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Authors

Khom, S
Steinkellner, T
Hnasko, TS
et al.

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NEUROSCIENCE

Alcohol dependence potentiates substance P/neurokinin-1 receptor signaling in the rat central nucleus of amygdala

S. Khom[1]*, T. Steinkellner[2], T. S. Hnasko[2,3], M. Roberto[1]*

Behavioral and clinical studies suggest a critical role of substance P (SP)/neurokinin-1 receptor (NK-1R) signaling in alcohol dependence. Here, we examined regulation of GABA transmission in the medial subdivision of the central amygdala (CeM) by the SP/NK-1R system, and its neuroadaptation following chronic alcohol exposure. In naïve rats, SP increased action potential–dependent GABA release, and the selective NK-1R antagonist L822429 decreased it, demonstrating SP regulation of CeM activity under basal conditions. SP induced a larger GABA release in alcohol-dependent rats accompanied by decreased NK-1R expression compared to naïve controls, suggesting NK-1R hypersensitivity which persisted during protracted alcohol withdrawal. The NK-1R antagonist blocked acute alcohol-induced GABA release in alcohol-dependent and withdrawn but not in naïve rats, indicating that dependence engages the SP/NK-1R system to mediate acute effects of alcohol. Collectively, we report long-lasting CeA NK-1R hypersensitivity corroborating that NK-1Rs are promising targets for the treatment of alcohol use disorder.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing condition defined by compulsive alcohol drinking, loss of control over alcohol intake, and the emergence of negative emotional states such as dysphoria, anxiety, and irritability during alcohol withdrawal (*1*). AUD originates from maladaptive processes in neurocircuits involved in reward, stress, and executive function including the basal ganglia, extended amygdala, and prefrontal cortex (*2*). Specifically, sensitization of stress systems is accompanied by decreased brain reward function (*3*). The central nucleus of the amygdala (CeA) is particularly sensitive to both acute and chronic alcohol and is critically implicated in the development of AUD (*4*). The CeA is a predominantly GABAergic nucleus modulated by pro- and antistress peptides, and increased CeA GABAergic transmission represents a hallmark of alcohol dependence across species (*5*–*7*).

Substance P (SP) and its primary molecular target, neurokinin-1 receptors (NK-1Rs), are widely expressed in brain areas mediating stress and AUD-associated behaviors including the amygdala (*8*, *9*). Furthermore, both preclinical and clinical studies implicate a critical involvement of SP/NK-1R systems in AUD (*10*). NK-1R antagonists or genetic ablation of the gene encoding for NK-1Rs (*Tacr1*) reduces alcohol reward and escalation in mice (*11*). Further, the NK-1R antagonist L822429 attenuates stress-induced reinstatement in rats (*12*) and decreases alcohol self-administration in outbred alcohol-preferring rats (*13*, *14*). These studies also identified the amygdala as a key brain region of SP action. Specifically, alcohol-preferring rats display augmented NK-1R expression accompanied by increased SP-binding affinity that likely contributes to their elevated alcohol preference (*13*). Accordingly, intra-CeA application of an NK-1R antagonist reduces alcohol self-administration in alcohol-preferring rats (*13*), and viral overexpression of NK-1R in the CeA emulates an alcohol-preferring phenotype (*15*).

NK-1R antagonists also ameliorate craving in detoxified alcoholics (*16*), and *Tacr1* polymorphisms are associated with the development of AUD in humans (*17*). These studies highlight a critical role of SP/NK-1R signaling in AUD and suggest potential for NK-1R antagonism in the treatment of AUD. However, it is unknown how chronic alcohol affects CeA SP/NK-1R signaling at a cellular level. Thus, given the critical role of the CeA in alcohol-related behaviors and its unique sensitivity to alcohol, we tested the hypothesis that alcohol dependence and withdrawal potentiate SP/NK-1R signaling in the medial subdivision of the CeA (CeM) using ex vivo electrophysiology, immunohistochemistry, and optical imaging in rat brain slices.

RESULTS

SP and NK-1Rs display subdivision-specific expression pattern in the CeA

Using immunohistochemistry, we first examined SP and NK-1R expression patterns in medial (CeM), lateral (CeL), and capsular (CeC) subdivisions of rat CeA (Fig. 1, A and B) and found that both SP and NK-1R were not homogeneously expressed within the three main CeA subdivisions. Specifically, NK-1R immunofluorescence was strongest in CeL, followed by comparable levels of expression in CeM and CeC [$F(2,18) = 64.06$, $P < 0.0001$, assessed by one-way analysis of variance (ANOVA) with post hoc Tukey mean comparison; Fig. 1C]. In contrast, SP immunofluorescence was highest in CeC, and no differences were observed between CeM and CeL subdivisions [$F(2,18) = 23.72$, $P < 0.0001$, assessed by one-way ANOVA with post hoc Tukey mean comparison; Fig. 1C].

SP enhances GABA release in the CeM of naïve rats

Given the critical role of the CeM, the major output of the amygdala (19), in AUD and its abundant NK-1R expression, we focused on functional assessment of SP actions on GABAergic synapses in this

[1]Department of Molecular Medicine, Scripps Research, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. [2]Department of Neurosciences, University of California, San Diego, 9500 Gilman Drive La Jolla, CA 92093, USA. [3]Research Service, VASDHS, 3350 La Jolla Village Drive, San Diego, CA 92161, USA.
*Corresponding author. Email: mroberto@scripps.edu (M.R.); skhom@scripps.edu (S.K.)

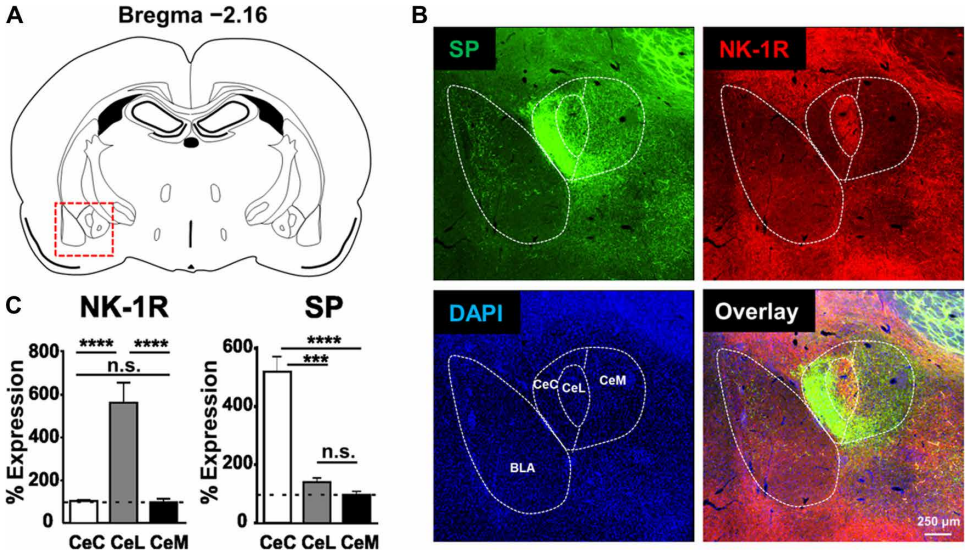

Fig. 1. Subregion-specific expression of SP and NK-1R in rat CeA. (**A**) Scheme highlighting neuroanatomy of CeA and basolateral amygdala (BLA) in a rat coronal brain section as used for immunohistochemistry. (**B**) Representative images of naïve rat amygdala stained for SP (green), NK-1R (red), and counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue). Scale bar, 250 μm. (**C**) Quantification of NK-1R (left) and SP (right) expression in the CeA subregions normalized to CeM expression levels; $n = 7$. ***$P < 0.001$ and ****$P < 0.0001$, one-way ANOVA (Tukey post hoc mean comparison). n.s., not significant.

