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Gq-DREADD Selectively Initiates Glial Glutamate Release and Inhibits Cue-induced Cocaine Seeking

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ABSTRACT

BACKGROUND: Glial cells of the central nervous system directly influence neuronal activity by releasing neuroactive small molecules, including glutamate. Long-lasting cocaine-induced reductions in extracellular glutamate in the nucleus accumbens core (NAcore) affect synaptic plasticity responsible for relapse vulnerability.

METHODS: We transduced NAcore astrocytes with an adeno-associated virus vector expressing hM3D designer receptor exclusively activated by a designer drug (DREADD) under control of the glial fibrillary acidic protein promoter in 62 male Sprague Dawley rats, 4 dominant-negative soluble N-ethylmaleimide-sensitive factor attachment protein receptor mice, and 4 wild-type littermates. Using glutamate biosensors, we measured NAcore glutamate levels following intracranial or systemic administration of clozapine N-oxide (CNO) and tested the ability of systemic CNO to inhibit reinstated cocaine or sucrose seeking following self-administration and extinction training.

RESULTS: Administration of CNO in glial fibrillary acidic protein-hM3D-DREADD transfected animals increased NAcore extracellular glutamate levels in vivo. The glial origin of released glutamate was validated by an absence of CNO-mediated release in mice expressing a dominant-negative soluble N-ethylmaleimide-sensitive factor attachment protein receptor variant in glia. Also, CNO-mediated release was relatively insensitive to N-type calcium channel blockade. Systemic administration of CNO inhibited cue-induced reinstatement of cocaine seeking in rats extinguished from cocaine but not sucrose self-administration. The capacity to inhibit reinstated cocaine seeking was prevented by systemic administration of the group II metabotropic glutamate receptor antagonist LY341495.

CONCLUSIONS: DREADD-mediated glutamate gliotransmission inhibited cue-induced reinstatement of cocaine seeking by stimulating release-regulating group II metabotropic glutamate receptor autoreceptors to inhibit cue-induced synaptic glutamate spillover.

Keywords: Astrocytes, Biosensor, Cocaine, DREADD, Glutamate, Reinstatement

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Addiction is characterized by an enduring vulnerability to relapse mediated by long-lasting drug-induced alterations in glutamatergic synaptic plasticity within the prefrontal cortex to nucleus accumbens core (NAcore) circuit (1–3). Animal studies reveal that cocaine exposure reduced levels of basal extracellular glutamate in the NAcore due to decreased glial cystine-glutamate exchange (4,5). Cocaine-induced reduction of extrasynaptic glutamate tone on presynaptic group II metabotropic glutamate autoreceptors (metabotropic glutamate receptor [mGluR] 2/3) increases synaptic glutamate release probability and contributes to synaptic glutamate spillover during cocaine- or cue-induced reinstatement of cocaine seeking (1,6,7). Withdrawal from cocaine exposure also increases expression of the activator of G-protein signaling 3 in the prefrontal cortex, which selectively binds to Giα-GDP and further inhibits the function of mGluR2/3 (8). Glutamate overflow is then further exacerbated by reduced rates of glutamate clearance that arise from cocaine-induced downregulation of the glial glutamate transporter 1 (1). These drug-induced perturbations of glutamate homeostasis potentiate glutamatergic synapses in the NAcore and promote excessive glutamate release in response to cue exposure, which induces cocaine-seeking behavior by activating postsynaptic glutamate receptors (1,9).

Glia maintain basal extracellular glutamate levels required for the regulation of synaptic plasticity by autoreceptors like mGluR2/3 (10). Gliotransmission occurs via several mechanisms including through anion channels, transporters, and via calcium ion (Ca^{2+})-dependent release (11,12). Gliotransmission is likely regulated by intracellular Ca^{2+} ($[Ca^{2+}]_i$) arising from Ca^{2+} stores from the endoplasmic reticulum (ER) and proceeding through a process requiring vesicular Ca^{2+}-dependent binding proteins (13). Studies with Ca^{2+} sensitive dyes reveal oscillations in astrocytic $[Ca^{2+}]_i$ (14,15), linked to release of astrocytic transmitters that modulate synaptic transmission and plasticity (13,16).

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We hypothesized that increasing glutamate release from astrocytes before reinstatement would restore tone onto mGluR2/3 autoreceptors and thereby inhibit the reinstatement of cue-induced cocaine seeking that occurs due to potentiated synaptic release of glutamate. We hypothesized that this manipulation would not drive reinstatement, due to the fact that presynaptic mGluR2/3 autoreceptors are more sensitive to endogenous levels of glutamate than other mGluR subtypes (17,18). To selectively stimulate astroglial release of Ca^{2+} from intracellular stores, we employed adeno-associated virus transduction in the NAcore to express the hM3D (Gq) designer receptor exclusively activated by a designer drug (DREADD) (19,20) under control of the glial fibrillary acidic protein (GFAP) promoter (21). Stimulating Gq-coupled DREADDs with clozapine N-oxide (CNO) (20) increases inositol 1,4,5-triphosphate (IP_3) signaling, which increases release of intracellular Ca^{2+} from the ER (21,22). We employed an in vivo glutamate biosensor to validate CNO-induced release of glutamate into the extracellular space and demonstrated that glutamate release from astroglia reduces cue-induced cocaine seeking by increasing stimulation of mGluR2/3 autoreceptors.

METHODS AND MATERIALS

Animal Housing and Surgery

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Association for the Assessment and Accreditation of Laboratory Animal Care International. Male Sprague Dawley rats (250 g to 300 g on arrival; Charles River Laboratories, Wilmington, Massachusetts) were individually housed in a temperature- and humidity-controlled environment with a 12-hour dark/light cycle. Experiments were conducted during the animals' dark cycle. Rats received food and water ad libitum and were allowed 1 week to acclimate to the vivarium before surgery. Rats were anesthetized with ketamine hydrochloride (100 mg/kg Ketaset; Fort Dodge Animal Health, Wyeth International, Overland Park, Kansas) and xylazine (10 mg/kg Rompun; Bayer, Bayer HealthCare, Bayer Inc., Mississauga, Ontario) and given ketorolac analgesic (2 mg/kg; Sigma, St. Louis, Missouri). They were implanted with intravenous jugular catheters, followed by virus microinjections (3,23). Briefly, 1 μL of rAAV5/GFAP-HA-hm3D-IRES-mCitrine (GFAP-Gq-DREADD) (University of North Carolina Vector Core, Chapel Hill, North Carolina) was infused bilaterally into the NAcore (anterior-posterior [AP] +1.5 mm; medial-lateral [ML] ±1.8 mm; dorsal-ventral [DV] −7.5 mm from bregma, at a 6° angle) using 33-gauge injectors (Plastics One, Inc., Roanoke, Virginia) at .1 μL per minute. NAcore adeno-associated virus transduction was also performed in GFAP promoter driven dominant-negative soluble N-ethylmaleimide-sensitive factor attachment protein receptor (dnSNARE) transgenic and wild-type littermates utilizing the methods described above, except mice did not receive jugular catheters or undergo cocaine self-administration (SA). Mice received .3 μL microinfusion of rAAV5/GFAP-HA-hm3D-IRES-mCitrine (AP +1.1 mm; ML ±1.2 mm; DV −4.2 mm from bregma) bilaterally.

