Heroin Cues Reveal Astroglial Heterogeneity in the Nucleus Accumbens Core

Anna Kruyer[1]* and Peter W. Kalivas[1]

[1] Department of Neuroscience, Medical University of South Carolina, Charleston, SC, USA.

*Correspondence should be addressed to A.K. (173 Ashley Ave, MSC510, Charleston, SC, 29425; 843-876-2246, kruyer@musc.edu).

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ABSTRACT

BACKGROUND: Cues predicting heroin delivery induce heroin seeking by initiating synaptic glutamate release in the nucleus accumbens core. The intensity of heroin seeking is negatively modulated by cue-induced increases in synaptic proximity of astrocytes. Glutamate-driven heroin seeking is also negatively regulated by compounds that promote glutamate uptake through the astrocytic transporter GLT-1. We hypothesized that the cue-induced increase in astrocyte synaptic proximity reduces heroin seeking by increasing GLT-1 synaptic proximity.

METHODS: Rats were trained to self-administer heroin or sucrose before undergoing extinction and cued reinstatement of heroin or sucrose seeking. We used confocal microscopy to assess expression and co-registration of GLT-1 with the synaptic marker Synapsin I in the nucleus accumbens core.

RESULTS: Extinction from heroin, but not sucrose self-administration, downregulated GLT-1. Heroin cues increased surface expression of GLT-1 in parallel with heroin seeking, but counter to expectations, the increase was not proximal to synapses identified by Synapsin I. In fact, astroglia showing cue-induced increased surface expression of GLT-1 constituted a distinct subpopulation of astroglia from those showing increased synaptic proximity. Supporting discrete mechanisms, preventing cue-evoked increases in astrocyte synaptic proximity by knocking down the astroglial-selective actin binding protein ezrin did not impact cue-induced increases in GLT-1 surface expression.

CONCLUSIONS: Our data demonstrate that heroin-paired cues elicit two transient adaptations in astrocytes in the nucleus accumbens core, restoration of synaptic proximity and increased surface expression of GLT-1. Each adaptation occurs in largely non-overlapping subpopulations of astrocytes, but both adaptations appear to dampen reinstated heroin seeking.

INTRODUCTION

Relapse to heroin use remains a leading cause of death in the United States. Decades of research demonstrate the importance of glutamate dysregulation in the nucleus accumbens core (NAcore) as a causative factor in relapse-like behavior in animal models[1]. Glutamate dysregulation results from chronic use of addictive drugs due in large part to changes in NAcore astroglia that express the glutamate transporter GLT-1 and conduct the bulk of glutamate uptake in brain[2,3]. Addictive substances, including alcohol, nicotine, psychostimulants, and opioids produce an enduring downregulation of GLT-1 on astrocytes in the NAcore[4]. Furthermore, astroglial processes that normally ensheath synapses and take up glutamate during synaptic transmission retract from NAcore synapses after withdrawal from cocaine, heroin, and methamphetamine, but not after sucrose self-administration and extinction[5-7]. Synaptic retraction of astroglial processes and downregulation of GLT-1 disrupt glutamate homeostasis, permitting spillover of synaptic glutamate and postsynaptic potentiation during seeking triggered by drug-, but not sucrose-associated cues[8].

We previously found that synaptic retraction of NAcore astroglia after heroin withdrawal is partially reversed during cue-induced heroin seeking and the transient restoration of synaptic proximity by NAcore astrocytes reduces the intensity of heroin seeking[6]. We hypothesized that the suppression of cued heroin seeking by astrocyte morphological plasticity results from enhanced synaptic proximity of GLT-1 on perisynaptic astroglial processes, serving to attenuate the spillover of synaptic glutamate that mediates cue-induced drug seeking. We examined synaptic proximity of GLT-1 by measuring co-registration of immuno-labeled GLT-1 with the presynaptic marker Synapsin I after 15- or 120-min of cue-reinstated heroin seeking. We found that GLT-1 expression in NAcore astrocytes and co-registration of GLT-1 with Synapsin I were both decreased after extinction from heroin self-administration. Although heroin cues did not alter GLT-1 levels in NAcore astroglia, the proportion of total GLT-1 on the astroglial surface transiently increased. Although heroin cues simultaneously increased synaptic proximity of

NAcore astroglia and GLT-1 surface expression, we found no increase in co-registration of GLT-1 with Synapsin I after 15-min of cued reinstatement. Moreover, astroglia with high heroin cue-induced surface expression of GLT-1 were not those demonstrating cue-induced increases in synaptic proximity. These findings indicate the presence of two discrete subpopulations of NAcore astroglia undergoing molecularly dissociable mechanisms of cue-induced plasticity that both decrease heroin seeking.