subdivision. Using whole-cell patch clamp, we recorded pharmacologically isolated $GABA_A$ receptor–mediated spontaneous inhibitory postsynaptic currents (sIPSCs) from CeM neurons ($n = 134$; fig. S1) from naïve rats. Application of SP at concentrations ≥10 nM rapidly and strongly increased sIPSC frequency [10 nM SP: 148 ± 13%, $n = 6/11$, $t(5) = 4.158$, $P < 0.01$; 30 nM SP: 151 ± 10%, $n = 7/13$, $t(6) = 4.823$, $P < 0.01$; 100 nM SP: 173 ± 17%, $n = 8/10$, $t(7) = 4.276$, $P < 0.01$; 300 nM SP: 143 ± 10%, $n = 9/12$; $t(8) = 4.670$, $P < 0.01$, compared to baseline using one-sample t test; Fig. 2, A and C] in CeM neurons, indicative of SP increasing CeM GABA release. SP also significantly augmented sIPSC amplitudes [30 nM SP: 121 ± 5%, $n = 7/13$, $t(6) = 4.080$, $P < 0.01$; 100 nM SP: 136 ± 13%, $n = 8/10$, $t(7) = 2.709$, $P < 0.05$, compared to baseline using one-sample t test; Fig. 2, B and D], suggesting enhanced postsynaptic γ-aminobutyric acid type A ($GABA_A$) receptor function. sIPSC kinetics were not affected by any SP concentration tested (Fig. 2, E and F). SP (100 nM) also significantly increased membrane input resistance from 488 ± 82 Megaohm to 644 ± 106 Megaohm ($n = 18$, $P < 0.01$, Wilcoxon test), indicative of a reduction of membrane conductance. Next, we recorded miniature IPSCs (mIPSCs; using 0.5 μM tetrodotoxin) to assess SP effects on action potential–independent GABAergic transmission. We found that SP did not alter mIPSCs ($n = 11$; Fig. 2G), suggesting that SP enhanced CeM GABAergic transmission in a network- and action potential–dependent manner.

Since the CeA is a predominantly GABAergic nucleus, enhanced CeM GABA transmission induced by SP could result from elevated neuronal activity in response to disinhibition. Thus, we tested effects of SP (100 nM) on a separate set of spontaneously firing CeM neurons using cell-attached configuration under basal conditions [artificial cerebrospinal fluid (aCSF)], and in the presence of blockers of synaptic transmission (glutamate/$GABA_B$ receptor blockers), and we found that SP similarly increased neuronal firing in both conditions [aCSF: 174 ± 30%, $n = 10/15$ cells, $t(9) = 2.496$, $P < 0.05$ versus glutamate/$GABA_B$ receptor blockers: 149 ± 23%, $n = 5/7$ cells, $t(4) = 2.965$, $P < 0.05$, compared to baseline using a one-sample t test and unpaired t test between conditions: $t(13) = 0.5443$, $P > 0.05$; Fig. 2H], suggesting that SP induces a local GABA release.

SP mediates CeM GABA release via an interaction with inward rectifiers

The physiological and behavioral effects of SP are mediated by G protein–coupled NK-1Rs (*10*, *18*). Downstream effects of SP-induced NK-1R activation effects have been mainly attributed to closure of G protein–coupled inwardly rectifying K^+ (GIRK) channels and/or the opening of nonselective cation channels (*19*–*22*), resulting from G_q-mediated activation of phospholipase C (PLC) and protein kinase C (PKC), respectively (*10*, *23*, *24*). Other noncanonical intracellular transduction pathways potentially involved in mediating molecular SP effects include activation of adenylate cyclase (AC) (*22*, *25*), phospholipase A2 (*26*), and G protein–independent Src kinase–mediated mechanisms (*27*).

Thus, to determine the intracellular mechanism(s) involved in mediating SP-induced GABA release, we assessed SP (100 nM) effects on sIPSCs in the presence of pharmacological blockers of PLC [5 μM U73122 for phosphatidylcholine-specific PLC (PC-PLC) (*24*) or 10 μM D609 for phosphatidylinositol-specific PLC (PI-PLC) (*23*)], PKC [200 nM Ro 32-0432 (*28*)], GIRK channels [500 nM Tertiapin Q (TQ) (*29*)], AC [10 μM Rp-cAMP (*28*)], or phosphotyrosine kinase [PTK; 30 μM genistein (*27*)].

Notably, only TQ (500 nM) significantly blocked SP effects on both sIPSC frequency [TQ + SP: 109 ± 12%, $n = 6$, $t(5) = 1.423$, $P > 0.05$, compared to pre-TQ + SP baseline using one sample t test and unpaired t test to SP alone: $t(12) = 2.860$, $P < 0.05$; Fig. 2I] and amplitudes [TQ + SP: 99 ± 6%, $t(5) = 0.5192$, $n = 6$, $P > 0.05$, compared to pre-TQ + SP baseline using one sample t test and unpaired t test to SP alone: $t(12) = 2.266$, $P < 0.05$]. SP-induced enhancement of sIPSC frequency and amplitudes in the presence of blockers of PI-PLC, PC-PLC, PKC, AC, or PTK did not significantly differ from SP alone