Immunohistochemical Staining

Animals were given pentobarbital (200 mg/kg, intraperitoneal [IP]) and transcardially perfused 4% formaldehyde. Brains were sliced at 80 μm and stained for 24 hours with anti-GFAP (Abcam, Bristol, United Kingdom; Cat: ab7260 Lot: GR158671-1), anti-NeuN (Millipore Merck KGaA, Darmstadt, Germany; Cat: MABN140 Lot: 2198089), and/or anti-hemagglutinin (HA) (Covance, Raleigh, North Carolina; Cat: MMS-101P Lot: E12BF00336) antibodies at 1:1000 in phosphate-buffered saline + 5% normal goat serum. GFAP, NeuN, and HA staining was visualized using Alexa Fluor 488 and 594 (Abcam). Images were obtained with a Leica SP6 confocal (Leica Microsystems, Wetzlar, Germany). Z-series images were taken at 1- to 3-micron intervals (Figure S1 in Supplement 1). For co-localization analyses (Figure 1B,D and Figure 2G), 63X Z-series datasets were imported into Imaris (Bitplane, Oxford Instruments, Oxfordshire, United Kingdom). Once thresholded, a co-localization channel was constructed that depicts pixels that contain both signals in white, as seen in Figure 1B,D and Figure 2G.

Enzyme-Based Microelectrode Array

R2 ceramic-based microelectrode arrays (MEA) were prepared for recordings as described previously (24–26). Briefly, recording sites were coated with a glutamate oxidase (United States Biological, Salem Massachusetts) containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri), .125% glutaraldehyde (Sigma-Aldrich), and 1% glutamate oxidase. Twenty-four hours later, sites were electroplated with an m-phenylenediamine dihydrochloride (Acros Organics, Thermo Fisher Scientific, Geel, Belgium).

Selectivity ratios for glutamate over ascorbic acid were calculated in addition to the limit of detection and linearity (R^2) for all glutamate microelectrode arrays (24–26). Electrodes that had R^2 values $<$ .99 or had limits of detection of $>$ 3 μmol/L were excluded. After calibration, MEAs were fitted with single-barrel glass capillaries with an inner tip diameter of ~10 μm. Pipettes were embedded in modeling clay and molten wax was applied to stabilize the assembly. The tip of the pipette was positioned 50 μm to 100 μm from the surface of the MEA (24,27). Following attachment, the pipette was filled with 875 μmol/L CNO solution (.3 mg/ml, .5% dimethyl sulfoxide [DMSO] in .9% sterile saline) or a 70 mmol/L potassium chloride (KCl) solution (70 mmol/L KCl, 79 mmol/L sodium chloride, and 2.5 mmol/L calcium chloride, pH 7.4). For ω-conotoxin-(Group 6 Alpha) GVIA experiments and electrochemical experiments using LY341495, a second pipette was positioned 300 μm lateral to the central pipette 50 μm to 100 μm from the surface of the MEA (Figure 2A). Effector solution pipettes were filled with 10 μmol/L ω-conotoxin GVIA or with 283 μmol/L LY341495. In all cases, pipettes were connected via tubing to a Picospritzer III (Parker Instruments, Parker Hannifin Corporation, Cleveland, Ohio) for delivery.

Rats were anesthetized with 30% urethane at 5 mL/kg and placed in a stereotax (David Kopf Instruments, Tujunga, California) on a heating pad (37°C). Rats underwent a craniotomy above the nucleus accumbens and were implanted with MEAs into either the left or right NAcore (AP +1.8 mm; ML ±1.5 mm; DV −7 mm versus bregma). Mouse procedures were performed as described above with the following exceptions. Mice were anesthetized with 15% urethane at 5 mL/kg and coordinates used for placement were AP +1.1 mm; ML ±1.2 mm; DV −4.2 mm versus bregma.

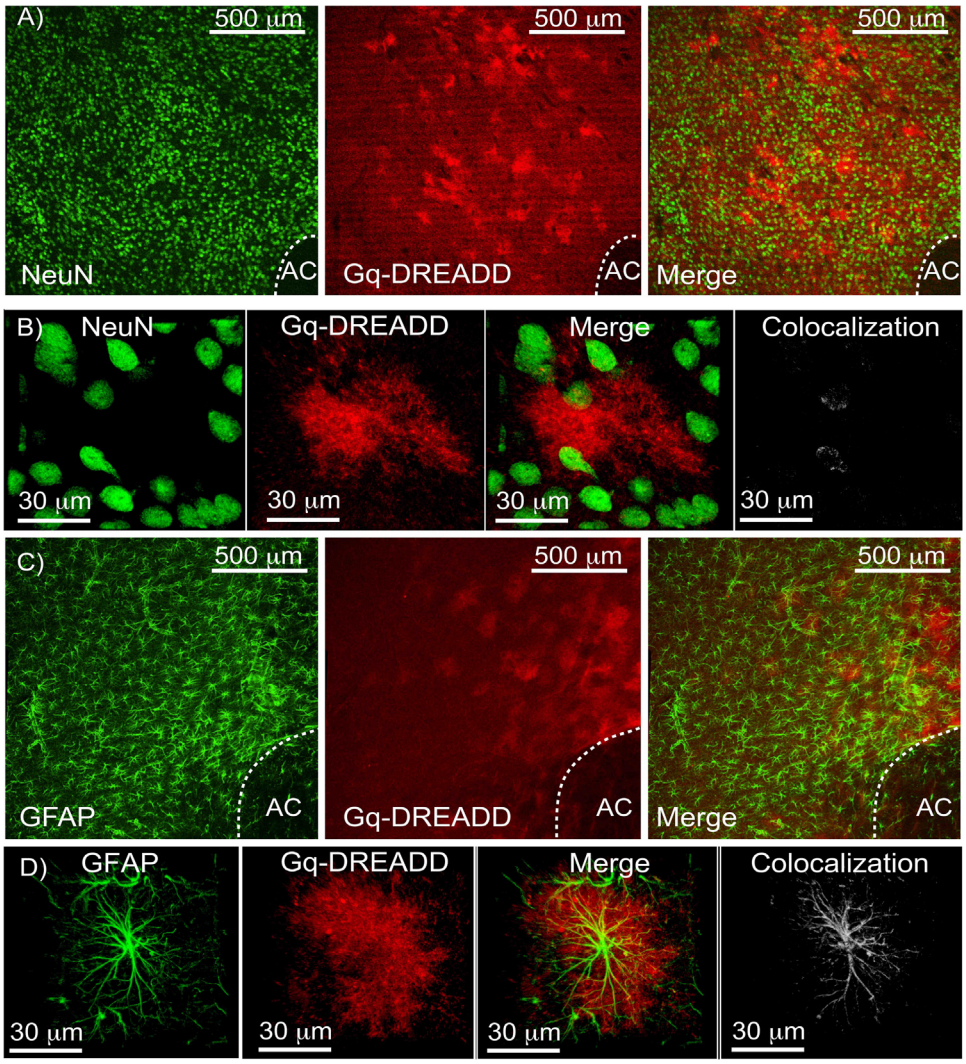

Figure 1. Astrocyte-specific expression of hM3D (Gq)-designer receptor exclusively activated by a designer drug (DREADD). **(A)** 10× images of a field of cells containing the accumbens with NeuN labeling in green, viral DREADD expression (anti-hemagglutinin [HA] tag) in red, and a red-green merged image. In each image, the anterior commissure is labeled AC. **(B)** 63× images of NeuN in green, viral DREADD expression (HA tag) in red, a merged image, and a three-dimensional (3-D) co-localization channel built by Imaris. 3-D co-localization analysis shows very little co-labeling (white) of the viral HA and NeuN signals. **(C)** 10× images of a field of cells containing the accumbens with glial fibrillary acidic protein (GFAP) labeling in green and viral DREADD expression (HA tag) in red, as well as a merged image. **(D)** 63× images with GFAP in green and viral DREADD expression (HA tag) in red, along with a merged image and a 3-D co-localization channel built by Imaris (white). Here, 3-D co-localization analysis displays co-labeling of the astrocyte-specific GFAP intermediate filament protein with viral HA staining.

