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Behavioral/Cognitive

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The importance of neuronal glutamate to synaptic transmission throughout the brain illustrates the immense therapeutic potential and safety risks of targeting this system. Astrocytes also release glutamate, the clinical relevance of which is unknown as the range of brain functions reliant on signaling from these cells hasn't been fully established. Here, we investigated system xc- (Sxc), which is a glutamate release mechanism with an *in vivo* rodent expression pattern that is restricted to astrocytes. As most animals do not express Sxc, we first compared the expression and sequence of the obligatory Sxc subunit xCT among major classes of vertebrate species. We found xCT to be ubiquitously expressed and under significant negative selective pressure. Hence, Sxc likely confers important advantages to vertebrate brain function that may promote biological fitness. Next, we assessed brain function in male genetically modified rats (*MSxc*) created to eliminate Sxc activity. Unlike other glutamatergic mechanisms, eliminating Sxc activity was not lethal and didn't alter growth patterns, telemetry measures of basic health, locomotor activity, or behaviors reliant on simple learning. However, *MSxc* rats exhibited deficits in tasks used to assess cognitive behavioral control. In a pavlovian conditioned approach, *MSxc* rats approached a food-predicted cue more frequently than WT rats, even when this response was punished. In attentional set shifting, *MSxc* rats displayed cognitive inflexibility because of an increased frequency of perseverative errors. *MSxc* rats also displayed heightened cocaine-primed drug seeking. Hence, a loss of Sxc-activity appears to weaken control over nonreinforced or negative-outcome behaviors without altering basic brain function.

Key words: astrocytes; autoshaping; cocaine; glutamate; set shifting; system xc

Significance Statement

Glutamate is essential to synaptic activity throughout the brain, which illustrates immense therapeutic potential and risk. Notably, glutamatergic mechanisms are expressed by most types of brain cells. Hence, glutamate likely encodes multiple forms of intercellular signaling. Here, we hypothesized that the selective manipulation of astrocyte to neuron signaling would alter cognition without producing widespread brain impairments. First, we eliminated activity of the astrocytic glutamate release mechanism, Sxc, in rat. This impaired cognitive flexibility and increased expression of perseverative, maladaptive behaviors. Notably, eliminating Sxc activity did not alter metrics of health or noncognitive brain function. These data add to recent evidence that the brain expresses cognition-specific molecular mechanisms that could lead to highly precise, safe medications for impaired cognition.

Introduction

Poor behavioral control contributes to addiction, cancer, and other chronic disorders (Verdejo-Garcia et al., 2015; Lange et al., 2017; Harrison and Wefel, 2018; Peters et al., 2019). Although relevant circuits have been identified, developing a treatment will require the discovery of molecular mechanisms that effectively and safely promote behavioral control. This is challenging, in part, because the bulk of neurotransmission is mediated by a small number of neurotransmitters, and individual neurotransmitter mechanisms regulate functionally distinct neural circuits. Hence, adverse and therapeutic effects can often stem from an

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identical mechanism. As a result, dose restrictions or other efforts to promote safety will likely also reduce efficacy.

The evolution of cognitive behavioral control involved, in part, the introduction of novel molecular mechanisms that increased signaling complexity in the brain (Grant, 2016; Ramos-Vicente et al., 2018; Santello et al., 2019). Critically, a new paralogue within the DLG (Discs-Large) family of postsynaptic scaffolding proteins was found to be required for complex cognition but not survival, simple learning, or other forms of brain function (Nithianantharajah et al., 2013). These and related results indicate the potential existence of cognition-specific signaling molecules (Grant, 2016), the existence of which could enable the rational development of highly precise therapeutics for the wide range of human disorders containing cognitive dysfunction. Hence, there is a need to understand the evolution of the major signaling systems in the brain.

Glutamate originated as a primitive signal that existed before the divergence of plants and animals (Chiu et al., 1999) and evolved into a network so complex that virtually every type of mammalian brain cell expresses a glutamate receptor, transporter, and/or release mechanism (Färber and Kettenmann, 2005; Volterra and Meldolesi, 2005; Cahoy et al., 2008; Ramos-Vicente et al., 2018; Verkhratsky and Nedergaard, 2018; Hogan-Cann et al., 2019; Suárez-Pozos et al., 2020). Hence, glutamate not only mediates signaling at most excitatory synapses, but it functionally couples many of the distinct types of intercellular communication in the mammalian brain. Astrocyte to neuron signaling may have been especially critical to phylogenetic gains in signaling complexity and cognitive behavioral control (Allen and Barres, 2005; Lee et al., 2014; Poskanzer and Yuste, 2016; Oberheim Bush and Nedergaard, 2017; Neuhofer and Kalivas, 2018; Santello et al., 2019; Oliveira and Araque, 2022).

The possibility that manipulating astrocyte to neuron signaling could alter cognition, possibly without producing widespread brain impairments, needs to be examined. System xc- (Sxc) is an ideal target to investigate this as it is an evolutionarily newer addition to the glutamate signaling network (Lewerenz et al., 2013), is expressed *in vivo* by astrocytes but not neurons (Ottestad-Hansen et al., 2018), and is a key determinant of glutamate signaling in the nucleus accumbens and other regions controlling behaviors (Baker et al., 2002; Moran et al., 2005; De Bundel et al., 2011; Williams and Featherstone, 2014).

We investigated Sxc using two broad strategies. First, we conducted phylogenetic analyses of Sxc to add to the emerging understanding of the evolution of the glutamate signaling network. We learned that Sxc is like other glutamatergic mechanisms because it is ubiquitously expressed in vertebrates and has been under significant selective pressure. Next, we created a novel rat model lacking Sxc activity. Eliminating Sxc activity increased approach behavior directed at a food-predictive cue, impaired cognitive flexibility, and enhanced cocaine-primed drug seeking in rats. Notably, each of these behaviors model impaired behavioral control in humans (Robbins, 1996; Robinson et al., 2014; Verdejo-Garcia et al., 2015). Unlike other glutamatergic mechanisms, eliminating Sxc was not fatal and did not impair development, physiological telemetry, general activity states, simple learning, object recognition, or other indicators of widespread disruption of brain function. Hence, we propose that Sxc belongs to a class of evolutionarily new molecular adaptations that contributed to the phylogenetic enhancement of cognition and, importantly, may not be required for noncognitive functions of the brain. Crucially, identifying cognitive-specific molecular mechanisms could enable the rational development of safe and effective treatments for cognitive impairment.

Materials and Methods

Phylogenetic comparisons of Slc7a11/xCT in vertebrates. mRNA protein coding sequences from the *Slc7a11* gene from 115 organisms were retrieved from GenBank (Benson et al., 2009). Partial and isoform sequences were left out of this study. Sequences were aligned by codon using MUSCLE (Edgar, 2004) in the MEGAX software package (Tamura et al., 2013) and reviewed for accuracy manually. Aligned sequences were run through MEGAX model selection software using a neighbor joining tree and a maximum likelihood statistical method. Gaps were treated by partial deletion and were not used for computing tree branch lengths. The K2+G (gamma distributed) model was used for construction of the phylogenetic tree. Pairwise distances were estimated with uniform rates and partial deletion of gaps. Bootstrap values were generated from 100 replications. A cutoff tree was computed with a minimum bootstrap value of 70. Sequence motifs for specific consensus phosphorylation sites were searched within the alignment, and conservational data were manually extracted based on the existence or absence of the site. Determination of sites under positive or negative selection was conducted using the Datamonkey Web server (Delport et al., 2010). Single Likelihood Ancestor Counting (SLAC; Kosakovsky Pond and Frost, 2005) analyses were conducted using a neighbor joining tree at a 0.05 significance level.

Animal care and usage. Male Sprague Dawley rats with a postnatal day (P) age range of P70–P150 were used in these studies. Rats were obtained from an internal colony of genetically modified rats generated using a Het/Het breeding approach, which allowed for the use of WT and mutant Sxc (*MSxc*) littermates in these studies. Outbreeding involved the use of Sprague Dawley rats purchased from Envigo, which occurred every three generations. After postnatal day 60, offspring were individually housed. Rats were maintained on a 12 h light/dark cycle with behavioral experiments occurring during the light cycle, unless otherwise stated. Housing conditions and experimental protocols were approved by Institutional Animal Care and Use Committees at Marquette University and the Medical College of Wisconsin and were conducted according to the National Institutes of Health guidelines.

For creation of *MSxc* genetically modified rats, zinc-finger nucleases (ZFNs) were designed to target the second exon sequence (TGCTAGCTTTTGTTC gagtcTGGGTGGAACTGCTG) and produce small deletions of a limited number of base pairs in the *Slc7a11* gene; capital letters represent binding sites for the individual ZFN monomers, on opposite strands. This mutation was predicted to disrupt Sxc activity because *Slc7a11* encodes the functional subunit of Sxc, xCT, and xCT is selective to Sxc. ZFNs were injected into the pronucleus of Sprague Dawley (Crl:SD) rat embryos by pronuclear microinjection of *in vitro* transcribed encoding messenger RNAs, and the resulting offspring were screened for mutations using a Cel-1 assay and validated by Sanger sequencing as previously described (Geurts et al., 2010), resulting in single-step, whole-animal disruption of *Slc7a11* (*MSxc* rats). Deletion of 39 consecutive base pairs (GAGGTCTTTGGTCCCT TGCTAGCTTTTGTTCGAGTCTGG) of exon 2 was confirmed by Sanger sequencing.