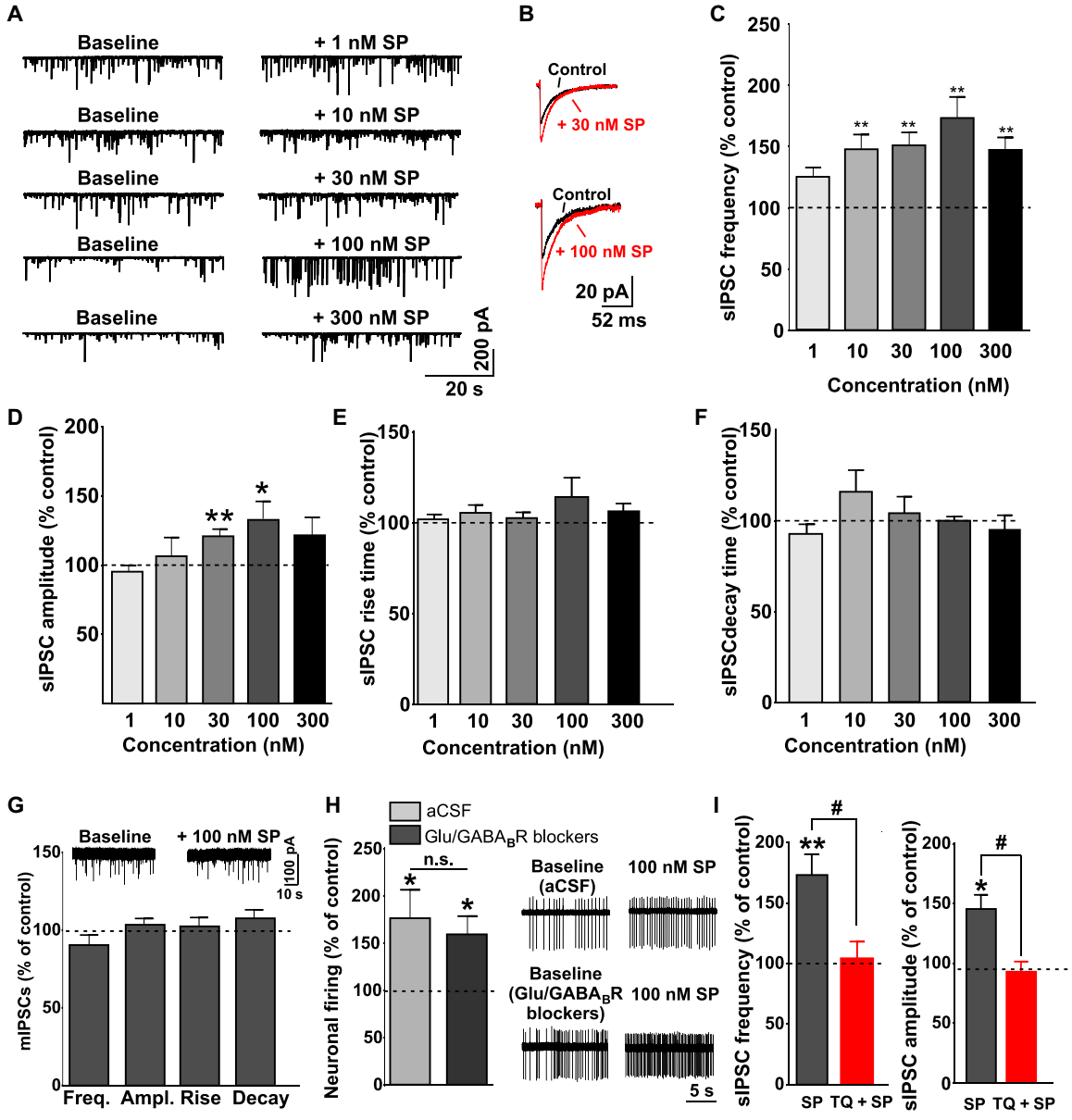

Fig. 2. SP increases action potential–dependent GABA release in the CeM of naïve rats. (**A**) Representative sIPSC recordings and (**B**) scaled averages before and during SP application. Bars represent means ± SEM of sIPSC properties (**C** to **F**; $n = 6$ to 14 cells). (**G**) Bars summarize effects of SP on mIPSCs ($n = 11$). Inset: Representative mIPSCs before and during SP (100 nM). (**H**) Percent change and representative traces of spontaneous action potential firing of CeM neurons under aCSF conditions ($n = 10$) and in the presence of blockers of synaptic glutamate transmission and $GABA_B$ receptors ($n = 5$) before and during SP. $GABA_B$ receptor, $GABA_BR$. (**I**) Bar graphs represent sIPSC frequencies and amplitudes in the presence of SP and SP + Tertiapin Q (TQ: 500 nM, $n = 6$, normalized to pre-SP + TQ baseline; note that TQ alone did not significantly alter sIPSC properties), respectively. *$P < 0.05$ and **$P < 0.01$, one-sample t tests compared to baseline conditions. #$P < 0.01$, unpaired t test between groups.

on CeA GABA transmission (data are summarized in table S1), indicating that SP-induced CeM GABA release is primarily mediated via an interaction with GIRK channels.

Alcohol dependence potentiates SP-mediated CeM GABA release but decreases expression of the SP/NK-1R system

We previously demonstrated that CeM GABAergic synapses are very sensitive to both acute and chronic alcohol exposure and that enhanced CeA inhibitory tone is a hallmark for alcohol dependence across species from rodents to nonhuman primates (*5*–*7*, *30*). In the present study, we also observed a significant increase in baseline sIPSC frequency in 84 neurons from dependent compared to naïve rats [naïve: 1.3 ± 0.1 Hz, n = 134 versus Dep: 1.7 ± 0.1 Hz, n = 84; unpaired t test: $t(216) = 2.287$, $P < 0.05$; fig. S1], corroborating augmented GABA release in dependence. Despite behavioral evidence for the involvement of the SP/NK-1R system in alcohol intake, studies at the cellular level are lacking. Thus, we first determined potential alterations of SP and NK-1R protein levels after chronic alcohol exposure by immunohistochemistry in CeA sections of naïve and dependent rats. A densitometric analysis of confocal images revealed

that both, SP and NK-1R staining, were significantly decreased in the CeM (Fig. 3, A and B), suggesting that alcohol dependence induces down-regulation of the CeM SP/NK-1R system [SP densitometry: naïve: 926 ± 82 arbitrary units (a.u.), $n = 7$; Dep.: 590 ± 87 a.u., $n = 6$; unpaired t test: $t(11) = 2.802$, $P < 0.05$. NK-1R densitometry: naïve: 2314 ± 333 a.u., $n = 7$; Dep.: 1144 ± 213 a.u., $n = 6$; unpaired t test: $t(11) = 2.839$, $P < 0.05$].

Next, we examined functional consequences of reduced NK-1R expression on CeM GABA transmission and found that 10 and 300 nM SP increased sIPSC frequency in dependent rats [Dep.: 10 nM SP: 151 ± 15%, $n = 4$, $t(3) = 3.439$, $P < 0.05$; 300 nM SP: 169 ± 15%, $n = 7$, $t(6) = 4.722$, $P < 0.01$, one-sample t test compared to baseline; Fig. 4, A to C] similar to naïve controls. However, in dependent rats, we found that 30 and 100 nM SP, respectively, induced a significantly larger increase of sIPSC frequency [30 nM SP: naïve: 149 ± 13% versus Dep.: 230 ± 19%, $n = 6/11$ neurons, $t(11) = 3.29994$, $P < 0.05$; 100 nM SP: naïve: 173 ± 17% versus Dep.: 228 ± 20%, $n = 8/14$ neurons, $t(14) = 2.18976$, $P < 0.05$; multiple t tests with post hoc Holm-Sidak comparison] compared to naïve controls (Fig. 4, B and C), suggesting increased agonist efficacy. Notably, chronic alcohol blunted the effects of SP on sIPSC amplitudes (Fig. 4D). Like in naïve animals, SP did not alter action potential–independent GABA transmission (mIPSCs) in dependent rats (Fig. 4, E and F).

SP further increases acute alcohol-induced CeM GABA release in naïve and dependent rats

Acute alcohol increases GABA transmission in the CeM by enhancing GABA release via recruitment of PKC (*31*), AC, L-type calcium channels (*28*), and corticotropin-releasing factor receptor 1 (CRF1) receptors (*28*, *32*). Here, we show that SP engages GIRKs to facilitate CeM GABA release (Fig. 2I). Hence, to determine potential interactions of acute alcohol and SP on GABA transmission, we compared effects of alcohol (44 mM) on sIPSCs before and after application of SP (100 nM). As shown in Fig. 5 (A and B), alcohol significantly increased sIPSC frequency, and subsequent co-application of SP in the presence of alcohol further enhanced this effect in both naïve [EtOH: 136 ± 12%, $n = 11$; one-sample t test to baseline: $t(10) = 3.068$, $P < 0.05$ versus EtOH + SP: 247 ± 21%, $n = 11$; one-sample t test: $t(10) = 6.862$, $P < 0.01$; between groups paired t test: $t(10) = 5.419$, $P < 0.001$] and dependent rats [EtOH: 145 ± 16%, $n = 11$; one-sample t test: $t(10) = 2.819$, $P < 0.05$ versus EtOH + SP: 390 ± 89%, $n = 11$; one-sample t test to baseline: $t(10) = 3.256$, $P < 0.01$; between groups paired t test: $t(10) = 3.166$, $P < 0.01$].

Acute alcohol blunted SP effects on sIPSC amplitudes in naïve rats (Fig. 5C) but facilitated postsynaptic responses to SP (i.e., significant increase of sIPSC amplitudes) in dependent rats (Fig. 5C). SP did not alter alcohol-induced prolongation of current rise and decay times in naïve rats (Fig. 5D); last, neither alcohol nor alcohol + SP affected sIPSC kinetics in the CeM from dependent rats.