METHODS

Self-administration

Experimental procedures involving animals were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Operant training was conducted as previously described[6]. Briefly, male Sprague Dawley rats (200-250g) were anesthetized with i.m. ketamine (100 mg/kg) and xylazine (7 mg/kg) and fitted with intrajugular catheters. Rats were trained to self-administer heroin during 3h sessions for 10d and presses on an active lever were paired with light and tone cues and i.v. heroin infusion. Animals trained to self-administer sucrose did not undergo catheter implantation and received sucrose (45 mg, Bio-Serv) in place of heroin along with cues during self-administration. Yoked controls were played cues when a paired rat received heroin or sucrose. Rats yoked to heroin self-administering animals also received i.v. saline infusions. After self-administration, animals underwent 10-12d of extinction training (3h/d) where active lever presses yielded no reward or cues. Extinguished rats and yoked controls were sacrificed 24h after the final extinction session. Reinstated animals were placed in the operant chamber for 15 or 120m 24h after the last extinction session and cues were restored to the active lever, but no reward was delivered.

Viral labeling

After catheter implantation or 5d before starting sucrose self-administration, rats received microinjections (1 µL/hemisphere, 0.15 µL/minute, 5 min diffusion) of a virus driving expression of membrane-targeted mCherry under control of the GFAP promoter (AAV5/GFAP-hM3dq-mCherry, University of Zurich) in the NAcore (+1.5mm AP, ±1.8mm ML, -7.0mm DV). Virus incubation occurred over the course of operant training (~4 wks).

Confocal imaging and image analysis

Animals were anesthetized with an overdose of pentobarbital (20 mg i.v. or 100 mg i.p.) and perfused transcardially with 4% PFA. Brains were incubated overnight in 4% PFA and sliced at 100 µm using a vibratome (Thermo Fisher). Slices containing the NAcore were permeabilized in PBS with 2% Triton X-100 for 1h at room temperature. Non-specific epitope binding was blocked by blocking in PBS with 0.2% Triton X-100 (PBST) and 2% NGS for 1h at room temperature before incubation in primary antibodies (1:1000; rabbit anti-Synapsin I, ab64581 and guinea pig anti-GLT-1, ab1783) for 48h in block. After washing in PBST, tissue was incubated overnight in biotinylated anti-guinea pig antibody (1:1000, BA-7000) in PBST and then overnight in fluorescently-labeled antibodies (1:1000, 488-streptavidin and 647-anti-rabbit, Thermo Fisher) in PBST. Tissue was washed in PBST and mounted onto glass slides before imaging with a Leica SP5 laser scanning confocal microscope. All images were acquired at 63x using an oil immersion objective lens, 1024 x 1024 frame size, 12-bit resolution, 4-frame averaging and a 1-µm step size. Z-stacks were iteratively deconvolved 10 times (Autoquant) and digital analysis of mCherry signal intensity relative to background was used to generate a digital model of each astrocyte (Bitplane Imaris). Rendered astrocytes were used to mask GLT-1 and Synapsin I signal that was not co-registered with the astroglial volume. Co-registration (astrocyte with Synapsin I, astrocyte with GLT-1, GLT-1 with Synapsin I) was determined based on thresholded signal intensity in each channel. Voxels containing signal intensity greater than noise in each channel were determined empirically using the colocalization module and were used to build a colocalization channel. The surface module was used to determine the net volume of co-registered signal. Synapsin and GLT-1 co-registration were normalized to the volume of the astrocyte from which they were generated. Surface-proximal GLT-1 was determined by excluding co-registered signal that was within the astrocyte volume, but >250nm from the membrane and was normalized to total GLT-1 from the same astroglial volume. Imaging and analyses were conducted blind to animal treatment.

Ezrin knockdown

After catheter placement, animals were fitted with bilateral cannulae above the NAcore (+1.5mm AP, ±1.8mm ML, -5.5mm DV). Starting on day 6 of extinction training, animals received infusions of an ezrin antisense or control oligo 1.5mm beyond the base of the guide cannulae (1µL per hemisphere, 0.5µL/min) for 3 consecutive days, according to[6]. After 3 additional days of extinction training, rats underwent cued reinstatement for 15-min prior to sacrifice.

Statistics

Data were analyzed using GraphPad Prism 7 and a D'Agostino-Pearson normality test followed by Kruskal-Wallis or Mann-Whitney tests when one or more groups were not normally distributed. Dunn's test was used for post hoc comparisons. Normally distributed data were analyzed using a 1- or 2-way ANOVA or a Student's t-test. Cumulative distributions were analyzed using Kolmogorov-Smirnov or Chi^2. A Pearson's coefficient was calculated for all correlations. In all cases, $p < 0.05$ was considered significant.