Genotyping. A 2 mm ear punch from the outer middle edge of the pinna of each rat was obtained at weaning (~P21). DNA was then isolated as described by Laird et al. (1991), sequenced in duplicate using a capillary benchtop sequencer (model 3730xl, Applied Biosystems) and analyzed using GeneMapper software.

Cell culture. Astrocyte cultures were generated from WT or *MSxc* postnatal day 3 rat pups. The striatum was dissected and dissociated using 0.25% trypsin EDTA (Invitrogen) and cultured in 75 cm^2 flasks in a humidified incubator at 37°C under 95% O_2/5% CO_2 in Eagles minimum essential medium (Invitrogen) supplemented with 5% fetal bovine serum/5% horse serum (Atlanta Biologicals), Glutamax (Invitrogen), β-mercaptoethanol (Sigma-Aldrich), and antibiotics/antimycotics (Invitrogen). To remove debris and nonastrocytic glia, flasks were agitated, and the

resulting monocell layer was resuspended with 0.25% trypsin EDTA. Cells were counted by hand via a cytometer and seeded in 24-well plates coated with poly-D-lysine and laminin at a density of 200,000 cells per well.

RT-PCR. Total RNA was extracted from NAc tissue samples using Trizol reagent and was subsequently treated with DNase (Life Technologies) to remove genomic DNA contamination. RNA purity and quantity were assessed using a Nano Vue Plus spectrophotometer (GE Life Sciences). RNA (1 μg) from each sample was reverse transcribed for PCR (Promega). PCR was conducted using GoTaq DNA polymerase (Promega). Primer sequences were as follows: *Slc7a11* (xCT) forward, 5′ TTT GGA GCC CTG TCT TAT GC 3′; *Slc7a11* reverse, 5′ ACC CAG ACT CGA ACA AAA GC 3′; *Gapdh* forward, 5′ CTC CCA TTC TTC CAC CTT TGA 3′; *Gapdh* reverse, 5′ ATG TAG GCC ATG AGG TCC AC 3′. Note, the xCT primer was designed to include the mutation site. Note, because this is a heritable germline mutation, the alterations in xCT will be equivalent in all tissues.

Glutamate release assay. Striatal astrocytes (DIV14) were incubated for 30 min at 37°C in Na^+-free buffer containing the following (in mM): 116 choline chloride, 13.4 $MgSO_4$, 1.68 KH_2PO_4, 2.34 $CaCl_2$, 5.49 dextrose, 11.9 HEPES, and 0.2% choline bicarbonate, titrated to pH 7.4 with CeOH. This buffer was used to prevent Na^+-dependent uptake of glutamate. L-cystine at (0, 12.5, 25, 50, 100, or 200 mM) was applied to drive cystine-glutamate exchange by Sxc. Media samples (100 μl) were collected for subsequent glutamate analysis using HPLC. Cells were then dissolved in 0.5% SDS, and total protein for each well was quantified using the BCA method.

Glutamate HPLC. The concentration of glutamate was quantified by comparing peak areas from samples and external standards using HPLC coupled to fluorescence detection. A 10 μl sample underwent precolumn derivatization with orthophthaladldehdye in the presence of β-mercaptoethanol using a Shimadzu LC10AD VP autosampler. Chromatographic separation was achieved using a Kinetex XB-C18 (50 × 4.6 mm, 2.6 μm; Phenomenex) and a mobile phase consisting of 100 mM Na_2HPO_4, 0.1 mM EDTA, 10% acetonitrile, pH 6.04. Glutamate was detected using a Shimadzu RF-10AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively. Glutamate content for each sample was normalized to total protein in the respective well and depicted as a net change from baseline.

Developmental impact of Slc7a11 mutation. To determine the lethality and developmental impact of eliminating Sxc function, we measured mortality rates and body weight gain. Mortality was assessed in two cohorts. First, we monitored survival rates in a large cohort ($N > 500$/genotype/sex) until postnatal day 70, which corresponds to adulthood in rat (McCutcheon and Marinelli, 2009). Next, we monitored survival rates for 1 year in a smaller cohort (N = 8–10/genotype/sex). Body weights were monitored postweaning until adulthood (i.e., postnatal day 70; N = 25–28/genotype).

Physiology telemetry. Adult WT and *MSxc* rats (N = 12/genotype) were implanted intraperitoneally with telemetry probes (Mini-Mitter) to remotely record locomotor activity while in their home cage. Activity data were collected every 5 min and averaged in 1 h bins.

Novel object recognition. During the first 2 d, adult WT and *MSxc* rats (N = 9/genotype) underwent 5 min habituation sessions, which involved placing the rats in a 50 × 25 cm bedding-free chamber outfitted with a camera. On the third day, rats were placed into the maze for 5 min to become familiarized with two identical objects that were placed in adjacent corners of the chamber. One hour later, rats were placed into the maze for 5 min, which had one familiar object and one novel object. The time spent interacting (looking/sniffing/climbing) with each object was recorded during each session on the third day. The placement and identity of each object were randomized to control for object and spatial preference.

Pavlovian conditioned approach. Adult WT and *MSxc* rats (N = 10 and 9, respectively) underwent autoshaping in operant chambers (Campden Instruments) equipped with touch-sensitive display screens (Horner et al., 2013). One week before testing, subjects underwent daily handling and were food deprived to achieve a target weight within 90–95% of their *ad libitum* mass at study onset. In the first of two habituation phases, rats were placed into the chamber until they consumed 20 chocolate-flavored sucrose pellets located in the food tray. This continued once per day until each of the sucrose pellets was consumed within 20 min. Next, rats underwent daily 30 min sessions during which the delivery of a sucrose pellet was paired with the onset of a compound stimulus comprised of a tray light and a 1 s, 3 kHz auditory cue. The pellet and compound cue were delivered on a variable interval (VI) schedule (ranging between 0 and 30 s). All rats met the criterion of consuming 40 rewards after 1 d. Approaches to each side of the food tray were recorded to detect potential side biases, which was defined as ≥70% of approaches to one side.

Rats then underwent pavlovian conditioned approach training over five daily sessions. Each session involved 40 trials (20 CS+ and 20 CS− presentations). The conditioned stimulus (CS)+ cue was located on the screen on the nonpreferred side of the rat if a preference was detected during habituation or was randomly determined for rats that did not display a side bias. Each trial was initiated once a 10–40 s VI had passed, and the subject broke the infrared beam in the back of the chamber. A visual stimulus was illuminated on the display screen for 10 s. In CS− trials, the intertrial interval began on termination of the cue. In CS+ trials, the termination of the cue was immediately followed by the compound stimulus (the tray light and a 1 s, 3 kHz auditory cue kHz) and the delivery of one sucrose pellet in the food tray. The intertrial interval (VI, ranging between 10 and 40 s) would then restart. Measures include approaches to each cue screen and the food tray. Physical contacts of the display screens were recorded but occurred too infrequently to be analyzed. After completing five daily autoshaping trials, rats underwent five sessions of omission testing. These trials were identical to the earlier trials with the sole exception that an approach to the CS+ cue resulted in the omission of the food reward.

Attentional set shifting. Adult rats (N = 6–7/genotype) were food restricted to 90% of their *ad libitum* body mass for 1 week before habituation to a maze. The maze contained four arms (60 × 20 × 12 cm), which were configured as a T maze by blocking off the arm opposite to starting location. Day 1 of testing required rats to perform a simple discrimination task in which a visual cue (a laminated paper with black and white stripes) was placed on the floor of the food-reinforced arm (45 mg sucrose). The starting arm of the maze and the placement of the visual cue was randomized with a fixed ratio on subsequent trials until the rat entered the correct arm on 10 consecutive trials. Day 2 of testing required rats to perform an extradimensional shift as the baited arm could be identified using a spatial/directional cue (e.g., the left or right arm) rather than a visual cue. Day 3 required an intradimensional shift because the baited arm was opposite to the day 2 assignment of the subject. Errors were distinguished based on whether they were committed before or after the subject displayed success in solving the current task (i.e., a threshold of 75% successful trials within a block of eight). Incorrect responses made before this threshold were considered perseverative errors that typically involve the animal using a past strategy. Incorrect responses after this threshold were deemed regressive errors as the subject had been successfully applying the new strategy. Animals were removed from the study if they took longer than 30 min to complete 15 trials during any day of testing.

Surgery. WT and *MSxc* rats that self-administered cocaine were implanted with chronic indwelling catheters. Anesthesia was maintained using 2–2.5% isoflurane using a precision vaporizer during the surgery. A silicon-tubing catheter (0.31 mm inner diameter, 0.64 mm outer diameter) was inserted into the right posterior facial vein until it terminated at the right atrium. The internal aspect of the catheter was sutured to the vein. The distal end exited 2 cm posterior to the scapula. The exit port consisted of a back-mounted 22 gauge guide cannula (Plastics One) attached to a polypropylene monofilament surgical mesh (Atrium Medical). Rats were allowed to recover for at least 7 d before self-administration testing during which time they were provided acetaminophen (480 mg/l) in their drinking water. Cefazolin (100 mg/kg, i.v.) was administered to rats displaying signs of an infection. Postsurgical pain and inflammation were managed using meloxicam (1.0 mg/kg, s.c.). Catheters were filled daily with a heparin solution

(83 IU/ml) with the distal cannula capped with a closed piece of Tygon tubing whenever the leash/delivery line assembly was disconnected.