Following implantation, animals underwent 20-minute acclimation to establish a stable baseline.

Pressure ejections of 875 μmol/L CNO dissolved in .9% sterile saline with .5% DMSO were performed using the Picospritzer III. Fifteen, 30, 60, or 100 nL of CNO solution was ejected ~ 50 μm to 100 μm from the surface of the microelectrode to stimulate Gq-DREADD. Following CNO ejection, recordings were allowed to return to baseline for 3 to 5 minutes before repeating the procedure and/or increasing the amount of CNO ejected.

Rats were administered either vehicle (1 mL/kg; 5% DMSO in .9% sterile saline) or CNO (3 mg/kg, IP) and recordings were allowed to proceed for 1 hour following injection of CNO or vehicle.

A dual pipette–MEA assembly (Figure 2A) designed to deliver ω-conotoxin GVIA and either KCl or CNO into the NAcore was constructed. Following application of 60 nL 70 mmol/L, KCl recordings were allowed to return to baseline for 3 to 5 minutes. We then puff applied 60 nL of 10 μmol/L ω-conotoxin GVIA (3.037 ng) immediately followed by application of 60 nL of 70 mmol/L KCl. Maximum glutamate peak height amplitude values were averaged between two and three paired observations for each recording across four separate animals. The same procedure was also carried out with 60 nL of .3 mg/mL (875 μmol/L) CNO solution substituted for 70 mmol/L KCl to evaluate the ability of ω-conotoxin GVIA to inhibit CNO-mediated glutamate release or 60 nL of 283 μmol/L LY341495 to evaluate the ability of

mGluR2/3 antagonists to impact CNO-mediated glutamate release (Figure S6 in Supplement 1).

Sixty nanoliters of .3 mg/mL (875 μmol/L) CNO solution was puff applied as described above in transgenic mice and wild-type age-matched littermates. Maximum glutamate amplitude was recorded and averaged across four CNO applications in four dnSNARE transgenic animals and four age-matched wild-type littermates.

Data Analysis

The FAST16 MKIII recording system (Pronexus Analytical AB, Stockholm, Sweden) saved amperometric data, time, and experimenter ejection marks. All data traces from the MEAs were analyzed using the FAST Analysis software (Jason Burmeister Consulting, LLC, Council Bluffs, Iowa). All data were passed through a low stringency wavelet low pass filter (using the Daubechies wavelet).

SA, Extinction, and Reinstatement

SA experiments occurred in operant chambers with two levers, a house light, and a cue light and tone (78 dB, 4.5 kHz) generator (Med Associates, Fairfield, Vermont). During 2-hour sessions, presses on the active lever resulted in a cocaine infusion (.2 mg in 50 μL, in .9% sterile saline) on a fixed ratio 1, with 20-second time-out; cocaine was provided by National Institute on Drug Abuse. Active lever presses during time-out did not result in drug infusion and inactive lever presses had no consequence. Tone and light cues were presented concurrently with the cocaine infusion. Following 10 consecutive SA sessions with >10 infusions, rats began extinction where active lever presses produced no drug infusion or cues. Animals extinguished for 14 days and until <25 active lever presses were made for 2 days. For cue-induced reinstatement, rats received two IP injections of CNO (3 mg/kg, IP) or vehicle (.9% sterile saline with 5% DMSO) at 90 and 30 minutes before the reinstatement session. In some experiments, animals also received the mGluR2/3 antagonist LY341495 (1 mg/kg, IP) (Tocris Bioscience, Bristol, United Kingdom) or its vehicle (.9% sterile saline with 5% DMSO) at the same time they received CNO, according to our previous studies showing blockade of mGluR2/3 with this treatment protocol (28,29). The reinstatement sessions were identical to an SA session, except that pressing the active lever resulted in the presentation of light and tone cues without cocaine delivery. Each rat underwent one reinstatement trial. Sucrose SA, extinction, and cued reinstatement were performed in the same manner with 45 mg sucrose pellets (Noyes pellets, Fisher Scientific) substituted for cocaine infusions.

Statistics

Reinstatement data were statistically analyzed using a two-way repeated measures analysis of variance with a Bonferroni posttest to compare values between treatment groups. Neurochemical measurements were compared using an analysis of variance or a Mann-Whitney *U* test. All statistical tests were conducted using Prism (Graphpad Software Inc., La Jolla, California).

RESULTS

Astrocyte-Specific Expression of Gq-DREADD

Injection of the GFAP-Gq-DREADD virus in the NAcore produced fields of transduced astrocytes, easily distinguished from neurons given their morphology and lack of co-localization with a NeuN neuronal marker (Figure 1A,B). The GFAP-Gq-DREADD virus produced an immunoreactivity pattern consistent with astrocyte labeling (30–32), and astrocyte-specific expression of Gq-DREADD was very likely given the apparent co-localization of the astrocyte-specific filament protein GFAP and viral HA signals (Figure 1C,D; see Figure S1 in Supplement 1 for individual images of the *Z*-series shown in Figure 1D; see Figure S6 in Supplement 1 for orthogonal views of Gq-DREADD expression). Gq-DREADD expression was consistently found in ~70% of the GFAP expressing astroglia at the site of virus microinjection (Figure S2 in Supplement 1).

Activation of Gq-DREADD with CNO Initiates Glutamate Release

Using in vivo electrochemical recordings, we sampled extracellular glutamate concentrations in real time following CNO administration in animals expressing Gq-DREADD in NAcore astrocytes. Puff application of CNO (875 μmol/L) yielded a reproducible, dose-dependent, transient increase in extracellular glutamate concentration (Figure 3A,B). Importantly, focal ejections of CNO produced no response when the MEA-pipette assembly was implanted in a control region (dorsal striatum) where viral DREADD expression was absent (Figure 3A). The dose-dependent increase in the maximum amplitude of glutamate release (μmol/L) versus CNO ejected (pmol) was linear (R^2 = .98 ± .03 across three separate trials; Figure 3C). Puff application of the vehicle for CNO (100 nL of .9% saline + .5% DMSO) produced no glutamate response (Figure 3B). Systemic injection (IP) of vehicle (.9% saline + 5% DMSO) caused little fluctuation in the baseline levels of glutamate, whereas CNO (3 mg/kg, IP) caused an increase in glutamate transients lasting for approximately 60 minutes (representative traces of vehicle and CNO recordings are shown in Figure 3D). The CNO-mediated glutamate response began approximately 10 minutes after systemic CNO administration [consistent with expected time for CNO to access the brain (19,33)], was maximal at approximately 20 minutes, and slowly decreased over the next 40 minutes (Figure 3E).

