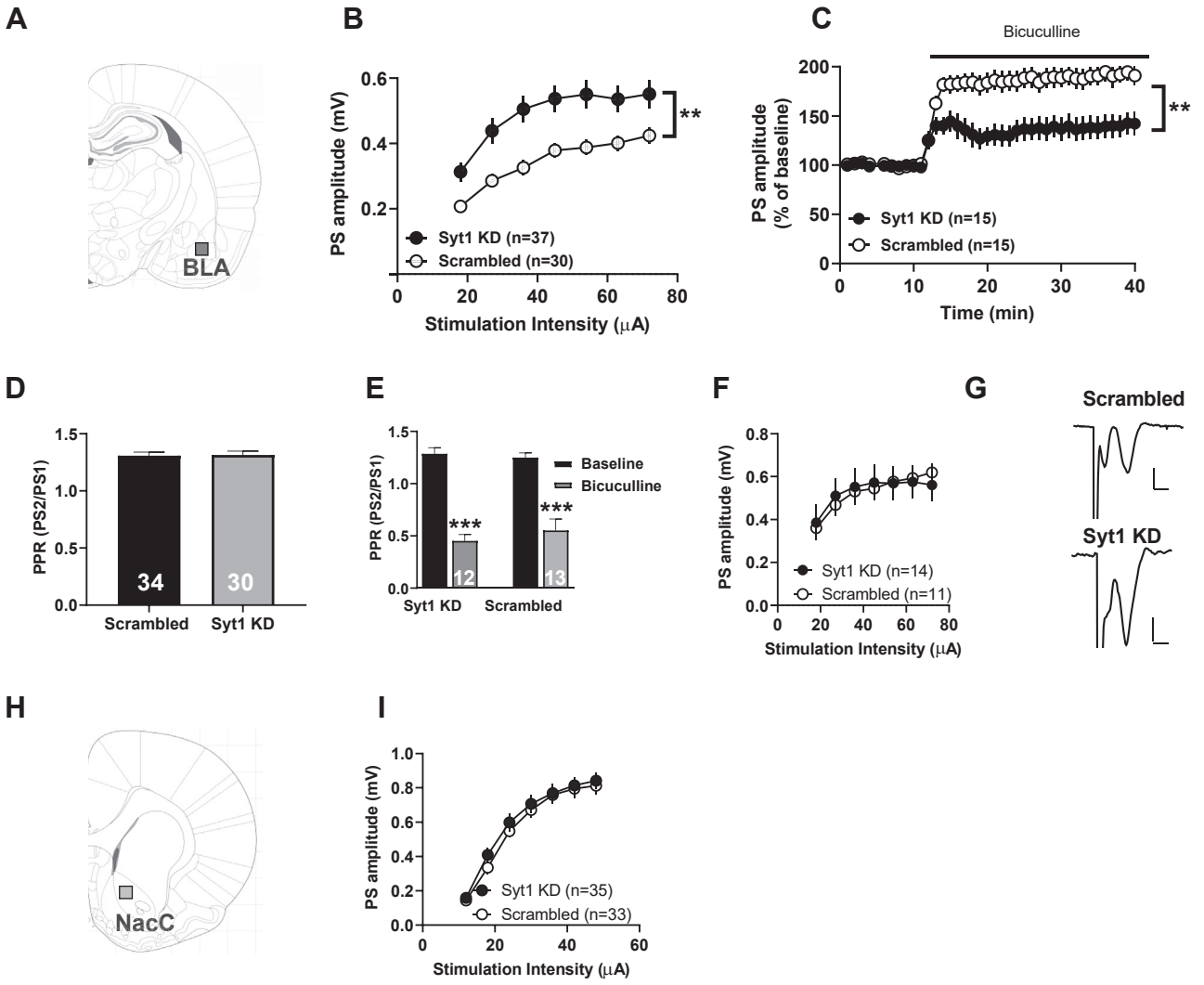

Figure 4. *Syt1* KD in the medial prefrontal cortex increases neurotransmission in the BLA. **(A)** Schematic drawing showing the area for electrophysiological recordings in the BLA. **(B)** Stimulus response curves demonstrates that downregulation of *Syt1* in the prelimbic cortex increased the amplitude of evoked potentials in the BLA. **(C)** Disinhibition induced by the $GABA_A$ receptor antagonist bicuculline (20 μM) was significantly reduced in BLA following *Syt1* KD in the medial prefrontal cortex. **(D, E)** PPR was not significantly modulated by *Syt1* KD and bicuculline significantly reduced PPR in both treatment groups. **(F)** *Syt1*-mediated effects on stimulus/response curves were completely blocked in brain slices incubated with the $GABA_A$ receptor antagonist bicuculline. **(G)** Example traces show evoked responses in the BLA in slices from scramble-treated control (upper trace) and *Syt1* KD (lower trace). Calibration: 2 ms, 0.2 mV. **(H)** Schematic drawing showing the area for electrophysiological recordings in the NAcC. **(I)** Downregulation of *Syt1* in the prelimbic cortex did not modulate the amplitude of evoked potentials in the NAcC. Data are presented as mean values ± SEM. *n* = number of recordings. Data are retrieved from 7 to 9 animals/treatment. $^{**}p < .01$; $^{***}p < .001$. BLA, basolateral amygdala; GABA, gamma-aminobutyric acid; KD, knockdown; NAcC, nucleus accumbens core; PPR, paired pulse ratio; PS, population spike.

suppresses glutamatergic transmission in neurons originating in this region, in turn weakening their ability to exert top-down control over subcortical targets of PL projections.

We therefore proceeded to investigate whether PL *Syt1* KD influences downstream brain regions and attempted to identify PL projections through which *Syt1* KD affects alcohol-addiction-like behaviors. We focused on the PL projections to the NAcC and BLA, as these have been implicated in control of alcohol drinking (20,21). A selective *Syt1* KD for the PL-BLA increased compulsive alcohol taking as previously observed following a history of alcohol dependence (6,8) and after a *Syt1* KD within the KD that was not projection specific. Although the BLA has an established role in the acquisition of drug-seeking behavior (36), to our knowledge, the present study is the first to show a role of the BLA in compulsive alcohol taking.

In support of a role for *Syt1* in regulating PL control of BLA neurotransmission, we found that *Syt1* KD in the PL resulted in increased BLA excitability. Previous studies suggest that disruption of GABAergic transmission intrinsic to the BLA can account for this observation (37). Principal cells of the BLA are