Cocaine self-administration and reinstatement. Adult WT and *MSxc* rats (N = 16 and 14, respectively) were trained to self-administer cocaine on an fixed ratio 1 schedule of reinforcement during once-daily 2 h sessions (Madayag et al., 2010). In contrast to the other experiments, rats in these studies were maintained on a reverse light cycle and were tested during their dark phase. During each session, a response on the active lever extinguished the house light, illuminated a cue light located above the active lever, and resulted in an infusion of cocaine (0.5 mg/kg/inf, i.v.). Acquisition training sessions continued until rats received at least 12 daily infusions, and intake over three consecutive sessions varied <15%. Subjects achieving the acquisition criterion then received 12 daily maintenance self-administration sessions, which was followed by extinction training. During extinction training, a response on the active lever extinguished the house light, illuminated a cue light located above the active lever, and resulted in an infusion of saline. Once rats met the extinction criterion of 15 or fewer presses/day, reinstatement testing was conducted. Reinstatement testing was identical to an extinction session except rats received a low dose of cocaine (3 mg/kg, i.p.) 10 min before testing. Hence, the reinstatement test assessed drug seeking (i.e., active lever pressing) triggered by a low dose of cocaine and reinforced by delivery of the cocaine-paired cue. Note, the dose of cocaine used was selected because it is typically ineffective in producing reinstatement in WT rats, thereby enabling us to assess increase reinstatement susceptibility in *MSxc* rats.

Experimental design and statistical analysis. Investigators were blinded to genotype for all behavioral procedures. All data are presented as means ± SEM with individual data points for independent samples and paired lines for dependent samples overlaid. Statistical analyses were conducted using IBM SPSS Statistics software (version 14.0). Statistical significance was determined using a mixed-model, repeated-measures ANOVA, varying genotype (*MSxc* vs WT) as the between-subject factor and session (1–5) as the within-subject factor. Fisher's least significant difference tests or Student's t tests were used to make planned pairwise comparisons when there were only two means to compare or when ANOVAs revealed an appropriate significant main effect or interaction. The Holm–Sidak procedure was used to contain familywise α level at 0.05 for multiple *post hoc* comparisons. Effect sizes were calculated using partial eta squared (η_p^2; ANOVA main effects and interactions) and Cohen's d for important pairwise comparisons (Student's t tests and Fisher leaset siginficant difference comparisons). Unique analytical details for each experiment, such degrees of freedom and follow-up statistical analysis are reported below in Results.

Results

Slc7a11/xCT arose following the divergence of protostomes and deuterostomes (Lewerenz et al., 2013), which establishes it as an evolutionarily new mechanism. Glutamate release from Sxc involves the antiporting of cystine (Fig. 1*A*). To better understand the evolution of Sxc, we conducted a phylogenetic comparison of xCT across 115 vertebrate species that were distributed across the major classes of vertebrates (i.e., Chondrichthyes or cartilaginous fish, Osteichthyes or bony fish, Reptilia, Aves, and Mammalia). We found that *Slc7a11*/xCT is expressed by every vertebrate species examined.

A SLAC analysis of *Slc7a11*/xCT revealed that it has been under negative selective pressure (Fig. 1*B*). Many codons displayed significantly more nucleotide mutations that did not alter amino acid sequence (synonymous mutations; dS) than mutations that altered amino acid sequence (nonsynonymous mutations; dN). There were 395 codons with a dN/dS ratio that achieved significance with an unadjusted p value of < 0.05 (p values ranged from 1.65-E15 to 0.046); 209 codons had a dN/dS ratio that achieved significance even when p values were adjusted using the Holm–Sidak procedure to control for the numerous *post hoc* comparisons (p values for comparisons deemed significant were 0.00,014 or lower).

The genetic divergence of xCT resulted in a gene tree (Fig. 1*C*) that broadly matched phylogenetic relationships among sampled vertebrate species (Irisarri et al., 2017). These findings suggest that although Sxc is a relatively newer release mechanism, it likely encodes a type of signaling that is functionally beneficial to vertebrates. Although we did not observe significant positive selection pressure at any site, the advantages of SLAC analyses can be offset by the conservative nature of the method and the need for larger datasets to achieve the power of other approaches (Kosakovsky Pond and Frost, 2005). Hence, additional work is needed to determine whether any xCT residues are under positive selective pressure.

Next, we examined the possibility that genetic divergence of *Slc7a11*/xCT may be further advancing the complexity of the mammalian brain by examining the species-dependent expression of potential Sxc regulatory sites. We observed multiple expression patterns (Table 1), which included sites that were more abundantly expressed in primates. For example, RIMS (Rab3 interacting molecules; S481) expression was limited to mammalian species. Within mammals, RIMS (S481) was expressed in only 39% of species, whereas it was present in 100% of primates.

Creating an Sxc loss-of-function rat model

We next eliminated Sxc function by using zinc-finger nuclease technology to excise 39 consecutive base pairs from exon 2 of the gene encoding xCT, *Slc7a11* (Fig. 2*A*). We found that the result of this mutation was the complete elimination of cystine-evoked glutamate release in cultured striatal glia obtained from *MSxc* rats (Fig. 2*B*). An ANOVA performed on glutamate release revealed an interaction between genotype and cystine concentration (n = 3–12/genotype/cystine concentration; $F_{(5,79)} = 2.67$, p = 0.028, $\eta_p^2 = 0.145$). Analyses of cystine-evoked glutamate release in WT produced a main effect of cystine ($F_{(5,63)} = 7.58$, p = 0.00,001, $\eta_p^2 = 0.376$), with significant increases in glutamate occurring at every nonzero concentration of cystine (Holm–Sidak, p value for the first comparison = 0.029, p values for all other comparisons were < 0.0003; all Cohen's d values > 1.53). In contrast, a significant increase in glutamate was not observed in *MSxc*-generated cells at any concentration of cystine ($F_{(5,16)}$ =1.39, p = 0.280, $\eta_p^2 = 0.303$). Consistent with these data, we also found that brain tissue from adult *MSxc* rats were devoid of xCT mRNA (Fig. 2*C*). Because this is a heritable germline mutation, xCT should be impacted in all tissues. Collectively, these data demonstrate that Sxc function is absent in *MSxc* rats, which renders this model an excellent tool to explore the functional role of Sxc in behavioral control.

Basic brain function is unaltered in rats with genetic disruption of Sxc

To investigate the physiological and behavioral impact of loss of Sxc function we first examined genotypic differences in developmental mortality rates, postnatal increases in body weight, exploratory behavior, and simple associative learning, which could indicate the range of brain function reliant on Sxc. We did not observe any fatalities in either WT or *MSxc* rats up to week 10 in a large cohort (N > 500/genotype) or during a period of 1 year in a second (n = 8–10/genotype). Growth rate to adulthood and home-cage locomotor activity also did not differ between genotypes. Analysis revealed no genotypic effects on growth rate (Fig.