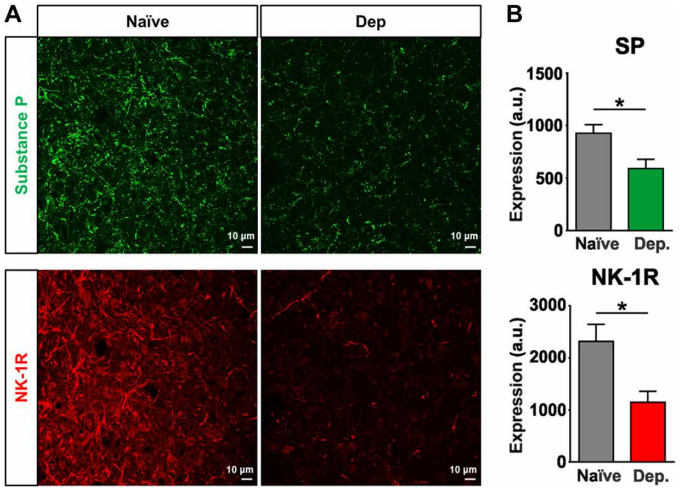

Fig. 3. Alcohol dependence decreases SP and NK-1R expression in the CeM. (**A**) Representative confocal images of SP (green) and NK-1R (red) of naïve (left) and dependent (right) rats. Scale bars, 10 µm. (**B**) Quantification of relative expression levels for SP (top) and NK-1R (bottom) of naïve ($n = 7$) and dependent animals ($n = 6$) expressed as arbitrary fluorescent units (a.u.). *$P < 0.05$, unpaired t test.

Endogenous SP regulates GABA transmission in the CeM under basal conditions in naïve and dependent rats

The NK-1R antagonist L822429 has been extensively used in rodent models to study the role of the SP/NK-1R system in addictive processes (*10*), but its effects on GABA transmission at a cellular level in rats have not been investigated. Thus, we first determined effects of L822420 (1 µM) on GABA transmission in the CeM of naïve rats and found that L822429 strongly reduced sIPSC frequency [71 ± 4%, $n = 15/18$ cells; one-sample t test: $t(14) = 8.492$, $P < 0.001$; Fig. 5, E and F] without altering postsynaptic sIPSC properties (i.e., sIPSC amplitude and kinetics), suggesting that endogenous SP regulates CeM GABA transmission under basal conditions (Fig. 1 for CeA SP expression). Furthermore, L822429 prevented SP-induced increases in sIPSC frequency, amplitude, or kinetics ($n = 6$; fig. S2), confirming the selective inhibition of SP/NK-1R signaling.

Despite significantly lower SP expression in the CeM of dependent rats (Fig. 3), L822429 decreased sIPSC frequency to a comparable extent as observed in naïve controls [67 ± 6%, $n = 10/14$ neurons; one sample t test: $t(9) = 5.923$, $P < 0.001$; Fig. 5, E and G], suggesting hypersensitive NK-1Rs. Postsynaptic $GABA_A$ receptor function was unaffected.

Next, we examined potential NK-1R involvement in the facilitatory effects of acute alcohol on CeM GABA release (Fig. 5H). We found that alcohol increased sIPSC frequency in the presence of L822249 in naïve rats [L822249 alone: 74 ± 5%, $n = 7$; one-sample t test to baseline: $t(6) = 5.127$, $P < 0.01$ versus L822429 + EtOH: 95 ± 11%, $n = 7$; one-sample t test to baseline: $t(6) = 0.6896$, $P > 0.05$; paired t test between groups: $t(6) = 2.812$, $P < 0.05$], but L822429 blocked facilitatory effects of acute alcohol in dependent rats [L822249 alone: 74 ± 7%, $n = 8$; one-sample t test to baseline: $t(7) = 5.134$, $P < 0.01$ versus L822429 + EtOH: 80 ± 7%, $n = 8$; one-sample t test to baseline: $t(7) = 4.367$, $P < 0.01$; paired t test between groups: $t(7) = 1.888$, $P > 0.05$; Fig. 5, I to K].

Neuroadaptive changes of the SP/NK-1R system persists after alcohol withdrawal

To assess the persistence of the effects of chronic alcohol exposure, dependent rats were withdrawn (WD) from alcohol vapor for 2 weeks before slice physiology. We recorded sIPSCs from 40 CeM neurons of withdrawn rats and found that the elevation of GABA transmission persisted during protracted withdrawal (fig. S1), suggesting long-lasting neuroadaptations at GABAergic synapses. A one-way ANOVA revealed significant differences in sIPSC frequencies [$F(2,257) = 4.403$, $P < 0.05$; fig. S1C], rise [$F(2,257) = 4.307$, $P < 0.05$; fig. S1E], and decay times [$F(2,257) = 9.778$, $P < 0.001$; fig. S1F] but not amplitudes

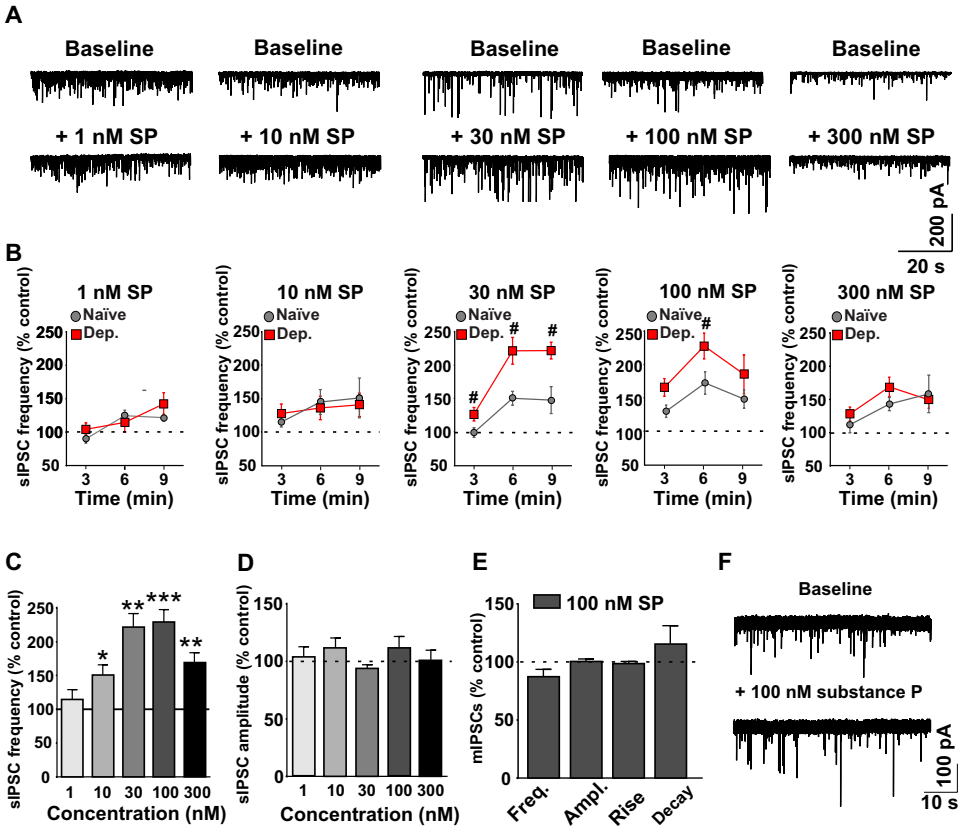

Fig. 4. SP effects on CeM GABA transmission are potentiated by alcohol dependence. (**A**) Representative sIPSCs during control and SP. (**B**) Time courses of SP effects in dependent (red lines) and naïve rats (gray lines) compared to baseline. Bars summarize sIPSC (**C**) frequencies, (**D**) amplitudes ($n = 4$ to 8), (**E**) mIPSC characteristics in the presence of SP ($n = 11$), and (**F**) representative mIPSCs before and during SP. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, one-sample t test compared to baseline conditions. #$P < 0.05$, multiple t tests between naïve and dependent (post hoc Holm-Sidak comparison).