RESULTS

Heroin self-administration constitutively reduced synaptic co-registration of GLT-1

In order to examine whether enhanced synaptic proximity of NAcore astroglia suppressed cue-induced heroin seeking through changes in synaptic proximity of GLT-1, NAcore astroglia were labeled with a membrane-bound fluorescent reporter prior to operant training. Rats were trained to self-administer heroin or sucrose and reward delivery was paired with light and tone cues (Fig S1A). Operant responding was extinguished and a portion of rats were reinstated by restoring conditioned cues to active lever pressing for 15 or 120 min (Fig S1B). Animals that received yoked saline delivery and cues served as controls for heroin-trained rats and animals that received yoked cues were controls for sucrose-trained rats. Some animals used to generate the data herein were included in a previous study[6], and mCherry transfected astrocytes from these animals were double-labeled for GLT-1 for further quantification in this report. New rats were also generated and Table S1 outlines the numbers of rats and cells that were used previously and herein co-labeled for GLT-1 or that were newly generated. NAcore slices from each treatment group (yoked, extinguished, 15-min reinstated, 120-min reinstated) were labeled for GLT-1 and the presynaptic marker Synapsin I and imaged using confocal microscopy (Fig 1). Total GLT-1 and co-registered GLT-1 and Synapsin I were quantified and individually normalized to the volume of each mCherry-labeled astrocyte. To estimate the proportion of surface-proximal GLT-1, we digitally isolated GLT-1 within 250 nm of the astroglial membrane and normalized this measure to total levels of GLT-1 from each astrocyte (Fig 1).

As previously reported[6], co-registration of the astroglial membrane with Synapsin I was reduced after withdrawal from heroin, but not sucrose (Fig S1C). We also found reductions in total astrocyte GLT-1 expression after extinction from heroin (Fig 2A), but not sucrose self-administration (Fig 2B), consistent with previous reports that heroin withdrawal is associated with reduced tissue levels of GLT-1 protein and glutamate uptake in the NAcore[9,10]. Although total GLT-1 was reduced in heroin extinguished rats, the proportion of surface-proximal GLT-1

was unaltered (Fig 2C, E). Surprisingly, the ratio of surface to total GLT-1 was reduced in the NAcore of rats extinguished from sucrose (Fig 2D). Synapsin I was not altered after extinction from heroin or sucrose[6]. However, consistent with a reduction in total GLT-1, the co-registration of GLT-1 and Synapsin I was reduced after extinction from heroin self-administration (Fig 2G), but not sucrose self-administration (Fig 2H).

GLT-1 surface expression was transiently elevated during cued heroin seeking.

Fifteen min of cued heroin reinstatement increased the co-registration of astroglia with Synapsin I (Fig S1C). In contrast, after 15 or 120 min of cued heroin reinstatement, total GLT-1 and GLT-1 co-registration with Synapsin I remained reduced to the same levels produced after heroin extinction (Fig 2A,G). However, compared to yoked saline and extinguished rats, surface-proximal GLT-1 was elevated during 15-min of cued heroin seeking and returned to extinction levels after 120-min of cue exposure (Fig 2C). The reinstatement-induced increase in surface proximal GLT-1 is shown by a shift in the proportion of astroglia having low surface GLT-1 and an increase in proportion of astroglia with higher levels of GLT-1 (Fig 2E). In contrast, although the proportion of surface-proximal GLT-1 was reduced after extinction in sucrose-trained rats (Fig 2D), reinstated sucrose seeking did not further alter surface proximal GLT-1 (Fig 2D, F).

Astrocyte heterogeneity in heroin cue-induced plasticity.

The fact that astroglial synaptic proximity was transiently induced by 15 min of cued heroin reinstatement, but the increase in surface proximal GLT-1 did not co-register with Synapsin I indicates two types of heroin cue-induced astroglial plasticity that may not co-exist in the same astrocytes. In order to more closely examine characteristics of astrocytes with high synaptic proximity, we used the median values of astrocyte co-registration with Synapsin I and GLT-1 surface proximity from yoked saline rats to separate astrocytes with high and low levels of each cellular trait. Astrocytes with low (<1.18% astrocyte volume) and high degrees of synaptic

proximity (>1.18% astrocyte volume) exhibited similar levels of surface-proximal GLT-1 in yoked saline rats (Fig 3A). When astrocytes from 15-min reinstated rats were subdivided using the same values described for saline-treated rats, we observed different surface GLT-1 expression in the two subpopulations. Astrocytes with a high degree of synaptic co-registration had lower levels of surface GLT-1, and vice versa (Fig 3B-C).