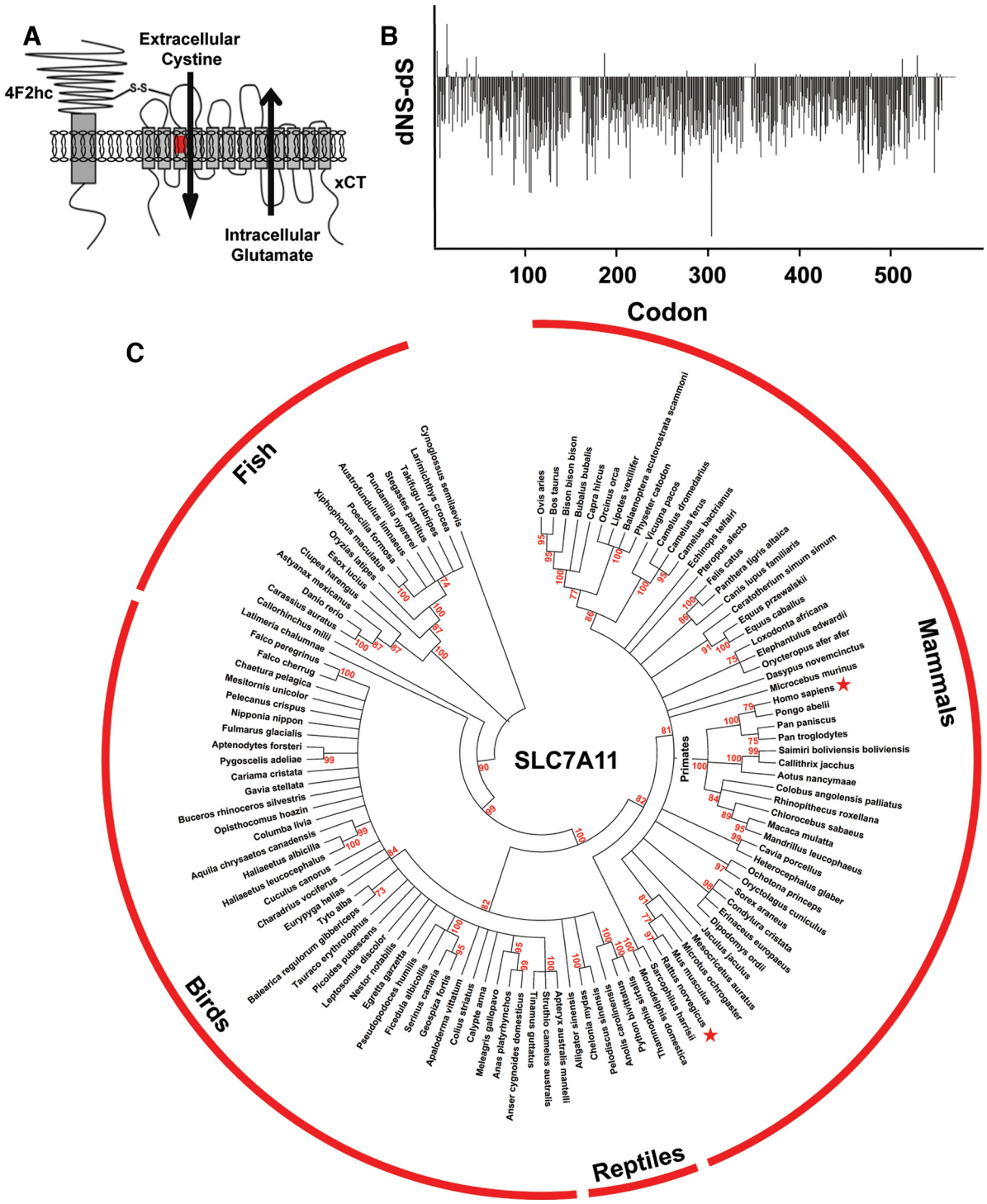

Figure 1. Phylogenetic comparison of *Slc7a11* in the major classes of vertebrate species. ***A***, The schematic depicts the two proteins that comprise Sxc, 4F2HC, and xCT and that Sxc releases glutamate through the heteroexchange of extracellular cystine. ***B***, A SLAC plot illustrating the difference in the number of nonsynonymous (dNS) and synonymous (dS) mutations at each of the *Slc7a11* codons across 115 vertebrate species. Most codons (391/569) had significantly more synonymous mutations (i.e., nucleotide mutations that did not alter amino acid sequence) than nonsynonymous mutations, which indicates that the *Slc7a11*/xCT has been under negative selection pressure. ***C***, A phylogenetic tree illustrating the xCT protein coding sequence relationship across 115 vertebrate species.

2*D*; n = 25–34/genotype; effect of genotype, $F_{(1,51)} = 0.331$, $p = 0.568$, $\eta_p^2 = 0.006$; week, $F_{(4,204)} = 2218$, $p < 7.57\text{E-}167$, $\eta_p^2 = 0.978$; genotype × time, $F_{(4,204)} = 5.27$, $p < 0.\ 0.000466$, $\eta_p^2 = 0.094$; p values were 0.401 or greater when comparing genotype at all weeks) or home-cage locomotor activity (Fig. 2*E*; n = 7–8/genotype; genotype × day, $F_{(7,91)} = 0.458$, $p = 0.862$, $\eta_p^2 = 0.034$; genotype, $F_{(1,13)} = 0.331$, $p = 0.57$, $\eta_p^2 = 0.025$; day, $F_{(7,91)} = 2.759$, $p = 0.012$, $\eta_p^2 = 0.175$). Additionally, there were no genotypic differences in time spent exploring a novel object (Fig. 2*F*; n = 9/genotype; $t_{(16)} = 0.216$, $p = 0.832$; Cohen's $d = 0.102$). Likewise, there were no genotypic effects in the ratio of time spent with the novel and familiar test objects on the test day (Fig. 2*F*; n = 9/genotype; $t_{(16)} = 0.439$, $p = 0.666$; Cohen's $d = 0.207$). These results demonstrate that *MSxc* rats do not exhibit disruptions in physiology or

in the performance of a basic memory task. Therefore, subsequent behavioral deficits are not likely to be caused by changes to more basic learning, activity, arousal, or physiological processes.

Associative learning is unaltered in rats with genetic disruption of Sxc

Next, we used a modified pavlovian conditioned approach task to investigate the impact of diminished Sxc activity on behavioral control. In this paradigm, subjects often display a dominant pattern of approach behavior directed toward a reward-predictive stimulus (i.e., sign tracking) or toward the food tray where the reward will soon be delivered (i.e., goal tracking). When goal tracking predominates, it is thought to reflect dominant behavioral control from executive functions such as response or attentional control, whereas when sign tracking predominates, it is thought to reflect behavior that is controlled by lower-order processes such as incentive salience or reward (Lovic et al., 2011; Koshy Cherian et al., 2017; Kuhn et al., 2018; Sarter and Phillips, 2018). Importantly, our procedure was modified to minimize neural processes contributing to CS+ approach behavior and the apparent magnitude of sign tracking (Moore, 2004). We used an illuminated CS+ rather than the insertion of a lever, which can trigger predatory or consummatory behaviors directed at the lever independent of the learned incentive properties.

Male WT ($n = 10$) and *MSxc* ($n = 9$) rats were first trained to associate the presentation of a visual stimulus (e.g., CS+) with the impending delivery of food into the food tray. In addition, rats were also trained that a second stimulus functioned as a CS− because it did not predict the delivery of food into the food tray. The ability of WT and *MSxc* rats to form this association can be determined by analyzing head entries into the food tray during the presentation of each stimulus (Fig. 3). An ANOVA conducted on the percentage of trials with a tray entry during CS+ presentation revealed a main effect of session ($F_{(4,68)} = 24.019$, $p = 2.06\text{E-}12$, $\eta_p^2 = 0.586$) with no main effect ($F_{(1,17)} = 0.022$, $p = 0.883$, $\eta_p^2 = 0.001$) nor an interaction ($F_{(4,68)} = 0.268$, $p = 0.898$, $\eta_p^2 = 0.016$) involving genotype. Subsequent *post hoc* analyses revealed the differences in tray entry between session 1 and sessions 2–5 to be significant (Fig. 3*A*; independent of genotype; Holm–Sidak, p values < 0.002; Cohen's d values > 0.925). In contrast, there were no significant changes in the percentage of trials with a CS− tray entry across training sessions (Fig. 3*B*; session, $F_{(4,68)} = 1.018$, $p = 0.405$, $\eta_p^2 = 0.056$; genotype, $F_{(1,17)} = 1.205$, $p = 0.288$, $\eta_p^2 = 0.066$; session × genotype, $F_{(4,68)} = 0.345$, $p = 0.847$, $\eta_p^2 = 0.020$). These data further establish that loss of Sxc does not alter a the capacity of a rat to form and discriminate simple pavlovian associations. Together, these results indicate that both genotypes assign incentive value specifically to the CS+ and subsequently approach the food tray at similar rates.

Sign tracking is augmented in rats with genetic disruption of Sxc

Given that WT and *MSxc* rats are equally adept at using a predictive cue to approach a goal, we next examined the percentage of trials in which subjects approached the visual CS+ (Fig. 4). Although both *MSxc* rats and WT rats approached the CS+ similarly during session 1, repeated training elicited significantly more CS+ approaches by the *MSxc* but not with WT rats. This observation is supported by a significant interaction between genotype and session when comparing the percentage of trials with a CS+ approach (Fig. 4*A*; $F_{(4,68)} = 4.341$, $p = 0.003$, $\eta_p^2 = 0.203$). An analysis across sessions of the percentage of trials with a CS+ approach revealed that WT rats did not change CS+ approach behavior across the five sessions ($F_{(4,36)} = 0.439$, $p = 0.779$, $\eta_p^2 = 0.047$). In contrast, *MSxc* rats had significantly more trials with a CS+ approach across the sessions ($F_{(4,32)} = 6.57$, $p = 0.\ 0.000563$; $\eta_p^2 = 0.451$) with significant increases on days 3 and 4 compared with session 1 (Holm–Sidak, p values ≤ 0.014; Cohen's d values > 1.650; note, session 5, Cohen's $d = 0.89$; $p = 0.034$ did not meet the p value adjusted by the Holm–Sidak test). *MSxc* rats also reliably approached the CS+ more frequently per session (Fig. 4*B*). ANOVA revealed a significant two-way interaction between genotype and session $F_{(4,68)} = 6.571$, $p = 0.00{,}016$, $\eta_p^2 = 0.279$). WT rats did not change the frequency of their CS+ approach behavior across sessions ($F_{(4,36)} = 0.593$, $p = 0.670$, $\eta_p^2 = 0.062$), but *MSxc* rats increased the total number of CS+ approaches across sessions ($F_{(4,32)} = 5.893$, $p = 0.001$, $\eta_p^2 = 0.424$; Holm–Sidak, sessions 3 and 4, $p < 0.019$, and session 5 $p = 0.021$; Cohen's $d = 1.246$; Fig. 4*B*). Pairwise *post hoc t* tests across genotype confirmed that although there were no differences during session 1 ($t_{(17)} = 0.595$, $p = 0.560$, Cohen's $d = 0.248$), *MSxc* rats committed significantly more CS+ approaches compared with WT rats on the last training day ($t_{(17)} = 2.396$, $p = 0.035$, Cohen's $d = 1.10$). Together, these results suggest that the loss of Sxc predisposes rats to approach reward-predictive cues.