(fig. S1D) between naïve, dependent, and withdrawn rats. Specifically, we found significantly augmented CeM sIPSC frequencies for both dependent and withdrawn rats compared to naïve controls (naïve: 1.3 ± 0.1 Hz versus Dep.: 1.7 ± 0.1 Hz versus WD: 1.9 ± 0.2 Hz) accompanied by prolonged sIPSC rise (naïve: 2.57 ± 0.04 ms versus Dep.: 2.71 ± 0.04 ms versus WD: 2.75 ± 0.09 ms) and decay times (naïve: 7.88 ± 0.27 ms versus Dep.: 9.47 ± 0.41 ms versus WD: 10.09 ± 0.50 ms).

Protracted withdrawal did not reverse dependence-induced neuroadaptations of SP/NK-1R signaling. Specifically, SP (100 nM) enhanced sIPSC frequency in withdrawn rats like in dependent rats [Dep.: 228 ± 20% versus WD: 210 ± 14%, $n = 10/15$ neurons; multiple t tests with Holm-Sidak post hoc comparison: $t(16) = 1.15021$, $P > 0.05$; Fig. 6, A to C] without affecting the properties of postsynaptic responses (Fig. 6C). Comparable to dependent rats, we found that acute alcohol (44 mM) increased sIPSC frequency [EtOH: 142 ± 12%, $n = 9/16$ neurons; one-sample t test: $t(8) = 3.458$, $P < 0.01$], suggesting a lack of tolerance of CeM neurons to acute alcohol effects. In addition, SP further increased GABA release in the presence of alcohol [EtOH + SP: 276 ± 44%, $n = 9$; one-sample t test compared to baseline: $t(8) = 3.971$, $P < 0.01$; paired t test between groups: $t(8) = 3.535$, $P < 0.01$; Fig. 6, D and E]. Neither alcohol nor SP affected postsynaptic properties (Fig. 6D). Conversely, the NK-1R antagonist L822429 (1 μM) decreased sIPSC frequency [67 ± 6%, $n = 7/9$ cells; one-sample t test: $t(6) = 5.989$, $P < 0.01$], again without affecting postsynaptic properties (Fig. 6G). Last, L822249 prevented facilitatory effects of acute alcohol on CeM GABA release [sIPSC frequency L822249 alone: 64 ± 8%, $n = 5$; one-sample t test compared to baseline: $t(4) = 4.647$, $P < 0.01$ versus L822429 + EtOH: 63 ± 10%, $n = 5$; one-sample t test: $t(4) = 3.755$, $P < 0.01$; paired t test between groups: $t(4) = 0.3196$, $P > 0.05$] in withdrawn rats, and alcohol in the presence of L822249 significantly decreased sIPSC amplitudes [82 ± 4%, one-sample t test: $t(4) = 3.418$, $P < 0.05$] without any further postsynaptic changes (Fig. 6I).

DISCUSSION

Excessive activation of brain prostress systems accompanied by compromised function of antistress systems is a hallmark of AUD (*3*, *33*), and the mainly GABAergic CeA is critically involved in mediating key aspects of negative emotional states associated with dependence (*5*–*7*).

The neuropeptide SP and its primary molecular target, the NK-1R, are highly expressed in brain stress and addiction neurocircuitries (*9*, *18*). Both preclinical and clinical studies strongly suggest a critical role of dysregulated SP/NK-1R signaling in AUD, implicating the amygdala as a crucial site of SP action (*10*).

Here, we sought to determine potential alterations in (i) the expression of SP and NK-1R in the CeM of dependent compared to naïve rats and (ii) the modulation of GABA transmission by the