Astroglial subtypes are evenly distributed in yoked saline, but not heroin-trained rats

To better characterize the distribution of astroglial subpopulations in yoked saline and heroin-trained rats, we applied the same median split strategy to create 4 subcategories of astroglia (High or Low Synapsin I co-registration at 1.18% astroglial volume and High or Low Surface GLT-1 at 5.11% total GLT-1). Stratifying astrocytes into subpopulations according to distribution of these two markers produced nearly equal populations in yoked saline rats (Fig 3D). We subdivided astrocytes from heroin-trained rats in the same manner and found that synaptic retraction by astroglia (Fig S1C) reduced the proportion of subpopulations characterized by high Synapsin I co-registration in heroin extinguished rats and after 120-min of cued heroin seeking (Fig 3D). Conversely, morphological plasticity after 15-min of active heroin seeking expanded the subpopulations with a high degree of synaptic proximity.

While synaptic proximity and surface expression of GLT-1 were positively correlated in astroglia from yoked saline rats (Fig 3E), these measures were negatively correlated after 15-min of cued heroin seeking, consistent with the finding that astrocytes undergoing morphological plasticity during heroin seeking did not exhibit a high degree of surface GLT-1 (Fig 3G). No correlation was found between these measures during extinction (Fig 3F). There was a trending negative correlation between surface GLT-1 in those astroglia designated as “high” (>5.11% total) and active lever pressing during 15-min of cued reinstatement (Fig 3H).

Abolishing morphological plasticity during cued reinstatement did not impact surface expression of GLT-1

To determine whether surface expression of GLT-1 remained elevated when astrocyte morphological plasticity was inhibited during cued seeking, we knocked down ezrin expression prior to reinstatement. Ezrin links actin with the cell membrane in astroglial peripheral processes[11,12], and ezrin knockdown using an antisense morpholino oligo reduces synaptic proximity of astroglia in the NAcore[6].

We trained rats to self-administer heroin and during extinction, an ezrin antisense oligomer was infused into the NAcore for 3 consecutive days (Fig 4A). We previously showed that this regimen of ezrin antisense oligo infusion knocks down ezrin in NAcore astroglia compared with a control oligo by 86% (N=19-24/6 cells/animal/group)[6]. Rats were reinstated for 15 minutes using heroin cues and ezrin knockdown increased active lever pressing (Fig 4B), as observed previously[6]. We quantified Synapsin I co-registration with NAcore astroglia and GLT-1 expression as described above. As expected after ezrin knockdown, Synapsin I co-registration with astrocytes was reduced (Fig 4C). We found no change in total GLT-1 expression after ezrin knockdown (Fig 4D), and a reduction in synaptic co-registration of GLT-1 (Fig 4E), likely the result of the synaptic retraction of astroglial processes produced by ezrin knockdown. Importantly, we found no impact of ezrin knockdown on the proportion of surface-proximal GLT-1 (Fig 4F), demonstrating that astroglial peripheral process extension is not required for surface diffusion of GLT-1.

Comparing synaptic co-registration and surface GLT-1 in NAcore astrocytes from rats that received the control oligo revealed a similar distribution as observed during 15-min of cued reinstatement (compare Fig 4G, I and Fig 3G, D). By reducing astrocyte co-registration with Synapsin I, ezrin knockdown abolished subpopulations characterized by high synaptic co-registration in all treatment groups (Fig 4H, I), resulting in a subpopulation distribution of astrocytes similar to heroin extinguished rats (see Fig 3D). Based on these results, we conclude

241 that heroin-associated changes in surface expression of GLT-1 on NAcore astroglia is
242 mechanistically distinct from changes in astrocyte morphological plasticity and the two cue-
243 induced adaptations occur in separate populations of NAcore astroglia.

DISCUSSION

NAcore astrocytes exhibit dynamic plasticity in synaptic proximity in response to heroin cues, and their increase in synaptic proximity dampens reinstated heroin seeking[6]. One mechanism whereby perisynaptic astroglia may reduce heroin seeking is by decreasing cue-induced synaptic glutamate spillover through synaptic localization of the astroglial glutamate transporter GLT-1[13,14]. Commensurate with this possibility, we found that the proportion of surface GLT-1 was elevated on NAcore astrocytes during cue-induced reinstatement of heroin seeking. However, the increase in surface GLT-1 during seeking did not give rise to increased co-registration of GLT-1 with Synapsin I. Instead, the transient increase in synaptic proximity by astroglia and the transient elevation in surface proximity of GLT-1 occurred in different subpopulations of NAcore astroglia, with only a small proportion of astrocytes in reinstated rats exhibiting both phenotypes. Importantly, either pharmacologically increasing GLT-1[9,15,16] or cue-induced increases in astroglial proximity to NAcore synapses[6] dampen cued heroin reinstatement. Thus, although our data reveal that NAcore astrocytes are equipped with two discrete and molecularly separable mechanisms of heroin cue-induced plasticity, both mechanisms serve a homeostatic function to dampen cue-induced relapse. How we expect that these mechanisms can decrease cued heroin seeking is described in detail below.