Table 1. Phylogenetic differences in potential Sxc regulatory sequences

		CaMKII				GSK-3	
Class	Total Species Analyzed	RLPS (S26)		RRLS (S481)		STSVS (S185)	
Cartilaginous fish	1	0	0%	0	0%	1	100%
Fringe-finned fish	1	0	0%	0	0%	1	100%
Ray-finned fish	14	0	0%	0	0%	5	36%
Reptiles	6	3	50%	0	0%	6	100%
Birds	39	31	79%	0	0%	39	100%
Mammals	42	19	45%	33	79%	42	100%
Primates	12	12	100%	12	100%	12	100%
Total	115	65	57%	45	39%	106	92%

The percentage of species within vertebrate classes that express designated xCT residues. The p values reflect the outcome of SLAC analyses, which determine whether the site was under selective pressure.

Cue discrimination is intact in rats with genetic disruption of Sxc

In pavlovian conditioned approach designs, rats learn to approach the CS+ but not the unrewarded CS−. Here, WT rats reduced CS− approach across sessions as expected. Interestingly, *MSxc* rats did not decrease CS− approach to the same extent, instead maintaining stable low levels of CS− approach (Fig. 4*C*). However, both groups showed intact cue discrimination learning. In contrast to their stable CS+ approach behavior, WT rats learned to further suppress CS− approach across training sessions. *MSxc* rats, on the other hand, increased CS+ approach behavior while maintaining stable and low levels of CS− approach across training. In support, ANOVA on the percentage of trials with a CS− approach confirmed this observation with a significant two-way interaction between genotype and session ($F_{(4,68)} = 2.947$, $p = 0.026$, $\eta_p^2 = 0.148$). Follow-up ANOVAs conducted on the groups separately revealed a significant effect of session for the WT ($F_{(4,36)} = 11.225$, $p = 0.000005$, $\eta_p^2 = 0.555$), but not *MSxc* ($F_{(4,32)} = 1.579$, $p = 0.204$, $\eta_p^2 = 0.165$) rats. *Post hoc* tests indicated that the percentage of trials with a CS− approach was significantly reduced during sessions 3–5 for the WT rats (Holm–Sidak, p values < 0.007; Cohen's d values > 1.642) but remained unchanged in the *MSxc* rats. Therefore, unlike WT rats, *MSxc* rats increased approach to the CS+ (Fig.

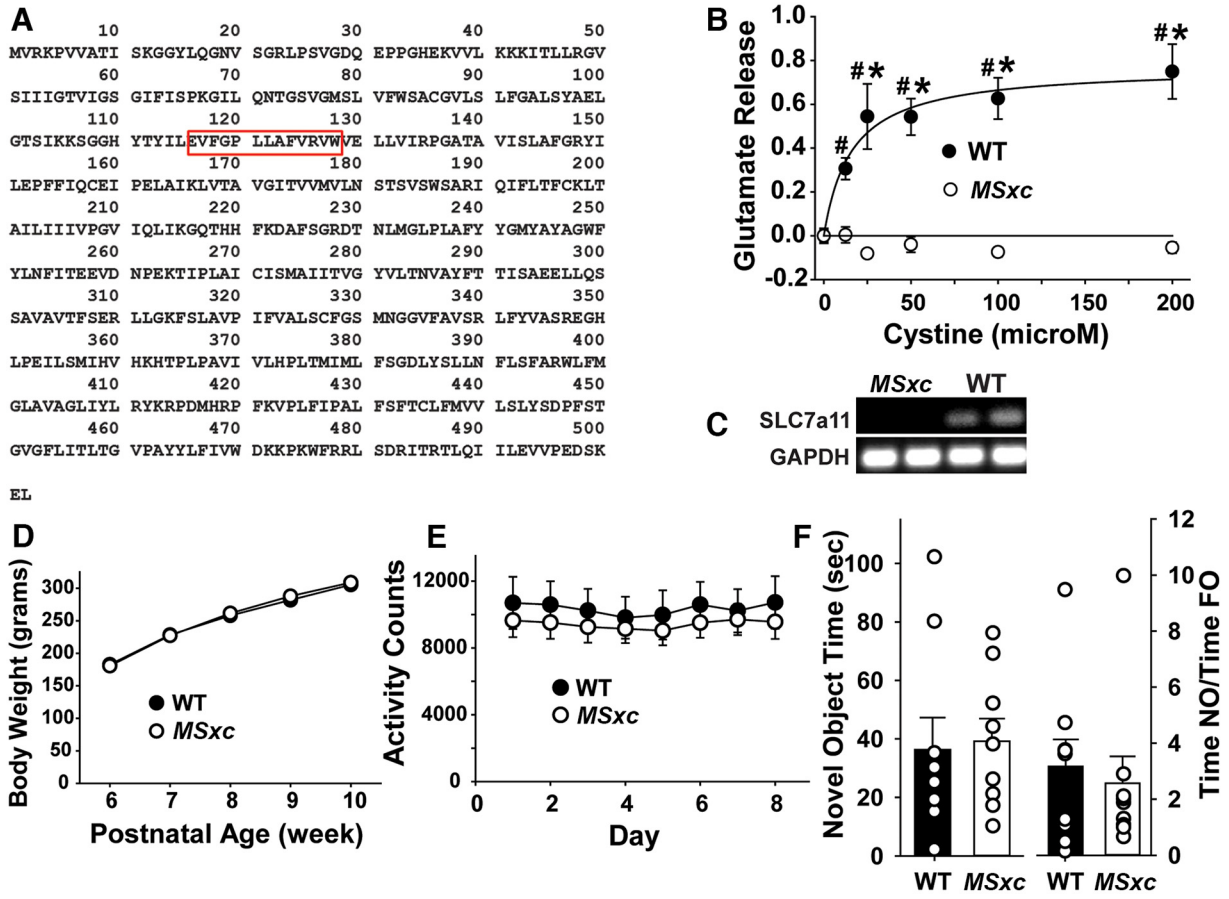

Figure 2. Mutating xCT protein eliminates Sxc function but does not produce generalized impairments in brain function. ***A***, The 13 amino acids deleted from rat *Slc7a11* are indicated by the red box. ***B***, Cystine application increases extracellular glutamate (mean ± SEM) in cultured striatal glia generated from WT but not *MSxc* rat, indicating a functional knockout of Sxc in *MSxc* tissue. The asterisk (*) specifies a significant difference from WT rats at the corresponding cystine concentration (Holm–Sidak, $p < 0.05$). The hashtag (#) indicates a significant difference from glutamate content at 0 cystine within genotype. ***C***, xCT mRNA was detected in brain tissue punches obtained from WT but not *MSxc* rats. ***D–F***, A loss of functional Sxc activity in *MSxc* rats did not alter growth rates (***D***), home cage activity measured using telemetry probes (***E***), or time spent with a novel object in the object recognition task (time interacting with novel object is depicted in ***F***, left; relative time spent with novel and familiar objects is depicted in ***F***, right).

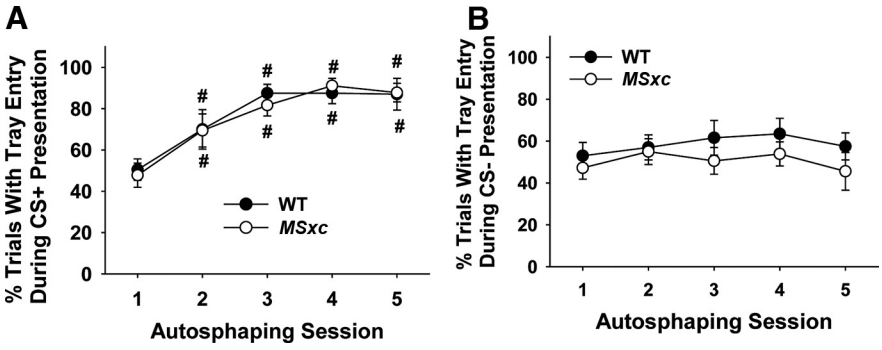

Figure 3. Pavlovian conditioning is not disrupted in *MSxc* rats. ***A–B***, Data depict the percentage of trials (mean ± SEM) with a tray entry during the presentation of CS+ (***A***) or CS− (***B***). The hashtag (#) indicates a significant difference from session 1 (Holm–Sidak, $p < 0.05$).

4*A*,*B*) and maintained a stable low level of approach to CS− (Fig. 4*C*).

MSxc rats display punished cue approach behavior

Increased CS+ approach behavior, sign tracking, exhibited by the *MSxc* rats could be considered maladaptive if it were to occur at the expense of reward consumption. However, *MSxc* rats, as observed in Figure 3, approached the goal tray at the same rate as WT controls. This ability to use environmental cues to predict biologically salient events and approach a primary reward is adaptive. Nevertheless, the *MSxc* rats approached the CS+ significantly more than the WT rats. This pattern of excess CS approach is not required to obtain the reward and can lead to negative outcomes. However, sign tracking observed in pavlovian conditioned approach can be made to be unambiguously maladaptive by using a punishment design. Hence, we modified the procedure to include punishment of CS+ approach by omitting the food reward on trials with a CS+ approach.