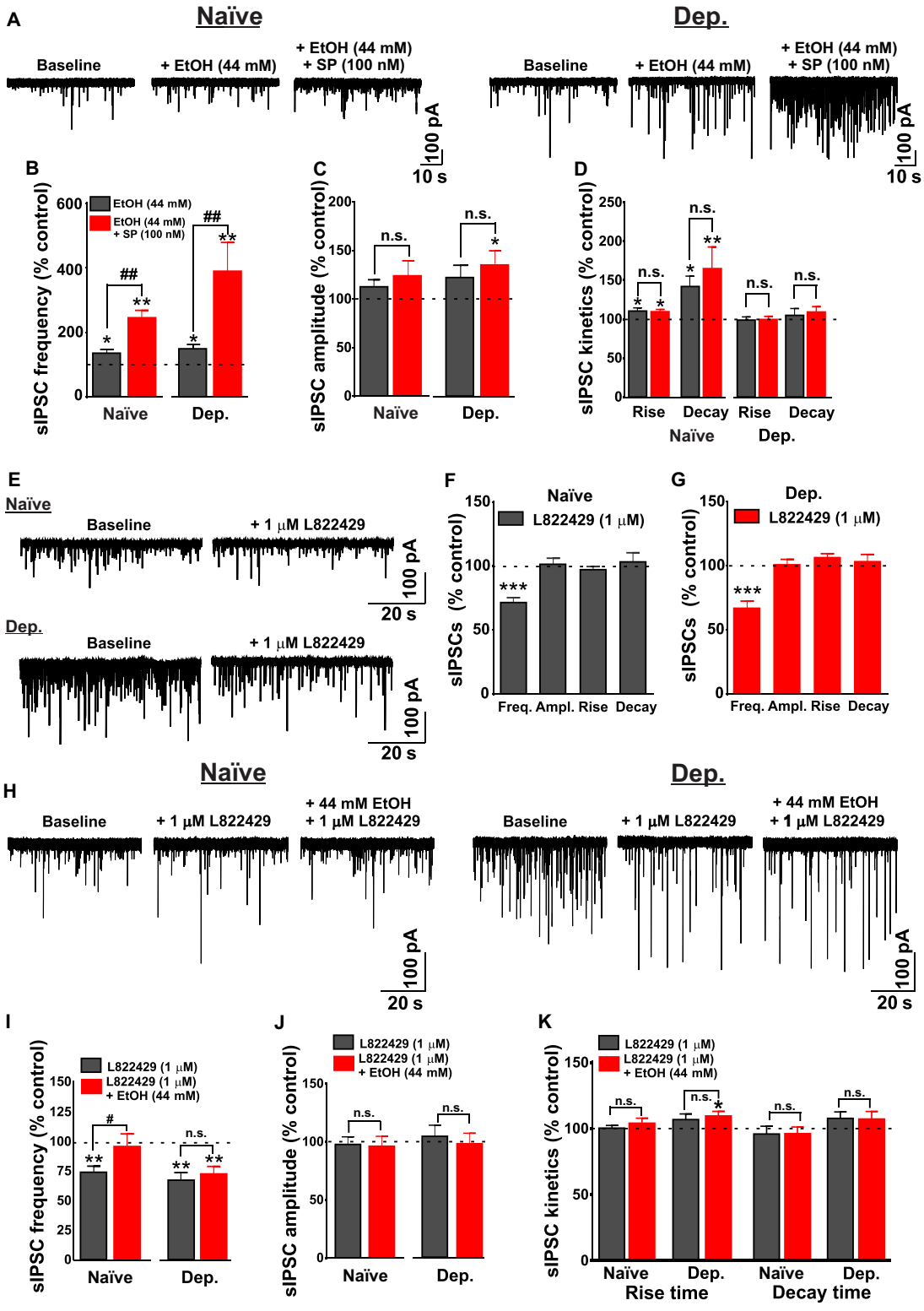

Fig. 5. Acute alcohol interactions with SP/NK-1R signaling. (**A**) Representative sIPSCs before, during alcohol, and alcohol and SP, respectively. Bar graphs represent means ± SEM of sIPSC (**B**) frequencies, (**C**) amplitudes, and (**D**) current kinetics in the presence of alcohol and alcohol + SP, respectively [$n = 11$ (naïve) and $n = 11$ (dependent)] compared to baseline control (dashed lines). (**E**) Representative sIPSCs before and during the NK-1R antagonist L822429. Bar graphs represent means ± SEM of sIPSC in naïve (**F**) ($n = 15$) and dependent (**G**) ($n = 10$) rats. (**H**) Representative sIPSCs before, during the NK-1R antagonist, and NK-1R antagonist and alcohol, respectively. Bars summarize sIPSC (**I**) frequencies, (**J**) amplitudes, and (**K**) current kinetics in the presence of L822429 and L822429 + alcohol, respectively [$n = 7$ (naïve) and $n = 8$ (Dep.)] compared to baseline control (dashed lines). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, one-sample t test compared to baseline conditions. #$P < 0.05$ and ##$P < 0.01$, paired t test between groups.

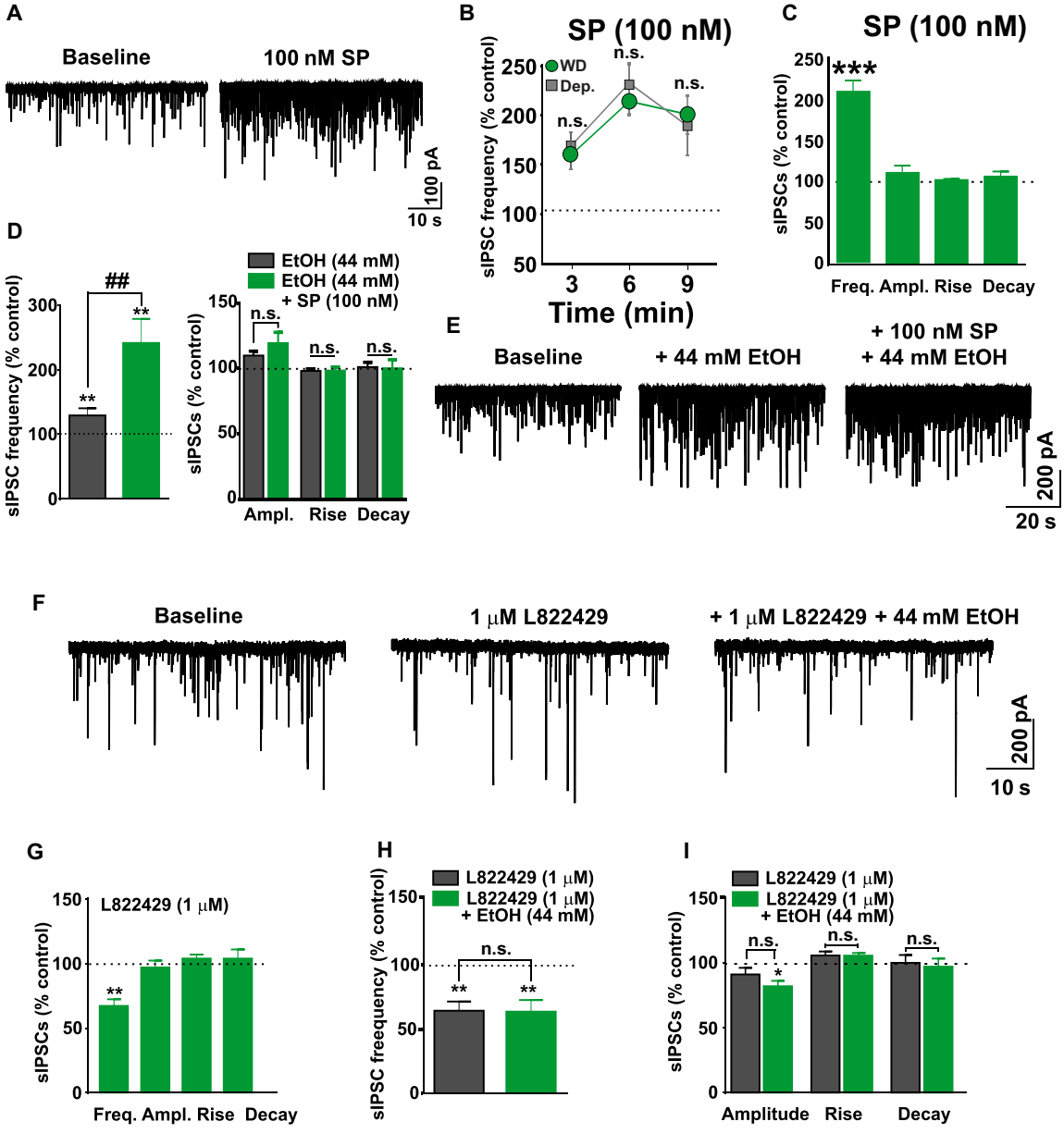

Fig. 6. SP/NK-1R signaling remains elevated after withdrawal. (**A**) Representative sIPSCs before and during SP. (**B**) Time course of SP action. (**C**) Bar graphs represent means ± SEM of sIPSC characteristics in the presence of SP ($n = 10$). (**D**) Bars summarize sIPSC frequencies and amplitudes in the presence of alcohol and alcohol + SP, respectively. (**E**) Representative sIPSCs before, during alcohol, and alcohol + SP, respectively. (**F**) Representative sIPSCs before, during L822249, and L82249 + EtOH, respectively. Bars summarize in (**G**) sIPSC characteristics in the presence L822249 ($n = 7$). Bars show sIPSC (**H**) frequencies and (**I**) postsynaptic measures in the presence of L822249 and L822249 + EtOH, respectively ($n = 5$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, one-sample t test compared to baseline conditions. ##$P < 0.01$, paired t test between groups.

SP/NK-1R system and its interaction with acute and chronic alcohol. Our study revealed that SP increased spontaneous action potential–dependent (but not action–potential independent) GABA transmission in the CeM of naïve rats, suggesting network-dependent effects of SP leading to a heightened inhibitory CeM tone, thus dysregulating downstream brain areas that are normally tightly under CeA inhibitory control. Since SP increased both sIPSC frequency and amplitude, pre- and postsynaptic mechanisms are presumably contributing to CeM GABA release. Further, SP increased spontaneous CeM neuronal firing under both aCSF conditions and in the presence of $GABA_B$/glutamate signaling inhibitors, suggesting a direct excitatory action of SP on CeM neurons. The intracellular downstream effects of SP have been mainly attributed to the closure of GIRK channels and/or the opening of nonselective cation channels (*19*–*21*, *23*), resulting from a G_q-mediated PLC and PKC activation (*10*, *24*). SP/NK-1R interaction has also been associated with noncanonical signaling pathways including activation of AC (*22*, *25*), phospholipase A2 (*26*), or G protein–independent phosphotyrosine kinase–mediated mechanisms (*27*). It is unknown which of these intracellular pathways is primarily responsible for SP-induced GABA