under a robust tonic GABAergic inhibition (38). This was weakened in slices from *Syt1* KD rats, as shown by a marked reduction in disinhibition observed following administration of the $GABA_AR$ antagonist bicuculline. Furthermore, bicuculline blocked the effects of the *Syt1* KD on the stimulus/response curves. Collectively, these findings suggest that *Syt1* KD in the PL leads to reduced GABAergic neurotransmission in the BLA by weakening glutamatergic PL inputs onto GABAergic interneurons within this structure. The precise synaptic mechanism through which this occurs is presently unclear. A decreased availability of SYT1 protein in terminals of PL-BLA inputs could account for decreased release probability of glutamate in synapses onto GABAergic interneurons within the BLA, thereby disinhibiting sensory-driven affective responses (39).

Increased BLA excitability resulting from decreased *Syt1* expression in BLA-projecting prelimbic mPFC neurons is broadly consistent with reports in prior literature. Specifically, changes in regulation of BLA excitability have been associated with behavioral consequences that include increased anxiety, dysregulation of emotional responsiveness, and stress-induced relapse to drug use, behaviors commonly observed in patients with addictive disorders (37,40,41). In preclinical studies, hyperexcitability of the BLA has been observed after chronic intermittent alcohol exposure (42). Collectively, these data suggest that a history of alcohol dependence may modulate neuronal activity of the BLA through *Syt1* modulation and that this mechanism contributes to behavioral consequences of dependence.

In contrast to the BLA, a selective *Syt1* KD within the PL-NAcC projection affected neither alcohol-related behaviors nor neuronal activity in the NAcC. The electrophysiological data from the NAcC did not show large variability between recordings, making it unlikely that a bias was produced by differences in localization of recording electrodes or striosomal organization. We cannot exclude the possibility that transfected neurons in the PL primarily project to other brain regions or that the extensive glutamatergic inputs from other brain regions masked effects on excitability in NAcC (43). However, because the projection to NAcC and BLA are distinct and nonoverlapping (44), and as our *Syt1* KD was performed in a confined region of the PL and did not spread to the infralimbic cortex (28), this manipulation was most likely insufficient to recruit prefrontostriatal circuits. Taken together with the absence of behavioral effects, the most parsimonious interpretation is that *Syt1* expression within the PL-NAcC projection is not involved in modulation of alcohol-related behaviors.

Conclusions

Our findings demonstrate a mechanistic role of *Syt1* in several behaviors that are characteristic of alcohol use disorder. We identified the PL-BLA projection as a brain circuit through which SYT1 regulates compulsive alcohol taking, a key behavior of clinical alcohol addiction (45). Together, our data suggest that dysregulation of the synaptic calcium sensor SYT1 in glutamatergic projections from the PL to the BLA is a mechanism through which a history of alcohol dependence causes long-term neuroadaptation that promotes addiction-like behaviors.

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