Synaptic glutamate spillover and cue-induced heroin reinstatement are reduced by two distinct forms of cue-induced astroglial plasticity

Reinstated seeking sustained by heroin-paired cues is associated with synaptic glutamate spillover that originates from prefrontal cortical synapses in the NAcore[17]. Glutamate spillover arises in large part from the downregulation of GLT-1 after the use of heroin and other addictive substances[18]. Importantly, synaptic glutamate spillover does not occur during cued sucrose seeking[18] because, in contrast to heroin or psychostimulants, the proximity of astroglia to NAcore synapses and the perisynaptic expression of GLT-1 are not reduced following sucrose

self-administration (Fig 5). Cue-induced spillover interacts with extrasynaptic glutamate receptors in at least two ways to promote motivated heroin seeking. The first is through access to extrasynaptic NMDA receptors containing the GluN2b subunit (NR2B) located on the postsynaptic annulus. NR2B density in the NAcore is elevated after heroin withdrawal and NR2B stimulation is increased by synaptic spillover after heroin self-administration[9]. Moreover, preventing NR2B stimulation inhibits heroin-induced seeking and synaptic potentiation[9]. A second mechanism by which synaptic glutamate spillover promotes heroin seeking is via diffusion of glutamate to metabotropic glutamate receptor-5 (mGluR5) located on the subclass of NAcore interneurons expressing neuronal nitric oxide synthase (nNOS). The ensuing production of nitric oxide (NO) promotes matrix metalloprotease activation and stimulation of β3-integrin receptors in the postsynapse to initiate transient synaptic potentiation (t-SP) and increase drug seeking[19-21]. To suppress t-SP and motivated heroin seeking, astroglia undergo two adaptations that reduce the impact of synaptic glutamate spillover (Fig 5), and as shown here, these adaptations occur largely in distinct populations of astroglia. For discussion purposes, we will refer to these two populations below as Type I (cued increase in synaptic proximity) and Type II (cued increase in surface GLT-1). Not only did we find that Type I and Type II adaptations occur in different cell types, but preventing Type I by knocking down ezrin did not alter the cue-induced induction of Type II.

Type I Astroglia: Cue-induced morphological plasticity with no increase in surface GLT-1

A portion of mature synapses throughout the brain are insulated by astroglia[22], and the perisynaptic astroglial membrane expresses the highest density of GLT-1 and other proteins, such as glutamate receptors and actin binding proteins, that permit astroglial plasticity in response to synaptic neurotransmission in order to maintain synaptic glutamate homeostasis[13,22,23]. At least in the hippocampus, the astroglial sheath is biased toward the postsynapse[24], thereby permitting access of synaptically released glutamate to presynaptic

296 mGluR2/3, autoreceptors that regulate release probability[25]. Withdrawal from addictive drugs,
297 including heroin, cocaine, or methamphetamine is associated with an enduring retreat of
298 astroglia from synapses in the NAcore[5-7], and the presentation of heroin-paired cues promotes
299 transient synaptic re-association in Type I astroglia (Fig 5). Although surface GLT-1 is not
300 altered by heroin cues in Type I astroglia, the synaptic re-association may limit access of
301 synaptic glutamate to perisynaptic NR2B and thereby dampen t-SP and heroin seeking. In
302 support of this idea, blocking the cue-induced re-association of astrocytes with NAcore
303 synapses by application of an ezrin-targeted antisense oligomer promotes cued-heroin
304 seeking[6]. While inhibiting diffusion of synaptic glutamate to NR2B on the postsynaptic annulus
305 is one hypothesis for how synaptic re-association by Type I astroglia could dampen heroin
306 seeking, lack of coincident upregulated surface GLT-1 may permit glutamate spillover to diffuse
307 to more distant sites critical for cued reinstatement, such as mGluR5 on nNOS interneurons.
308
309 **Type II Astroglia: Cue-induced increases in surface GLT-1 with no change in synaptic**
310 **proximity**
311 A portion of astroglia responded to cue-induced glutamate spillover by increasing surface GLT-
312 1, but this increase did not co-register with Synapsin I, indicating that surface GLT-1 in
313 reinstated animals is distant from the synapse. Type II astroglia permit glutamate spillover to
314 access glutamate receptors near the pre and postsynaptic annulus to reduce glutamate release
315 probability (presynaptic mGluR2/3) and promote t-SP (postsynaptic NR2B). However, diffusion
316 of glutamate to more distal sites would be reduced by increased surface GLT-1[9]. Notably, by
317 inhibiting glutamate access to nNOS interneurons and the consequent mGluR5-mediated
318 increase in NO, extrasynaptic GLT-1 could dampen t-SP and cued heroin seeking[19]. Indeed,
319 pharmacologically upregulating GLT-1 with drugs such as ceftriaxone not only inhibits access of
320 synaptic glutamate to heroin-upregulated NR2B, but reduces both t-SP and cue-induced drug
321 seeking[26-28].