Low Sensitivity of CNO-Mediated Glutamate Release to N-Type Ca^{2+} Channel Blockade

To verify that DREADD-mediated glial glutamate release was dependent on the release of Ca^{2+} from internal stores, we evaluated the ability of ω-conotoxin GVIA (an N-type Ca^{2+} channel antagonist) to inhibit potassium ion (K^+)-mediated and CNO-mediated glutamate release. MEAs were placed in the NAcore with two pipettes fixed to the biosensor, one for 70 mmol/L KCl or 875 μmol/L CNO and another for the application of 10 μmol/L ω-conotoxin GVIA (Figure 2A). Puff application of 60 nL KCl produced peaks with average amplitude of 4.7 μmol/L ± 4.7 and application of 60 nL 875 μmol/L CNO produced peaks with an average amplitude of 2.7 μmol/L ± 2.0

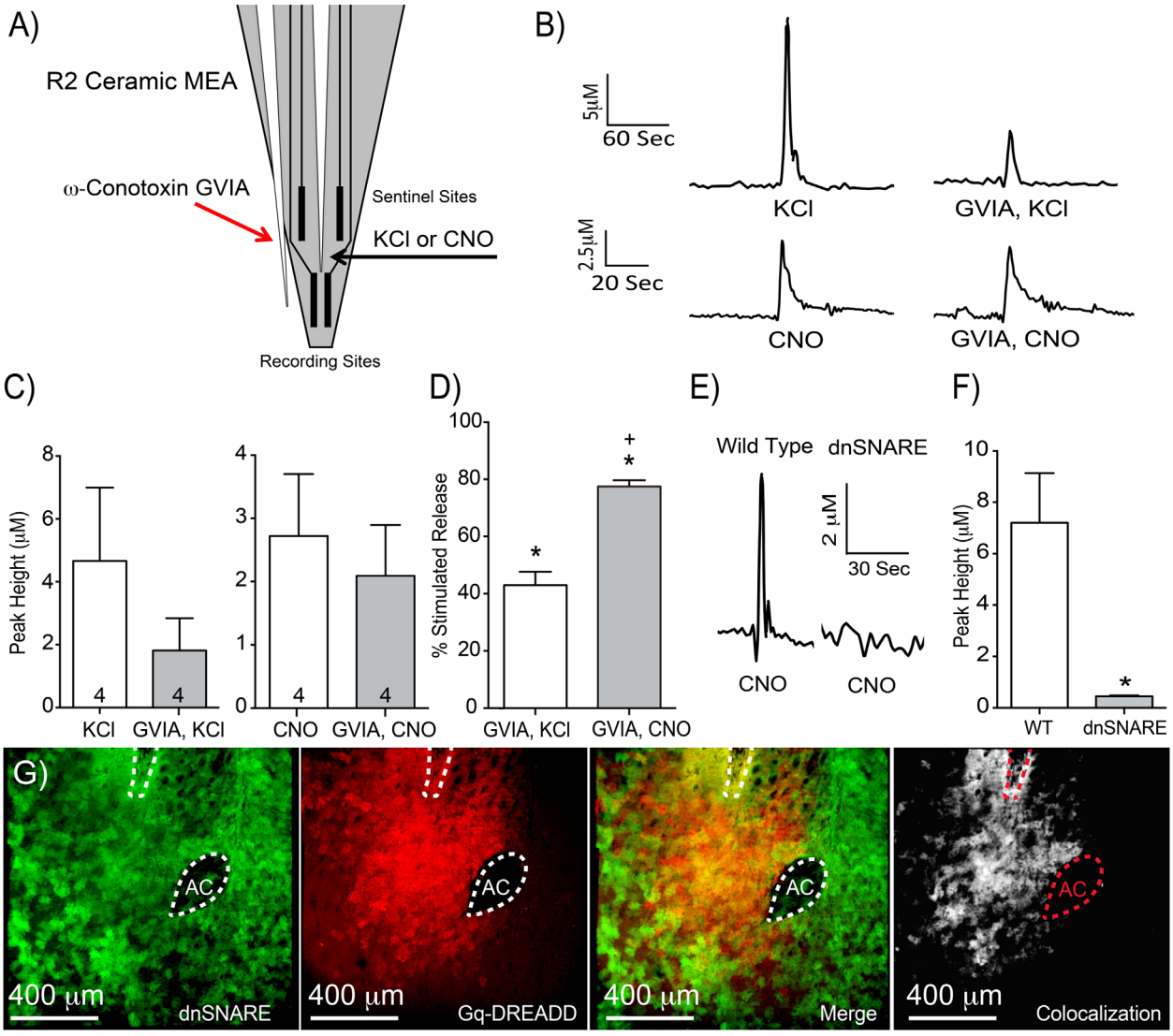

Figure 2. Clozapine N-oxide (CNO)-mediated glutamate release was less sensitive to N-type calcium ion channel blockade than potassium ion (K^+)-mediated glutamate release and required soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent gliotransmission. **(A)** A schematic of the dual pipette microelectrode array (MEA) assembly depicting which pipette contained each solution. **(B)** Representative traces of potassium chloride (KCl) and CNO peaks with and without GVIA inhibition. For all solutions, ejection volumes were 60 nL. **(C)** Left, maximum peak height for KCl experiments. Right, maximum peak height for CNO experiments. For both KCl and CNO experiments, peak heights from two to three matched observations were averaged across four separate animals. **(D)** Percentage of the uninhibited prior response is shown for both KCl and CNO experiments. ω-conotoxin GVIA significantly reduced peak height of K^+-mediated release (one-sample *t* test $^*p = .0012$) as well as CNO-mediated release (one-sample *t* test $^*p = .0019$). K^+-mediated glutamate release was more sensitive to ω-conotoxin GVIA than CNO-mediated glutamate release, Mann-Whitney test $^+p = .029$. **(E)** Representative 60 nL CNO peaks in wild-type and dominant-negative SNARE (dnSNARE) transgenic animals with glial fibrillary acidic protein-hM3D (Gq)-designer receptor exclusively activated by a designer drug (DREADD) expression in the nucleus accumbens core. **(F)** Maximum amplitude of CNO-mediated glutamate release in dnSNARE transgenic mice and wild-type (WT) littermates. Maximum amplitude of four CNO peaks was averaged across four separate animals for both wild-type and dnSNARE transgenics. Glutamate peak height was significantly higher in wild-type littermates when compared with dnSNARE transgenic mice Mann-Whitney test $^*p = .029$. **(G)** Gq-DREADD viral expression, dnSNARE expression, and MEA placement were confirmed ex vivo using Z-series confocal imaging. The representative micrographs depict dnSNARE in green, viral transduction of nucleus accumbens core astrocytes with Gq-DREADD in red, a merged image, and a three-dimensional co-localization analysis of the two signals in white. White signal shows the three-dimensional co-registration of the Gq-DREADD and dnSNARE signals in the Z-series. In these images, the anterior commissure is labeled AC and is surrounded by a broken white line. The damage created by the MEA probe, located in a field of virally transduced cells, is also surrounded by a broken white line.