Indeed, CS+ approach behavior persisted in *MSxc* rats under punishment conditions (Fig. 5*A*,*B*), whereas goal approach was similar. This observation was supported by ANOVAs revealing no significant changes in the percentage of trials with a goal-tray entry elicited by the CS+ (omission session, $F_{(4,68)} = 1.074$, $p = 0.376$, $\eta_p^2 = 0.059$; genotype, $F_{(1,17)} = 1.427$, $p = 0.249$, $\eta_p^2 = 0.077$; session × genotype, $F_{(4,68)} = 0.506$, $p = 0.732$, $\eta_p^2 = 0.029$) or CS− (omission session, $F_{(4,68)} = 1.213$, $p = 0.314$, $\eta_p^2 = 0.067$; genotype, $F_{(1,17)} = 0.905$, $p = 0.355$, $\eta_p^2 = 0.051$; session × genotype, $F_{(4,68)} = 0.736$, $p =$

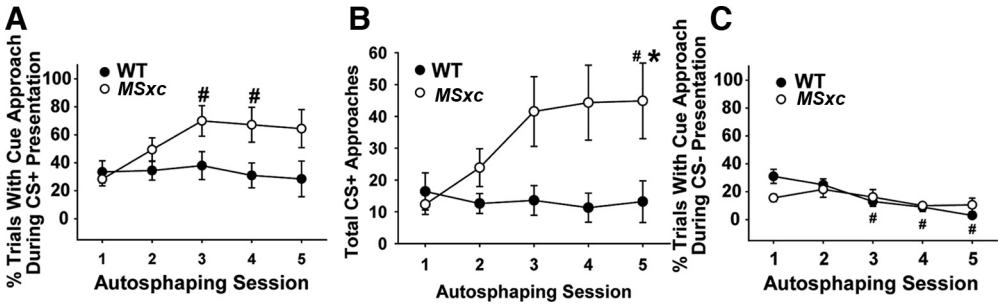

Figure 4. *MSxc* but not WT rats increase CS+ approach behavior following repeated testing. ***A–C***, Data depict the percentage of trials (mean ± SEM) with an approach to the CS+, total CS+ approaches (***B***), and percentage of trials with an approach to the CS− across the five training sessions (***C***). The hashtag (#) indicates an effect of session relative to session 1 (Holm–Sidak, $p < 0.05$). The asterisk (*) indicates a difference from WT within the indicated session (Holm–Sidak, $p < 0.05$).

0.571, $\eta_p^2 = 0.042$). CS+ approach behavior, on the other hand, remained significantly elevated in *MSxc* rats despite punishment. Although the percentage of trials with a CS+ approach was elevated marginally by disruption of Sxc (Fig. 5*A*), total CS+ approaches across omission sessions were significantly increased (Fig. 5*B*). In support, an ANOVA conducted on the percentage of trials with a CS+ approach yielded only a significant main effect of session (session, $F_{(4,68)} = 9.076$, $p = 0.000006$, $\eta_p^2 = 0.348$; genotype, $F_{(1,17)} = 2.705$, $p = 0.118$, $\eta_p^2 = 0.137$; session × genotype, $F_{(4,68)} = 0.663$, $p = 0.620$, $\eta_p^2 = 0.038$). *Post hoc* tests indicated that the percentage of trials with a CS+ approach significantly decreased across omission sessions 2–5 for both groups overall (Holm–Sidak, $p < 0.002$; Cohen's $d > 0.393$). Nevertheless, an ANOVA conducted on the total number of CS+ approaches during omission sessions revealed a significant main effect of genotype ($F_{(1,17)} = 6.187$, $p = 0.024$, $\eta_p^2 = 0.267$) and session ($F_{(4,68)} = 7.633$, $p = 0.000039$, $\eta_p^2 = 0.310$) in the absence of an interaction ($F_{(4,68)} = 1.707$, $p = 0.158$, $\eta_p^2 = 0.091$), indicating that *MSxc* rats maintained a significantly higher rate of CS+ approach behavior compared with WT rats even while significantly reducing CS+ approach behaviors across the omission sessions. This observation was supported by *post hoc* tests of the main effect of omission session that show total CS+ approaches were reliably reduced during omission sessions 2–5 compared with session 1 (Fig. 5*B*: Holm–Sidak, $p < 0.01$; Cohen's $d > 0.292$).

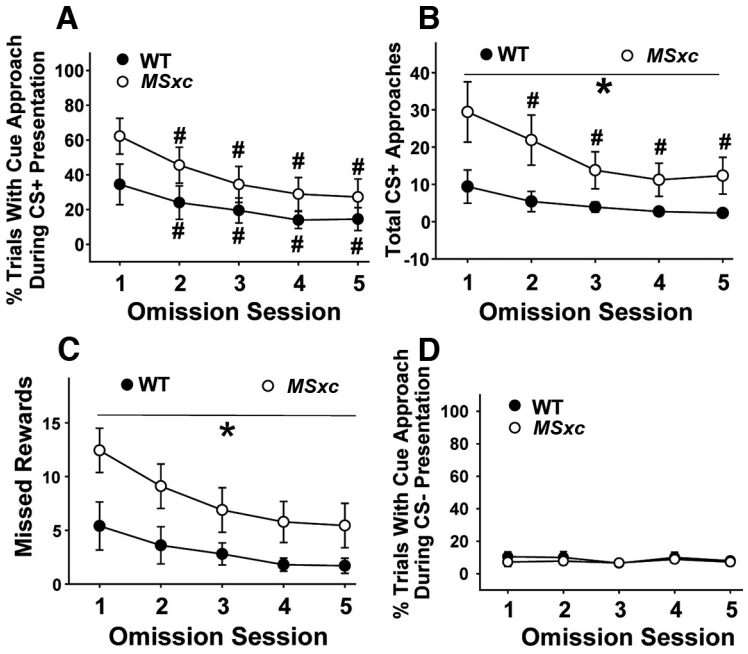

Figure 5. Persistent CS+ approach behavior in *MSxc* rats persist despite the punishment of this behavior during omission training. ***A***, ***B***, Data depict the percentage of trials (mean ± SEM) with a CS+ approach and (***B***) total CS+ approaches per session. ***C***, These data reflect the total number of missed rewards during approaches toward CS+ during omission training. The hashtag (#) indicates an effect of session relative to session 1 (Holm–Sidak, $p < 0.05$). ***D***, These data depict the percentage of trials with a CS− approach during omission training. The asterisk (*) indicates a significant difference from WT (ANOVA, $p < 0.05$).

Critically, this resistance to punishment exhibited by the *MSxc* rats while sign tracking leads to a significant failure to obtain the food rewards across the omission sessions (Fig. 5*C*). An ANOVA conducted on the total number of missed food rewards revealed significant main effects of genotype ($F_{(1,17)} = 5.86$, $p = 0.027$, $\eta_p^2 = 0.256$) and session ($F_{(4,68)} = 8.317$, $p = 0.000016$, $\eta_p^2 = 0.329$) but no interaction ($F_{(4,68)} = 0.832$, $p = 0.509$, $\eta_p^2 = 0.047$). *Post hoc* tests of the session effect indicate a reliable decrease in the number of missed rewards across sessions for both groups (session 1 > sessions 2–5). Despite this shared decrease, the *MSxc* rats missed significantly more food rewards under punishment conditions compared with WT rats (average total missed rewards ± SEM, *MSxc* = 39.67 ± 8.56 vs WT = 15.3 ± 5.67; $t_{(17)} = 0.2.42$, $p = 0.013$, Cohen's $d = 1.11$). Collectively, these data indicate that punishment of CS+ approach behavior by reward omission does not reduce the increased sign-tracking behavior exhibited by the *MSxc* rats. Therefore, failure to suppress the perseverative CS+ approach behavior then directly leads to a negative outcome, the loss of food.

In contrast to the increased CS+ approach behaviors, reward omission had no discernable effect on approach to the CS− (Fig. 5*D*). In support, there were no significant main effects or interactions when analyzing percent trials with a CS− approach (session, $F_{(4,68)} = 0.583$, $p = 0.676$, $\eta_p^2 = 0.033$; genotype, $F_{(1,17)} = 0.348$, $p = 0.563$, $\eta_p^2 = 0.020$; session × genotype, $F_{(4,68)} = 0.192$, $p = 0.942$, $\eta_p^2 = 0.011$) or the total number of CS− approaches during omission training (data not shown; session, $F_{(4,68)} = 0.870$, $p = 0.487$, $\eta_p^2 = 0.049$; genotype, $F_{(1,17)} = 0.523$, $p = 0.479$, $\eta_p^2 = 0.030$; session × genotype, $F_{(4,68)} = 0.723$, $p = 0.579$, $\eta_p^2 = 0.041$). Together, these findings highlight the selective nature of the deficit imposed by genetic disruption of astrocytic glutamate release.

Impaired Sxc promotes negative outcome behaviors such as increased sign tracking and resistance to punishment.