Potential non-neuronal sources of extracellular glutamate may also be subject to regulation through increased surface GLT-1, including calcium-dependent release from astroglia[29,30] and glutamate extruded by the cystine-glutamate antiporter in exchange for cystine uptake, an essential amino acid needed for glutathione synthesis and regulation of cellular redox status[31]. These sources do not appear to contribute directly to cued increases in glutamate transmission since inhibiting prefrontal cortical inputs to the NAcore is sufficient to abolish cue-dependent increases in extracellular glutamate[18]. Instead, stimulating calcium-dependent astroglial glutamate release by increasing intracellular calcium selectively in NAcore astroglia or stimulating the cystine-glutamate antiporter with N-acetylcystine decreases reinstated drug seeking by activating mGluR2/3[30-32], arguing that reducing these nonsynaptic contributions to extracellular glutamate are not consequential to how we hypothesize increasing GLT-1 inhibits heroin seeking. Another interesting source of extracellular glutamate is activation of astroglial glutamate release by stimulating mu opioid receptors[33]. It is possible that the presumed elevation in astroglial glutamate release produced by mu opioid receptor stimulation during heroin self-administration could induce some of the enduring synaptic and astroglial adaptations seen after heroin withdrawal in the NAcore. This possibility is supported by the fact that mu receptor mediated release of astroglial glutamate stimulates NR2B located on the postsynaptic annulus.

Two distinct forms of astroglial plasticity to negatively regulate heroin seeking

It is unclear what contingencies in individual astroglia lead to cue-induced deployment of one or the other mechanism of astroglial plasticity described here. However, having two distinct subpopulations of cue-induced astroglial plasticity poses the intriguing possibility that the astroglial subpopulations may be differentially associated with the two principle cell types in the NAcore, D1- and D2-receptor expressing medium spiny neurons (D1- and D2-MSNs)[34]. Supporting this possibility, astrocytes in the dorsal striatum are selectively tuned to one or the

other neuronal subtype, responding with calcium flux during electrophysiological stimulation of either D1- or D2-MSNs, but not both[35]. If the increase in surface GLT-1 and astrocyte synaptic proximity also segregate across D1- and D2-MSNs, the two distinct forms of astroglial plasticity have the potential to utilize the opponent behavioral outputs from D1- and D2-MSNs to dampen cued heroin seeking. Thus, one process could reduce the capacity of D1-MSNs to promote cued drug seeking, and the other augment D2-MSN suppression of drug seeking[36,37].

Astroglial plasticity during sucrose versus heroin seeking

The data shown here provide insight into unique astroglial adaptations that follow from operant training with sucrose versus heroin. While intake of the two reinforcers is quite different, with rats pressing significantly more for sucrose than for heroin (Fig S1A), sucrose did not down-regulate GLT-1 and does not produce changes in synaptic proximity by NAcore astroglia[6,10]. Interestingly, although we found no changes in co-registration of GLT-1 with Synapsin I in sucrose-trained or reinstated animals, we observed a reduction in surface expression of GLT-1 after extinction from sucrose self-administration compared to yoked controls. The marked reductions in synaptic insulation and GLT-1 expression by NAcore astroglia after heroin extinction compared with the modest reduction in GLT-1 surface expression after sucrose extinction may contribute to the more perseverative extinction responding after exposure to addictive drugs compared with natural rewards (see Fig S1A and [38]). Alternatively, the unique constitutive adaptations after sucrose versus heroin use may permit the dramatic astroglial morphological plasticity and increases in surface GLT-1 produced by heroin- but not sucrose-paired cues.

Conclusions and clinical implications

We found that astroglia in the NAcore exhibit heterogeneous forms of plasticity capable of shaping cue-induced heroin seeking. Moreover, both astroglial morphological plasticity and

GLT-1 surface expression are transient events that involve proteins selectively expressed in astroglia (e.g. ezrin and GLT-1), providing potential routes for experimental manipulation and perhaps selective therapeutic interventions into one or the other process. Indeed, pharmacological treatments such as ceftriaxone and N-acetylcysteine that elevate GLT-1 are effective at reducing drug seeking in rodent models of relapse[15,39,40]. While ceftriaxone also restores synaptic insulation by astroglia in preclinical studies using cocaine[5], this has not been evaluated for N-acetylcysteine, which has proven only marginally effective at reducing relapse in human trials[41-43]. Given that astroglia engage two distinct processes for dampening cue-induced drug seeking, the underwhelming efficacy in relapse prevention by drugs restoring GLT-1 might be improved in combination with drugs that promote synaptic proximity by NAcore astroglia.