(Figure 2B,C). Puff application of ω-Conotoxin-GVIA before K^+ application significantly decreased the amplitude of depolarization-induced glutamate peaks to an average of 43% of the K^+ alone response, while ω-Conotoxin reduced CNO peaks to 77.5% of the CNO alone maximum amplitude (Figure 2D). Consistent with a Gq mechanism and the recent report that GFAP driven Gq DREADD activation elevated $[Ca^{2+}]_i$ in astroglia (34), these data indicate that CNO-

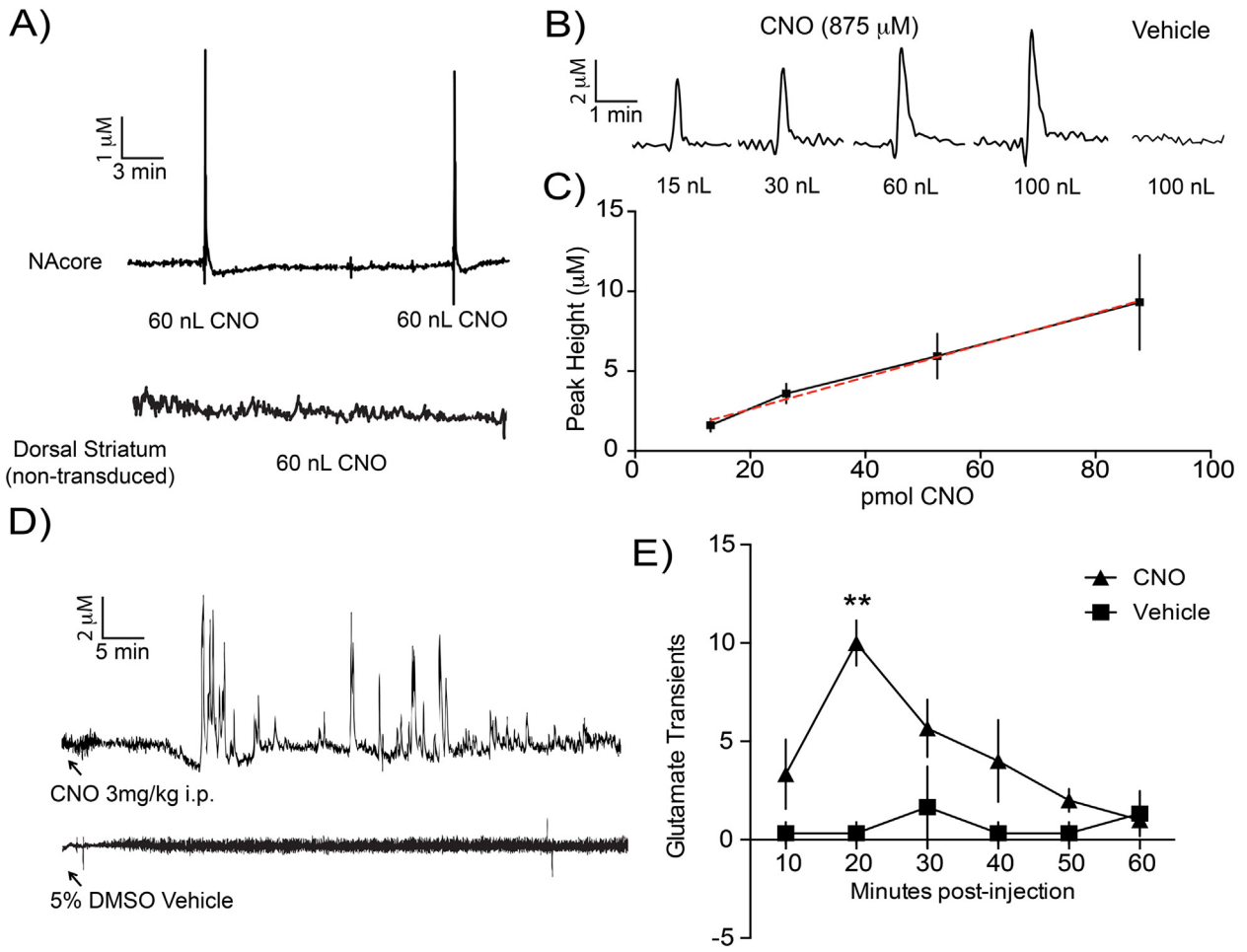

Figure 3. Activation of glial-designer receptor exclusively activated by a designer drug with clozapine-N-oxide (CNO) initiated glutamate release. **(A)** Representative glutamate peaks elicited by CNO puff application in the nucleus accumbens core (NAcore) and the lack of a response in the dorsal striatum (nontransduced control region). **(B)** Representative glutamate peaks elicited by puff application of increasing amounts of CNO. **(C)** Graph of maximum glutamate peak amplitude in μmol/L versus pmol of CNO ejected, $n = 3$ animals. **(D)** Top, following an intraperitoneal (IP) injection of 3 mg/kg CNO, glutamate spiking is observed for approximately 60 minutes. Bottom, vehicle recording shows very little activity in the nucleus accumbens after an injection of 1 ml/kg saline + 5% dimethyl sulfoxide (DMSO). **(E)** CNO induced glutamate spiking following IP administration was quantified as number of peaks per 10 minutes, with a peak defined as having an amplitude >3 times the height of the variability in the baseline recording. $n = 3$ each for CNO and vehicle. Two-way repeated measures analysis of variance revealed a significant effect of CNO treatment ($F_{1,2} = 25$; $p = .038$), time ($F_{5,10} = 5.602$; $p = .10$), and a significant interaction ($F_{5,10} = 3.950$, $p = .031$). Bonferroni posttests revealed that glutamate transients were significantly higher at 20 minutes after CNO injection when compared with vehicle, $^{**}p < .01$.

mediated release of glutamate relies on internal Ca^{2+} stores. Also consistent with this interpretation, previous reports show minor involvement of plasmalemmal Ca^{2+} channel conductances on vesicular glial glutamate release (13,35).

Genetic Inhibition of Gliotransmission Blocked CNO-Mediated Glutamate Release in the NAcore

To validate CNO-mediated vesicular glutamate release, glutamate MEAs were positioned in fields of GFAP-Gq-DREADD transduced astrocytes in the NAcore of transgenic mice selectively expressing dnSNARE in astrocytes or in age-matched wild-type littermates. The GFAP-Gq-DREADD virus produced an HA immunoreactivity pattern that overlapped substantially with dnSNARE expression (Figure 2G; Figure S3 in Supplement 1). Ejection of 60 nL CNO into dnSNARE transgenic mice produced peak heights of .44 μmol/L ± .04, whereas application of 60 nL CNO in wild-type littermates produced much larger peak heights of 7.27 μmol/L ± 1.93. These results demonstrate that CNO-mediated glutamate release required SNARE-dependent vesicular release.