release. Thus, to investigate putative signaling mechanisms involved, we systematically blocked downstream signaling pathways including various isoforms of PLC (PC-PLC and PI-PLC) and PKC, as well as AC and phosphotyrosine kinase. Blocking PLC and PKC, which are thought to be the canonical second messenger systems linked to NK-1R, did not prevent SP-induced GABA release. Furthermore, our studies in CeA slices did not confirm an involvement of AC or phosphotyrosine kinase on SP-mediated GABA release. However, we found that SP significantly increased membrane input resistance, suggesting a reduction of membrane conductance. This was further corroborated by the GIRK inhibitor TQ blocking SP effects on CeM GABA release. However, we do not rule out that SP/NK-1R interaction also activates other signaling pathways, but their potential activation apparently does not play a critical role in mediating SP-induced GABA release in the CeM of naïve rats. Thus, the apparent discrepancy between published results on SP/NK-1R signaling and our studies may arise from the studied end point (i.e., modulation of GABA release) and the used model system. Many studies on SP/NK-1R signaling were performed using isolated systems, such as oocytes or cell culture, and we used ex vivo brain slices representing a more physiological preparation where many of the local neuronal connections are still intact. In summary, on the basis of our findings in CeA slices, we identify GIRK channels as critical for mediating SP-induced GABA release.

A main finding of our study is that the SP/NK-1R system is persistently altered during alcohol dependence and withdrawal. It is important to consider that the extended chronic intermittent alcohol vapor exposure has predictive validity as a model of alcohol dependence in humans, mimicking neuroadaptations at the molecular, cellular, and behavioral levels (*34*). Our laboratory has also shown that this paradigm produces consistent and reproducible alterations in multiple neuropeptidergic systems in the CeA despite the age of the rats and the associated potential technical challenges performing ex vivo slice electrophysiology in these animals (*4*, *5*, *28*, *32*, *35*).

Specifically, we found larger SP-induced GABA release in the CeM of dependent and withdrawn rats compared to naïve controls. This apparent increase in SP efficacy was accompanied by a down-regulation of NK-1R expression in dependent rats, suggesting that these receptors become hypersensitive in alcohol dependence. We also found that SP expression was reduced in dependent rats, suggesting that reduction of the neuropeptide could induce receptor hypersensitivity. NK-1Rs have been shown to rapidly internalized upon SP binding in the rat amygdala following immobilization stress (*36*). Similarly, fear provocation in phobic patients reduced NK-1R availability in the human amygdala (*37*). Our results show that the effects of SP on CeM GABA release are transient (reaching their peak after 3 to 6 min) and returned to baseline after prolonged application (>12 min) possibly due to NK-1R internalization. Hence, we hypothesize that our model of chronic intermittent alcohol exposure triggers intermittent SP release during withdrawal periods and induces receptor internalization and degradation, thereby decreasing overall NK-1R levels. A recent study showing that intermittent, but not continuous, alcohol access reduces amygdala *Tacr1* mRNA levels (*38*), further underlines a critical role of withdrawal on NK-1R expression and is thus in line with our data revealing that chronic alcohol decreased CeM NK-1R protein. The NK-1R antagonist revealed comparable SP regulation of CeM GABA transmission under basal conditions in both dependent and withdrawn rats compared to naïve. On the basis of these findings, we suggest that NK-1Rs become hypersensitive as a compensatory mechanism in that they exert similar regulation of CeM GABA transmission in response to lower SP levels. Similar hypersensitive states have been reported for other G protein–coupled receptors including D1 dopamine receptors (*39*), muscarinic acetylcholine receptors (*40*), or α2-adrenoceptors (*41*). Yet, we can only speculate on the molecular mechanisms underlying NK-1R hypersensitivity in alcohol dependence as it could occur at multiple levels including recruitment of additional intracellular signaling pathways, alterations in G protein coupling, and/or even enhanced agonist binding affinity. In naïve rats, GIRKs are pivotal in mediating SP effects on GABA release. Chronic alcohol and withdrawal might dysregulate CeM SP/NK-1R signaling in a manner that activation of NK-1Rs results in a broader recruitment of intracellular pathways, hence leading to the observed larger CeM GABA release. Moreover, it is also important to consider that albeit we observed similar functional effects of the SP/NK-1R system on CeA GABA release in dependent and withdrawn rats, the molecular mechanisms underlying NK-1R hypersensitivity might differ between these two states.

CeM GABAergic synapses are highly sensitive to acute and chronic alcohol exposure, and we have previously shown that acute and chronic alcohol increase CeM GABA release (*5*, *30*). Here, we discovered that SP and acute alcohol have additive effects on CeM GABA transmission (i.e., SP further increased alcohol-induced GABA release) in naïve, dependent, and withdrawn rats indicative of recruiting distinct second messenger systems by SP and acute alcohol. As described above, we found that L822429 decreased CeM GABA transmission in naïve, dependent, and withdrawn rats to a similar extent. Furthermore, the NK-1R antagonist blocked acute alcohol-induced GABA facilitation in dependent and withdrawn rats but not in naïve rats. Similar results were reported with CRF1 antagonists, which blocked the network-dependent alcohol effects only in CeM of dependent rats (*28*). These findings are particularly important since elevated GABA transmission is critically linked to AUD, which persists in protracted abstinence (2 weeks after cessation of alcohol exposure). Recent findings in nonhuman primates also revealed elevated CeA GABA transmission in abstinent animals with intermittent drinking history (*7*) and highlights the relevance of increased GABA signaling as a common molecular mechanism in alcohol dependence across species (*5*–*7*, *35*).

In conclusion, we report hypersensitive NK-1Rs in the CeM of alcohol-dependent rats. Given the critical role of the CeA in alcohol dependence, our study on neuroadaptations of the CeM SP/NK-1R system further expands our knowledge of cellular mechanisms involved in the pathophysiology of AUD. Specifically, our preclinical studies highlight that the SP/NK-1R system regulates amygdala function and may play a key role in the development and maintenance of alcohol dependence, further corroborating that targeting NK-1Rs to reduce elevated CeA inhibitory tone might alleviate behaviors associated with AUD. Thus, this system may represent a promising therapeutic target for treatment of AUD.

MATERIALS AND METHODS

All procedures were approved by the Scripps Research Institutional Animal Care and Use Committee and are consistent with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. In this study, we used a total of 209 male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 225 to

250 g upon arrival. Rats were group-housed (two to three per cage) in standard plastic cages in a temperature- and humidity-controlled room and were maintained under a reverse 12-hour light/12-hour dark cycle with ad libitum access to food and water.

Chronic intermittent alcohol exposure

We induced alcohol dependence in 93 rats using the standard chronic intermittent alcohol inhalation method in their home cages, exposing them 14 hours daily with alcohol vapor (10 hours air) over a period of 5 to 7 weeks. We determined blood alcohol levels (BALs) one to two times per week from tail blood samples (average BAL: 151 ± 5 mg/dl). Twenty-eight dependent rats were subsequently subjected to protracted withdrawal for 2 weeks (14 ± 1 days). Alcohol-naïve controls were treated similarly, except that they were exposed to air only. Animals used for this study were age-matched (5 to 6 months old).

Slice preparation and electrophysiological recordings

Preparation of acute brain slices and electrophysiological recordings were performed as previously described (*5*, *30*, *32*). We decapitated deeply anesthetized rats (3 to 5% isoflurane anesthesia) and rapidly isolated their brains in ice-cold oxygenated high-sucrose cutting solution (composition: 206 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM glucose, and 5 mM Hepes). We cut 300-μm coronal slices containing the medial subdivision of the central amygdala (CeM) using a Leica VT 1000S vibratome and incubated them for 30 min in warm (37°C), oxygenated aCSF (composition: 130 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 1.5 mM $MgSO_4$, 24 mM $NaHCO_3$, and 10 mM glucose), followed by another 30-min incubation at room temperature. Dependent rats were euthanized during the last hour of their daily alcohol vapor exposure. Slice were cut and stored in ethanol-free solutions, and thus, the slices from dependent animals technically underwent acute in vitro withdrawal (1 to 10 hours).