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DISCLOSURES

The authors declare no competing financial interests.

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FIGURES AND LEGENDS

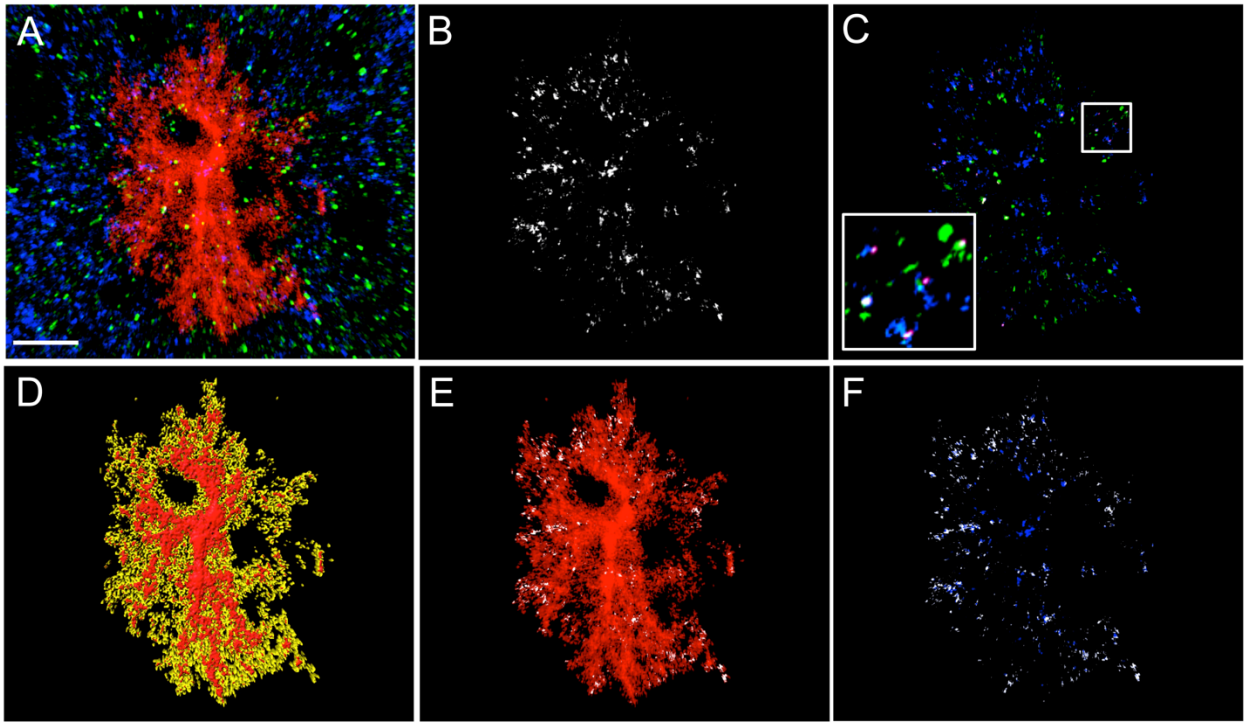

Figure 1. Workflow used for confocal analysis of astroglial morphology and surface-proximal GLT-1. (**A**) Z-series depicting an NAcore astrocyte transfected with AAV5/GFAP-hM3d-mCherry (red) and immuno-labeled for Synapsin I (green) and GLT-1 (blue). (**B**) GLT-1 immunoreactivity co-registered with mCherry in (**A**) is shown in white. (**C**) Co-registration of GLT-1 (blue) with Synapsin I (green) from the region occupied by the astrocyte in (**A**) is shown in pink. (**D**) Digital rendering of the astroglial surface (yellow) was used to identify GLT-1 signal within 250 nm of the cell membrane (**E**-**F**, white) relative to total GLT-1 from the same astrocyte (**F**, blue). Bar in (**A**)= 10 µm.