CNO Activation of NAcore Glial DREADD Inhibited Cue-Reinstated Cocaine Seeking via mGluR2/3 Activation

To determine whether DREADD-mediated glial glutamate release impacts cue-induced cocaine seeking, we employed a rat operant model of cocaine SA followed by extinction training and cued reinstatement (Figure 4A). Administration of CNO

(3 mg/kg, IP) 15 minutes before a SA session had no effect on the number of active or inactive lever presses compared with a vehicle injection (Figure 4B). Following extinction, cue-induced reinstatement trials were conducted. Animals were injected with either vehicle or CNO (3 mg/kg) 90 minutes and 30 minutes before the reinstatement session. CNO significantly reduced active lever pressing during reinstatement compared with vehicle injection (Figure 4C), without affecting inactive lever pressing (Figure S4 in Supplement 1). Importantly, significant cue-induced reinstatement was observed in nontransduced control animals treated with CNO (extinction = 14 ± 2.4, reinstatement = 116 ± 28, n = 6, paired t test, p = .015). However, it is important to note that the results from Figures 4B and 4C are not directly comparable due to the fact that the CNO treatment paradigm was not identical. To test the hypothesis that the inhibitory effect of CNO was mediated by increased activation of mGluR2/3, reinstatement trials were conducted after animals were given consecutive injections of CNO (3 mg/kg) + LY341495 (1 mg/kg) or vehicle + LY341495 (1 mg/kg). Rats treated with LY341495 + vehicle displayed significant reinstatement, indicating that blocking mGluR2/3 did not, by itself, affect reinstated cocaine seeking (Figure 4C). However, when LY341495 was administered in concert with CNO, the CNO-induced reduction in reinstated lever pressing was reversed (Figure 4C). To determine if the effect of LY341495 on CNO was caused by inhibiting CNO-induced

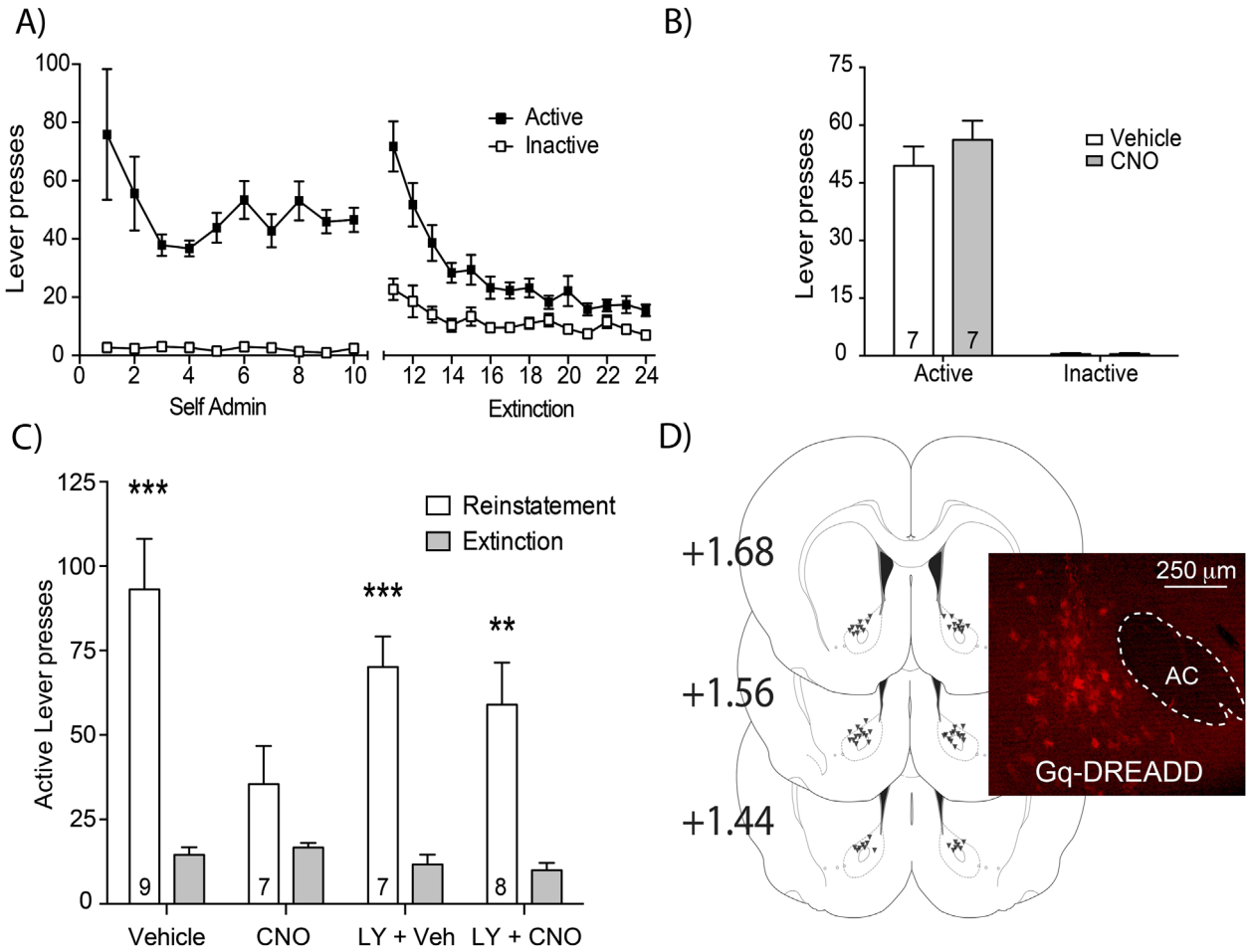

Figure 4. Activation of hM3D (Gq) signaling in astrocytes inhibited cue-induced cocaine reinstatement via metabotropic glutamate receptor 2/3. **(A)** Cocaine self-administration (Self Admin) and extinction data (X axis is days). **(B)** Two-way repeated measures analysis of variance revealed no significant effect of intraperitoneal injection of clozapine N-oxide (CNO) on active or inactive lever pressing during cocaine self-administration ($F_{1,12}$ = 2.575; p = .135), a significant difference between active and inactive lever presses ($F_{1,12}$ = 132.6; p < .0001), and no interaction ($F_{1,12}$ = 2.575; p = .1346). Bonferroni posttests revealed no significant differences in active versus inactive lever presses in CNO and vehicle treated animals, p > .05. **(C)** Active lever presses for extinction and reinstatement for vehicle (Veh), CNO, LY341495 (LY), and LY341495 + CNO groups. Two-way repeated measures analysis of variance revealed a significant effect of treatment ($F_{3,27}$ = 3.126; p = .042), significant effect of reinstatement ($F_{1,27}$ = 70.5, p < .0001), and a significant interaction ($F_{3,27}$ = 4.174, p = .015). Bonferroni posttests revealed that cue-induced reinstatement increased active lever pressing significantly (compared with extinction) in the vehicle control group, as well as in the LY341495 and LY341495 + CNO groups, but not in the CNO group, $^{**}p$ < .01, $^{***}p$ < .001. **(D) I**njection sites for rAAV5/GFAP-HA-hm3D-IRES-mCitrine viral infusion. The terminal points of the injection sites are represented as small black triangles with the coordinates shown relative to bregma. The representative micrograph shown details the location of the tip of the injection cannula relative to the anterior commissure (AC) and expression of the viral anti-hemagglutinin tag. $^{**}p$ < .01, $^{***}p$ < .001 compared with extinction. DREADD, designer receptor exclusively activated by a designer drug.

glutamate release, extracellular glutamate was measured using glutamate biosensors (as described above) and application of LY341495 did not prevent CNO from increasing extracellular glutamate (Figure S5 in Supplement 1). Figure 4D shows the location of the injection cannula termination in the NAcore. Stimulating glial glutamate transmission did not produce nonspecific reductions in reinstated operant responding, as we found no effect of CNO treatment on cued reinstatement of sucrose seeking (Figure 5C; Figure S4 in Supplement 1 for inactive lever pressing).