Genetic disruption of Sxc impairs cognitive mechanisms regulating behavior

The prolonged increase in CS+ approach behaviors exhibited by *MSxc* rats could indicate perseverative or inflexible behavioral. To test this, we next assessed the capacity of WT ($N = 6$) and *MSxc* rats ($N = 7$) to flexibly adopt behavioral strategies in an attentional set-shifting paradigm. On day 1 of the task, rats were required to use visiouspatial learning to identify the arm of a maze baited with food. On days 2 and 3, rats needed to display cognitive flexibililty involving extradimensional set shifting on day 2 (i.e., transition from a visual to a directional cue) and intradimensional set shifting on day 3 (reverse the direction from day 2) to receive optimal reinforcement. The impact of genotype depended on the task or day (day × genotype, $F_{(2,22)} = 3.78$, $p = 0.039$, $\eta_p^2 = 0.256$). On test day 1, WT and *MSxc* rats required the same number of trials to learn that a visual cue indicated the location of a food reward (Fig. 6*A*; $t_{(11)} = 0.348$, $p = 0.735$, Cohen's $d = 0.193$), which revealed that *MSxc* rats retained the capacity to form simple associations. On test days 2 and 3, *MSxc* rats required significantly more trials than WT rats to reach the criterion to employ an extradimensional shift in strategy (day 2, $t_{(11)} = 3.84$, $p = 0.003$, Cohen's $d = 2.134$) or an intradimensional shift (day 3, $t_{(11)} = 2.96$, $p = 0.013$, Cohen's $d = 1.648$). The impaired performance on these days was primarily because of an increase in perseverative errors. This observation was supported by ANOVAs revealing a significant interaction between genotype and error type ($F_{(2,22)} = 4.245$, $p = 0.028$, $\eta_p^2 = 0.278$) and a significant effect of genotype in the number of perseverative errors (Fig. 6*B*; $t_{(11)} = 3.39$, $p = 0.006$, Cohen's $d = 1.887$), but not regressive errors (Fig. 6*B*; $t_{(11)} = 0.859$, $p = 0.409$, Cohen's $d = 0.478$). The increase in perseverative errors indicates that *MSxc* rats persisted in applying ineffective strategies before searching for new approaches, but the lack of a genotypic effect indicates equivalent performance between WT and *MSxc* rats once the rule of the new day had been identified (as defined by a rate of success $\geq 75\%$ over a block of eight trials).

Genetic disruption of Sxc increases drug-induced reinstatement of drug seeking

The behavioral deficits displayed by *MSxc* rats could indicate that a loss of Sxc may increase the risk for other maladaptive behaviors that are characterized by compulsive or perseverative behavior drug seeking (Tunstall and Kearns, 2015; Versaggi et al., 2016). If disruption of Sxc leads to a general disruption of top-down control of behavior and/or enhanced incentive sensitization, then *MSxc* rats would be predisposed to developing compulsive drug seeking. To determine the extent to which Sxc disruption affects drug seeking behaviors, WT ($n = 16$) and *MSxc* rats ($n = 14$) were trained to self-administer cocaine (Fig. 7). Drug seeking was then extinguished and evaluated during a reinstatement test that included a priming dose of cocaine coupled with response-contingent presentation of the cocaine-paired cue. Loss of Sxc had no effect on the acquisition or maintenance of cocaine self-administration. First, WT and *MSxc* acquired cocaine self-administration at the same rate across days [mean (+SEM) acquisition sessions 8.7 + 1.15 (WT) and 10.3 + 0.97 (*MSxc*); $t_{(28)} = -1.046$, $p = 0.305$, Cohen's $d = 0.383$] revealing again that these rats do not differ on simple forms of learning. Next, the number of infusions self-administered by WT and *MSxc* rats during daily 2 h sessions were comparable (Fig. 7*A*). Analyses of this data revealed a main effect of session ($F_{(11,308)} = 2.237$, $p = 0.013$, $\eta_p^2 = 0.074$) in the absence of a significant main effect ($F_{(1,28)} = 0.002$, $p = 0.962$, $\eta_p^2 = 0.00,008$) or interaction involving genotype ($F_{(11,308)} = 1.062$, $p = 0.392$, $\eta_p^2 = 0.037$). Finally, there were no significant differences in intake relative to session 1 that would indicate an escalation of drug intake in either WT or *MSxc* rats (Holm–Sidak, p values > 0.106). These data indicate that a loss of Sxc does not alter sensitivity to the reinforcing properties of cocaine in *MSxc* rats.

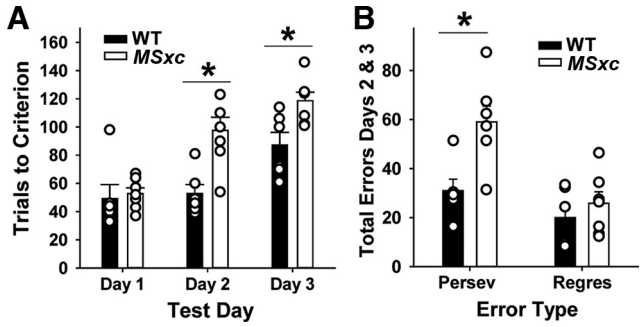

Figure 6. A loss of Sxc activity impairs cognitive flexibility. ***A***, The number of trials (mean + SEM) subjects needed to meet the solution criteria on day 1 (the arm baited with food was indicated by the location of a visual cue), day 2 (the baited arm was indicated by a spatial directional cue, e.g., right or left arm), and day 3 (the baited arm was indicated by a spatial cue that was in the direction opposite of day 2). ***B***, The data (mean + SEM) depict the number of perseverative (persev; i.e., errors reflecting rules from prior test sessions) and regressive errors (regres errors; errors committed after the subject learned the rule for the current test day). The asterisk (*) indicates a significant difference from WT ($p < 0.05$).

Likewise, there was no effect of genotype on extinction learning. Analysis of these data did not reveal an effect of genotype in the number of trials needed to achieve the extinction criterion [mean (+SEM) extinction sessions 7.2 + 1.04 (WT) and 7.4 + 1.2 (*MSxc*); $t_{(28)} = -0.107$, $p = 0.915$, Cohen's $d = 0.39$] or in the pattern of reduced responding across the first seven extinction sessions (Fig. 7*B*; genotype, $F_{(1,10)} = 0.100$, $p = 0.758$). A main effect of extinction session was present ($F_{(6,60)} = 7.624$, $p = 0.000004$, $\eta_p^2 = 0.433$), but there was no interaction between session and genotype ($F_{(6,60)} = 1.227$, $p = 0.305$, $\eta_p^2 = 0.109$). *Post hoc* analyses revealed that the number of operant responses on the previously cocaine-paired lever was significantly reduced on sessions 6 and 7 relative to session 1 (Holm–Sidak, $p < 0.002$, Cohen's $d > 1.417$).

We then tested the hypothesis that eliminating Sxc function would make rats more vulnerable to relapse. To do this, rats were injected with a subthreshold dose of cocaine before a reinstatement test. This dose (3.0 mg/kg, i.p.) does not reinstate drug seeking in WT rats following extinction (Fig. 7*C*). Analyses of operant responding exhibited by WT and *MSxc* rats on the last extinction session and reinstatement test day revealed a significant interaction ($F_{(1,28)} = 8.179$, $p = 0.008$, $\eta_p^2 = 0.226$). WT and *MSxc* did not differ during the last extinction session ($t_{(28)} = 0.242$, $p = 0.811$, Cohen's $d = 0.089$), but *MSxc* rats exhibited significantly more lever presses during reinstatement testing than WT rats ($t_{(28)} = 2.723$, $p = 0.011$, Cohen's $d = 0.997$). Furthermore, only *MSxc* rats reinstated previously extinguished operant responding as this behavior was significantly higher during the reinstatement test compared with their last extinction session ($t_{(13)} = -5.805$, $p = 0.000061$, Cohen's $d = 1.55$). As expected, WT rats did not reinstate cocaine seeking ($t_{(15)} = -1.640$, $p = 0.122$, Cohen's $d = 0.41$). Therefore, these data demonstrate that *MSxc* rats have increased vulnerability to reinstate cocaine seeking.

Discussion

Here, we learned that eliminating Sxc activity increased approach behavior directed at a food-predictive cue, impaired cognitive flexibility, and enhanced cocaine-primed drug seeking in rats. Notably, each of these behaviors model impaired behavioral control in humans (Robbins, 1996; Robinson et al., 2014; Verdejo-Garcia et al., 2015). Unlike other glutamatergic mechanisms, eliminating Sxc was not fatal and did not impair development, physiological telemetry, general activity states, simple learning, object recognition, or other indicators of widespread disruption of brain function. Based on present and past results, we propose that Sxc belongs to a class of evolutionarily new molecular adaptations that contributed to the phylogenetic enhancement of cognition in humans, and, importantly, may not be required for noncognitive functions of the brain. If substantiated by further studies, the identification of cognitive-specific molecular mechanisms would be a significant advance that could enable the rational development of highly safe and effective treatments for substance abuse and disorders featuring cognitive impairments.