We recorded from 304 CeM neurons using either whole-cell voltage clamp or cell-attached current clamp mode. Neurons were visualized with infrared differential interference contrast optics using a 40× water immersion objective (Olympus BX51WI) and a charge-coupled device camera (EXi Aqua, QImaging). All recordings were performed in gap-free acquisition mode with a 10-kHz sampling rate and 10-kHz low-pass filtering using a MultiClamp700B amplifier, Digidata 1440A, and pClamp 10 software (Molecular Devices).

We pulled patch pipettes from borosilicate glass (3 to 5 Megaohm; King Precision) and filled them for whole-cell recordings with a KCl-based internal solution [composition: 135 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM Hepes, 2 mM Mg-ATP (magnesium adenosine triphosphate), and 0.2 mM Na-GTP (sodium guanosine triphosphate); pH 7.2 to 7.4 adjusted with 1 M NaOH, 290 to 300 mOsm] or a K-gluconate–based internal solution for cell-attached recordings (composition: 145 mM K-gluconate, 0.5 mM EGTA, 2 mM $MgCl_2$, 10 mM Hepes, 2 mM Na-ATP, and 0.2 mM Na-GTP; pH 7.2 to 7.4 adjusted with 1 M NaOH, 290 to 300 mOsm). We pharmacologically isolated action potential–dependent $GABA_A$ receptor–mediated sIPSCs by adding blockers of glutamatergic transmission [20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 30 μM DL-2-amino-5-phosphonovalerate (AP-5)] and $GABA_B$ receptors (1 μM CGP55845A) to the bath solution, and we additionally added 0.5 μM tetrodotoxin to study action potential–independent $GABA_A$ receptor–mediated transmission (mIPSCs). We held neurons at −60 mV. Neurons with an access resistance of >15 Megaohm or with a change of >20% during the recording, as monitored by frequent 10mV pulses, were excluded. Spontaneous action potential firing of CeM neurons was recorded in current-clamp mode in either aCSF or in the presence of $GABA_B$ and glutamate blockers as described above. All drugs were applied by bath perfusion.

Drugs

We purchased SP, AP-5, CGP55845A, DNQX, Ro 32-0432 hydrochloride (3-[(8*S*)-8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-10-yl]-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione hydrochloride), TQ, D609 [*O*-(octahydro-4,7-methano-1*H*-inden-5-yl) carbonopotassium dithioate], U73122 [1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione], and genistein from Tocris Bioscience. Rp-cAMP (Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt) was purchased from Sigma-Aldrich, and ethanol was purchased from Remet. L829429 was synthesized by K. Rice at the Drug Design and Synthesis Section, Chemical Biology Research Branch, National Institute on Drug Abuse, NIH, Bethesda, MD.

Stock solution of all drugs were prepared in either distilled water or dimethyl sulfoxide and added to the bath solution to achieve final concentrations.

Immunohistochemistry

At the last day of the daily exposure, we anesthetized seven naïve and six dependent rats using isoflurane (3 to 5%) and transcardially perfused them with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Brains were postfixed in 4% PFA overnight at 4°C, transferred to 30% sucrose for 48 to 72 hours for cryoprotection, and frozen in isopentane chilled on dry ice. We cut coronal sections (30 μm) using a cryostat (CM3050S, Leica) and collected them in PBS containing 0.01% sodium azide. Free-floating sections were washed three times for 5 min in PBS and blocked for 1 hour in PBS containing 5% normal donkey serum and 0.3% Triton X-100 (blocking buffer), followed by incubation with primary antibodies [1:1000; rabbit anti–NK-1R (*42*); Sigma S8305; 1:100; guinea pig anti-SP; Abcam ab10353] overnight at 4°C. The following day, we washed the sections three times (15 min) in PBS and incubated them with secondary antibodies in blocking buffer for 2 hours at room temperature. We purchased secondary antibodies (5 μg/ml) conjugated to Alexa Flour 488 (donkey anti–guinea pig) and Alexa Flour 594 (donkey anti-rabbit) from Jackson ImmunoResearch. After secondary antibody incubation, sections were rinsed three times in PBS for 15 min, mounted onto glass slides (Thermo Fisher Scientific), and coverslipped using Fluoromount-G mounting medium (Southern Biotech) containing 4′,6-diamidino-2-phenylindole (0.5 μg/ml). Images were captured using a Zeiss Axio Observer Epifluorescent microscope or a Leica SP5 confocal microscope.

Imaging and densitometry

We quantified SP and NK-1R fluorescence in coronal sections containing the rostro-caudal extents of the CeA (roughly bregma points, −1.5 to −2.9 mm). Sampled sections consisted of 275 μm by 275 μm images that were acquired at a dorsal, central, and ventral spot within the CeM using a Leica SP5 Confocal Microscope at a 2048 × 2048 pixel resolution with a 40× objective lens. The number of pixels containing SP or NK-1R immunofluorescence was measured per unit area using ImageJ (NIH). Data are expressed as arbitrary units (a.u.).

Data analysis and statistical analysis

We analyzed frequency, amplitude, rise, and decay time of s/mIPSCs semiautomatically using MiniAnalysis software (Synaptosoft), followed by a visual confirmation of each event. s/mIPSCs of <5 pA were excluded. We analyzed spontaneous action potential firing semiautomatically using pClamp, followed by a visual confirmation of each action potential. Data were normalized to baseline, combined to 3-min bins, and pooled for each experimental condition. Neurons displaying ≥15% change of baseline in the presence of drug were considered as drug sensitive. We applied parametric tests for datasets following a Gaussian distribution (see details below) and a two-tailed Wilcoxon matched-pairs signed rank test for nonparametric data (membrane input resistance). Specifically, we analyzed single drug effects using two-tailed one-sample *t* tests (Figs. 2, C to I; 4, C to E; 5, B to D, F and G, and I to K; and 6, C and D and G to I, and fig. S2). We used unpaired *t* tests to compare drug/treatment effects across two conditions (Figs. 2, H and I, and 3B; fig. S2; and table S1) and paired *t* tests when comparing the effect of two drugs on the same cell (Figs. 5, B to D and I to K, and 6, D, H, and I). We used multiple *t* tests followed by Holm-Sidak assessment to correct for multiple comparisons to analyze data shown in Fig. 4B and Fig. 6B and, last, one-way ANOVAs followed by a Tukey post hoc test (Fig. 1C) or Dunnet post hoc test (fig. S1) when comparing three groups.

Data are reported as means and SEM. *n* indicates the number of either recorded cells or animals. All data were derived from at least six different animals. $P < 0.05$ was set as criterion for statistical significance. GraphPad Prism (v.6) was used to conduct the statistical analysis (La Jolla, CA, USA).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/12/eaaz1050/DC1

Fig. S1. Alcohol dependence induces long-lasting neuroadaptations of $GABA_A$ receptor–mediated transmission in the CeM.

Fig. S2. The NK-1R antagonist L822429 blocks SP effects on CeM GABA transmission.

Table S1. Summary of SP effects on sIPSC frequencies and amplitudes in the presence of blockers of potential SP/NK-1R intracellular signaling pathways in the CeM of naïve rats.

View/request a protocol for this paper from *Bio-protocol*.

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