DISCUSSION

Our data demonstrate that Gq-DREADD-mediated enhancement of NAcore glial-glutamate release was SNARE-dependent and that the inhibitory effect of glial-glutamate on reinstated cocaine seeking was mediated by mGluR2/3. Several groups have shown that activation of Gq-DREADD elevates $[Ca^{2+}]_i$, potentially leading to enhanced gliotransmission (21,34). Here, we show that activating Gq-DREADD in NAcore astrocytes promotes the release of glutamate. In support, hippocampal studies reveal a parallel mechanism whereby photolysis of caged astroglial calcium elevated $[Ca^{2+}]_i$, initiated glutamate release, and decreased synaptic transmission in neighboring neurons by stimulating presynaptic mGluR2/3 (36). Moreover, activation of NAcore mGluR2/3 receptors is an established mechanism to inhibit reinstatement (37–39); however, see Bauzo *et al.* (40). In addition, promoting glutamate release into the extracellular space by stimulating cystine-glutamate exchange with N-acetylcysteine also inhibited reinstated cocaine seeking in an mGluR2/3-dependent manner (18,28). Thus, mGluR2/3 agonist, N-acetylcysteine and GFAP-Gq-DREADD, activation inhibits reinstated cocaine seeking by activating presynaptic mGluR2/3 autoreceptors, causing reduced release probability at NAcore synapses (18,28) (Figure 6). Further supporting an involvement of glutamate gliotransmission in the regulation of addictive behavior, a recent report from the Bowers lab demonstrated that stimulating glial Gq-DREADD in the NAcore inhibited the motivation to seek ethanol (34). While we used cell morphology and characteristics of glutamate release by CNO (dnSNARE mice and relative insensitivity to N-channel blockers) to show that Gq-DREADD was expressed in glia, a very minor contribution by neuronal Gq-DREADD cannot be 100% ruled out.

Astroglial Regulation of Synaptic Plasticity

Gq-DREADD-mediated NAcore glial glutamate release could also potentially activate extrasynaptic mGluR5, which promotes reinstated cocaine seeking (9,41). While it is possible that this signaling cascade was engaged, its effects on reinstatement were likely countermanded by the presynaptic actions of Gq-DREADD-mediated glutamate release on mGluR2/3 autoreceptors, which are reported to be more sensitive than other mGluR subtypes (17). Apart from glutamate, activation of Gq signaling in astrocytes and the subsequent increase in $[Ca^{2+}]i$ could also potentially cause release of additional small molecule transmitters, including taurine, adenosine triphosphate (ATP), and D-serine (11,12,42,43). Despite the fact that the reversal of CNO-mediated inhibition of reinstatement by antagonizing mGluR2/3 indicates the involvement of glutamate, it may be possible that other gliotransmitters contributed to the effect of CNO on reinstated cocaine seeking. As an example, glial release of glutamate and D-serine contribute to the induction of *N*-methyl-D-aspartate (NMDA)-dependent slow inward currents, and blocking NMDA receptors attenuates both nicotine and heroin seeking (44–46).

dnSNARE Mice

We observed that CNO-mediated (glial) glutamate release was less sensitive to N-type Ca^{2+} channel blockade than K^+ depolarization-mediated (neuronal) glutamate release, consistent with CNO-mediated glutamate release occurring via the mobilization of internal Ca^{2+} from ER stores (47). However, our data also support a partial role for influx of external Ca^{2+}, as CNO-mediated glutamate release was impacted by N-type Ca^{2+} channel blockade. We also observed that CNO-mediated glutamate release in GFAP-dnSNARE transgenic mice was significantly blunted when compared with

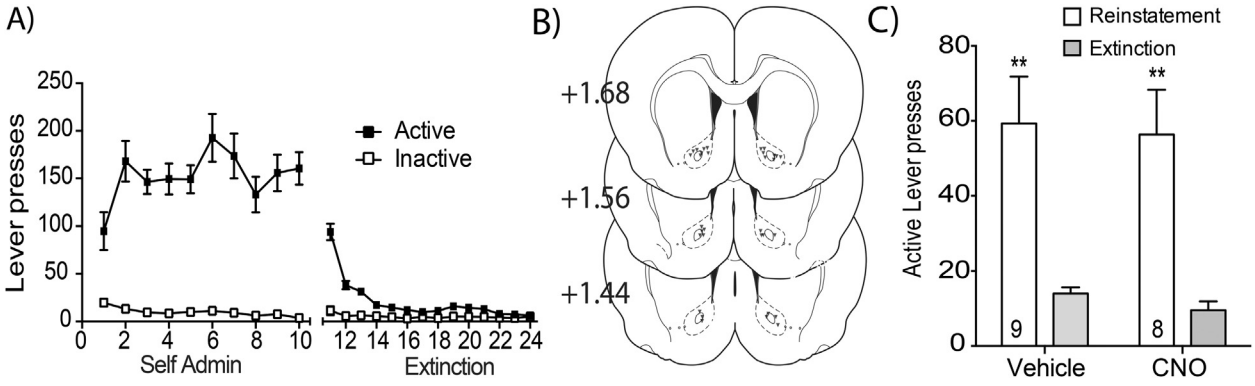

Figure 5. Clozapine N-oxide (CNO) activation of glial fibrillary acidic protein-hM3D-designer receptor exclusively activated by a designer drug in the nucleus accumbens core had no effect on cue-induced reinstatement of sucrose seeking. **(A)** Sucrose self-administration (Self Admin) and extinction data (X axis is days). **(B)** Injection sites for rAAV5/GFAP-HA-hm3D-IRES-mCitrine viral infusion. The terminal points of virus injection sites are represented as small black triangles with the coordinates shown relative to bregma. **(C)** Active lever presses for extinction and reinstatement for vehicle and CNO groups. Two-way repeated measures analysis of variance revealed no significant effect of CNO treatment ($F_{1,15} = .174$; $p = .682$), significant reinstatement ($F_{1,15} = 28.31$, $p < .0001$), and no significant interaction ($F_{1,15} = .008$, $p = .930$). Bonferroni posttests revealed that cue-induced reinstatement increased active lever responding significantly (compared with extinction) in the vehicle group, as well as the CNO group, $^{**}p < .01$.