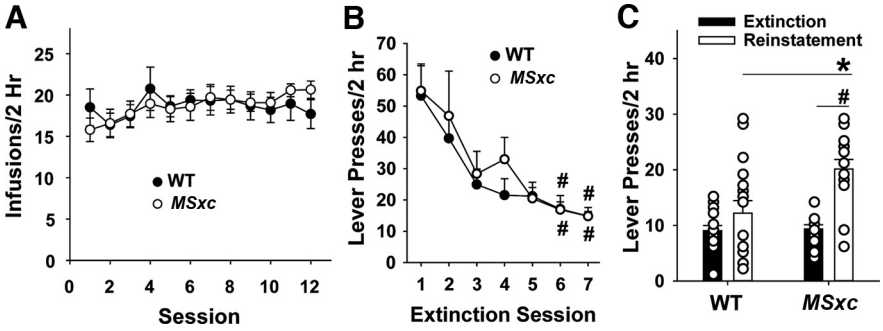

Figure 7. *MSxc* rats self-administer cocaine normally but are more vulnerable to cocaine-primed reinstatement. ***A***, Data depict the number of cocaine infusions received during the 12 maintenance sessions. ***B***, These data display the pattern of reduced responding between WT and *MSxc* across the first seven extinction sessions. ***C***, Depicts the number of active lever presses during the last day of extinction and during the cocaine reinstatement test (3 mg/kg, i.p.). The hashtag (#) indicates an effect of session relative to session 1 of the designated experimental phase (Fig. 7*B*) or the last extinction session within genotype (Fig. 7*C*; Holm–Sidak, $p < 0.05$). The asterisk (*) indicates a genotypic difference during reinstatement (Holm–Sidak, $p < 0.05$).

The evolution of cognition, in part, involved the introduction of novel molecular mechanisms that increased signaling complexity (Grant, 2016; Ramos-Vicente et al., 2018; Santello et al., 2019). Hence, there is a need to understand how the major signaling systems in the brain evolved. Glutamate existed before the divergence of plants and animals as a primitive signal (Chiu et al., 1999); it then evolved a complex network of receptors, transporters, and/or release mechanisms expressed by virtually every type of cell in the mammalian brain (Färber and Kettenmann, 2005; Volterra and Meldolesi, 2005; Ramos-Vicente et al., 2018; Verkhratsky and Nedergaard, 2018; Hogan-Cann et al., 2019; Suárez-Pozos et al., 2020). Sxc is an example of a glutamate mechanism that could have expanded signaling complexity because it enables astrocyte–neuron communication (Moran et al., 2005; Ottestad-Hansen et al., 2018). As it originated after the divergence of deuterostomes and protostomes (Lewerenz et al., 2013), it is not expressed by most animals. Despite its recent origin, we found the Sxc subunit xCT to be expressed by every vertebrate species we examined. We also learned that the amino acid sequence of xCT has been highly protected from mutations to the *Slc7a11* gene. Given the high expression pattern among vertebrates and the existence of significant selective pressure, Sxc function is likely critical to the brain and may contribute to biological fitness. Finally, we also learned that Sxc may still be advancing signaling complexity as we were able to identify potential xCT regulatory sites that were expressed by primates at a higher rate than other species.

In direct assessments of brain functions, we found that *MSxc* and WT rats did not differ on basic health and telemetry metrics or on measures of general activity states, reinforcement, classical conditioning, operant conditioning, extinction learning, object recognition memory, or discrimination of reward-paired stimuli. These findings are consistent with prior observations that $xCT^{-/-}$ mice and adult WT rats treated with systemic administration of Sxc pharmacological inhibitors do not display widespread impairments in brain function (De Bundel et al., 2011; Lutgen et al., 2014; McCullagh and Featherstone, 2014; Williams and Featherstone, 2014). Importantly, these findings are also consistent with clinical studies showing that the Sxc enhancer *N*-acetylcysteine enhances cognitions without producing traditional CNS side effects (Rapado-Castro et al., 2017; Conus et al., 2018; Sepehrmanesh et al., 2018). Hence, manipulations of Sxc activity do not produce widespread changes in brain function that are often observed with manipulations of other glutamate mechanisms (Tanaka et al., 1997; Wojcik et al., 2004; Christie et al., 2010).

An interesting pattern of results emerged when WT and *MSxc* rats were tested in behavioral paradigms that require the integration of simple learning with sophisticated forms of cognition (i.e., cognitive functions that are phylogenetically enhanced in humans). In the pavlovian conditioned approach, *MSxc* and WT rats appropriately increased their goal tray entries during the presentation of the CS+ but not the CS−. This demonstrates that WT and *MSxc* rats have an equivalent capacity to discriminate reward-paired stimuli. However, *MSxc* rats approached the CS+ more frequently than WT rats. Sensitized CS+ approach behavior in *MSxc* rats persisted even when the behavior was punished, which clearly indicates the maladaptive nature of the response. Transitioning approach behavior from goal to sign tracking could be because of increased incentive salience of the CS+ cue, as well as a weakening of response inhibition (Stepien, 1974; Flagel et al., 2008; Kuhn et al., 2018; Sarter and Phillips, 2018; Anselme and Robinson, 2020), which is an example of a phylogenetically enhanced form of cognitive control in humans and other species (Moore, 2004; Ardila, 2008; Grant, 2016; Dukas, 2017; van Duijn, 2017).

In attentional set shifting, *MSxc* and WT rats were equally adept at learning that a visual stimulus indicated the arm of a maze baited with food during day 1 of testing. However, the performance of *MSxc* rats was significantly worse than that of WTs on test days 2 and 3, which both required behavioral flexibility. During test day 2, an extradimensional-shift (e.g., from a visual to a fixed-directional cue) was required to obtain optimal reinforcement. During test day 3, an intradimensional shift or reversal learning was required as the arm of the maze baited with food on this day was opposite to that on day 2. Behavioral inflexibility displayed by *MSxc* rats was caused by perseverative but not regressive errors. Hence, *MSxc* rats were slower to abandon ineffective strategies and identify new strategies but were no longer impaired once an improved approach was identified. Hence, this pattern of results is unlikely to be explained by impairments in simple learning or memory (e.g., the inability to identify and pair

environmental cues with reward location). Instead, the deficits in *MSxc* rats are most likely to involve impairments in complex cognitive control processes (Robbins, 1996; Gilmour et al., 2013; Brown and Tait, 2016).

In the cocaine self-administration and reinstatement paradigm, *MSxc* rats displayed higher levels of cocaine-primed reinforcement, which involved increased levels of operant lever pressing despite the lack of cocaine reinforcement. This could arise because of increased incentive salience of the cue, especially because our test included response-contingent presentation of the cocaine-paired cue (but in the absence of continued cocaine infusions/reinforcement). However, *MSxc* rats did not differ from WT in the acquisition, maintenance, or extinction of cocaine-reinforced operant responding, which argues against an explanation involving altered incentive salience or other neural processes affecting reward, operant learning, and extinction. Given the likely involvement of cognitive control in suppressing cocaine seeking (Jentsch and Taylor, 1999; Schoenbaum et al., 2004, 2006; Connolly et al., 2012; Everitt and Robbins, 2016), these findings likely also reflect impairments in phylogenetically enhanced forms of cognitive control (Moore, 2004; Ardila, 2008; Grant, 2016; Dukas, 2017; van Duijn, 2017).

Relatedly, there is a need to understand the mechanistic basis of altered behavior in *MSxc* rats. Sxc integrates two major biological processes. In addition to glutamate signaling, Sxc activity has an impact on redox signaling/oxidative stress protective mechanisms because of the uptake of cystine, which contributes to the synthesis of the key cellular redox regulator and antioxidant glutathione (Miura et al., 1992; Dringen, 2000; Shih et al., 2006). It will be difficult to distinguish how each of these biological processes contributes to the behavioral deficits in *MSxc* rats as they are highly integrated. For example, knock-out mice lacking the glutathione synthesizing enzyme glutamate-cysteine-ligase exhibit deficits in glutamate signaling (Kulak et al., 2012). Second, the activity of NMDA receptors are regulated by extracellular redox-sensitive domains (Choi and Lipton, 2000). Third, the extracellular metabolism of glutathione can be a source for glycine and cystine (Bannai, 1986; Sato et al., 2002), which regulate NMDA receptors and Sxc activity, respectively. Hence, altered Sxc activity could disrupt signaling between astrocytes and neurons by decreasing glutamate release or by altering glutathione-dependent signaling mechanisms.

The possibility that altered Sxc activity selectively affects complex behaviors is consistent with past results using $xCT^{-/-}$ mice and acute pharmacological inhibition of Sxc in adult rats (De Bundel et al., 2011; Lutgen et al., 2014; McCullagh and Featherstone, 2014; Williams and Featherstone, 2014). The latter finding suggests that the behavioral impairment in *MSxc* rats likely involves aberrant glutamate signaling rather than long-term developmental impairments. Notably, clinical studies report that the Sxc enhancer *N*-acetylcysteine is largely devoid of CNS side effects (Pendyala and Creaven, 1995; Tenório et al., 2021) and is capable of restoring behavioral control in a range of human CNS disorders (Grant et al., 2009; Froeliger et al., 2015; Oliver et al., 2015; Schulte et al., 2018; Woodcock et al., 2021).

There are multiple implications of these results. First, our data support the idea that astrocytes contributed to the evolution of cognition (Oberheim Bush and Nedergaard, 2017; Santello et al., 2019). Second, parallel findings with Sxc and novel paralogues of the DLG family of synaptic proteins support the idea that molecular mechanisms enabling advances in signaling complexity can have functions in the brain that are restricted to cognition (Emes et al., 2008; Nithianantharajah et al., 2013; Grant, 2016). The existence of cognitive-specific signaling mechanisms could lead to the development of highly effective and highly safe treatments for substance abuse and related psychiatric syndromes.

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