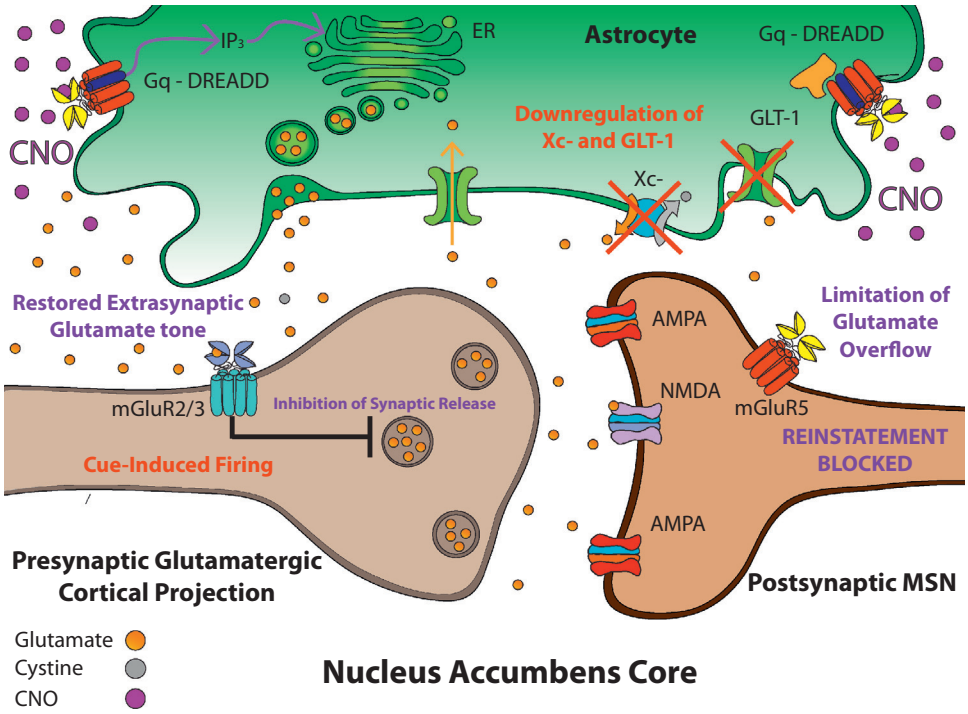

Figure 6. Summary of the mechanism of glial fibrillary acidic protein-hM3D (Gq)-designer receptor exclusively activated by a designer drug (DREADD)-mediated reduction of cue-induced reinstatement. Following cocaine self-administration and extinction training, the cystine-glutamate exchanger (Xc-) is downregulated, contributing to reduced extracellular basal glutamate and decreased tone on presynaptic metabotropic glutamate receptors 2/3 (mGluR2/3) (blue). Cocaine exposure also downregulates glial glutamate transporter 1 (GLT-1) (green), which exacerbates glutamate overflow in response to cue exposure. Glutamate overflow normally activates postsynaptic glutamate receptors including metabotropic glutamate receptor 5 (mGluR5) (red), leading to the reinstatement of cocaine seeking. The activation of Gq-DREADD on astrocytes by clozapine N-oxide (CNO) causes the production of inositol 1,4,5-triphosphate (IP_3), resulting in the release of calcium ions from the endoplasmic reticulum (ER), leading to the vesicular release of glutamate. Glutamate release from astrocytes enhances extracellular glutamate concentration, restoring tone on presynaptic mGluR2/3 receptors, which limits synaptic glutamate release, leading to lower levels of glutamate overflow and the inhibition of cue-induced cocaine seeking. AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; MSN, medium spiny neuron; NMDA, *N*-methyl-D-aspartate.

wild-type littermate control animals, which is consistent with data shown by others demonstrating inhibited hippocampal glial adenosine release in GFAP-dnSNARE mice (48). Interestingly, the GFAP dnSNARE mice did not display cue-induced reinstatement of cocaine seeking (49). This suggests that while increasing glial release of glutamate locally in the NAcore inhibits reinstated cocaine seeking, a global loss of gliotransmission impairs reinstated cocaine seeking (49).

As is the case with any model, the tetracycline-controlled transcriptional activation, tet-off based dnSNARE transgenic mice have potential caveats including chromosomal positioning effects and the possibility of leaky transgene expression from the tTA-tetO system. Contrary to what we observed in the NAcore, a recent report indicates that the dnSNARE mice show off target neuronal expression of the dnSNARE transgene in the cerebral cortex (50). These results provoke further debate on astrocytic Ca^{2+}-dependent vesicular exocytosis and highlight that additional examination of the dnSNARE model is required to determine its usefulness moving forward.

Ca^{2+} Dependent Release of Glutamate from Astroglia

There are several mechanisms that astrocytes utilize to release neuroactive molecules (10), with Ca^{2+}-dependent vesicular exocytosis being one of the more debated and intriguing modalities (13). In light of the oscillation of $[Ca^{2+}]_i$ observed in astrocytes (51), gliotransmission is thought to occur via the mobilization of internal calcium stores, most likely as a result of the activation of Gq signaling and the production of the second messenger IP_3 (13). Several studies support the role of astroglial IP_3 signaling in the modulation of synaptic plasticity by glial cells (52,53), yet others report a lack of IP_3-mediated effects on synaptic activity (54). However, there is general consensus that transmitters released from astrocytes can alter synaptic activity (36,55–57) and that astrocytes express the vesicular glutamate transporter and synthesize the proteins necessary for the assembly of the SNARE complex required for vesicle fusion (16,58–60). In the work presented above, it is interesting that the increased levels of glutamate observed over the 60 minutes after systemic CNO administration were composed of transients, reminiscent of quantal release events. Clearly, the time course of the CNO-induced glutamate transients exceeded expectations for synaptic quanta (61), implying either a more prolonged fusion-release process than synaptic vesicular release or an accumulation of glutamate from the closely timed release of glutamate from multiple glial vesicles. Unfortunately, the sensitivity of the

biosensors (~1 μmol/L) does not permit a more detailed quantification of the glutamate transients.

Investigations of exocytotic release of glutamate from astrocytes and its role as a signaling mechanism have been contradictory. Woo *et al.* (62) have shown that astroglial activation of Gq-coupled receptors induces slow, nonvesicular glutamate release in hippocampal astrocytes through the glutamate-permeable Ca^{2+}-activated anion channel Best1. However, recent reports provide evidence supporting vesicular release of ATP in response to elevations of $[Ca^{2+}]_i$ (60). Lalo *et al.* (60) observed a diminished burst of purinergic currents using sniffer cell technology following protease-activated receptor 1 receptor (Gq) activation when slices were taken from dnSNARE mice, indicating that Ca^{2+}-mediated release of ATP occurs through SNARE-dependent vesicular exocytosis. Recent data from this group also support vesicular release of glutamate from astrocytes, whereby elevation of $[Ca^{2+}]_i$ resulted in NMDA-receptor mediated miniature excitatory postsynaptic currents that were absent in slices prepared from dnSNARE mice (63). Additional experimentation is required to characterize precisely which signaling cascades are responsible for increasing $[Ca^{2+}]_i$, how this mechanistically leads to the release of glutamate, and if there are differences in the mechanisms underlying Ca^{2+}-mediated gliotransmission across brain regions.

Conclusions

Our study demonstrates that the acute release of glutamate from glia can inhibit cue-induced reinstatement of cocaine seeking in an mGluR2/3-dependent manner. As such, mGluR2/3 agonists appear an attractive target for cocaine relapse prevention medication. However, most studies have shown that mGluR2/3 agonists also decrease other motivated behaviors, such as food seeking (37,64,65), although this was not seen with a positive allosteric mGluR2/3 agonist (38). The fact that there was no effect on sucrose reinstatement following Gq-DREADD activation poses the possibility that stimulating mGluR2/3 selectively by enhancing glial glutamate release at a specific site of pathology in the brain (i.e., the NAcore) may yield a pharmacogenetic therapy lacking the side effects elicited by direct small molecule mGluR2/3 agonists that would act throughout the brain.

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