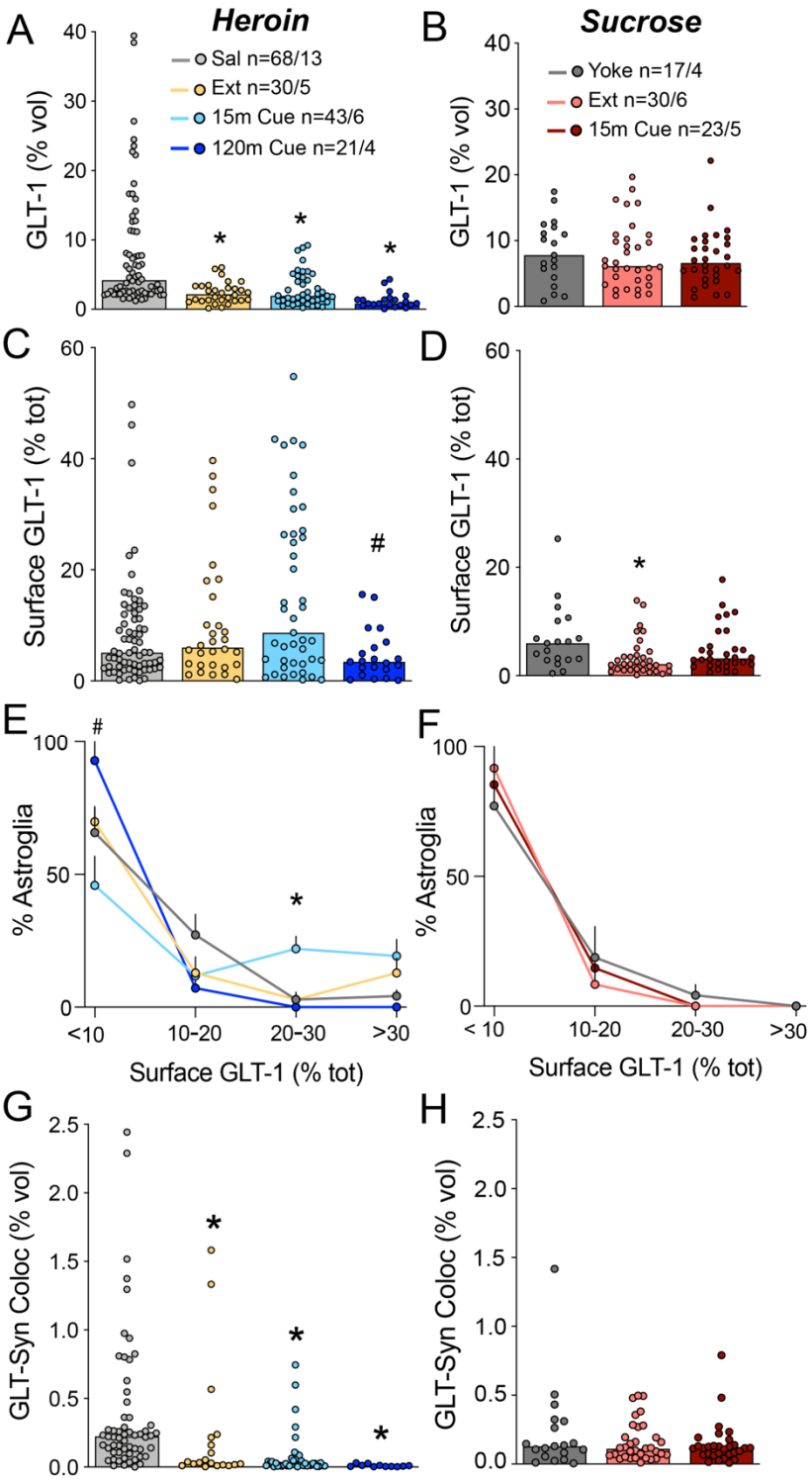

Figure 2. Surface expression of GLT-1 was transiently elevated during active heroin seeking. (**A**) Withdrawal from heroin self-administration produced a downregulation of GLT-1 on NAcore astroglia, whether or not rats were reinstated (Kruskal-Wallis=50.1, p<0.0001). (**B**)

GLT-1 expression was unchanged after operant training with sucrose (Kruskal-Wallis=0.7336, p=0.693). (**C**) Surface-proximal GLT-1, shown here as percent of total GLT-1 from each astrocyte, was increased during active seeking (15m Cues) compared to extinguished seeking (120m Cues, Kruskal-Wallis=9.848, $p<0.05$). (**D**) This measure was also significantly reduced after extinction from sucrose self-administration (Kruskal-Wallis=8.056, $p<0.05$). A greater proportion of astrocytes exhibited high levels of surface-proximal GLT-1 after 15-min of heroin cues compared to yoked saline controls (**E**, 2-way ANOVA, $F_{6,48}=2.904$, $p<0.05$), but no difference was observed during reinstated sucrose seeking (**F**, 2-way ANOVA, $F_{6,36}=0.469$, $p=0.827$). Co-registration of GLT-1 with the presynaptic marker Synapsin I analyzed in a subgroup of the same cells was found to be reduced in heroin- (**G**, Kruskal-Wallis=54.15, $p<0.0001$; N=58/10 Sal, 19/4 Ext, 43/6 15m Cue, 11/3 120m Cue), but not sucrose-trained rats (**H**, Kruskal-Wallis=0.3724, $p=0.830$). N shown in (**A**-**B**) as cells/animals. *$p<0.05$ compared to yoked control, #$p<0.05$ compared to 15-min reinstated.

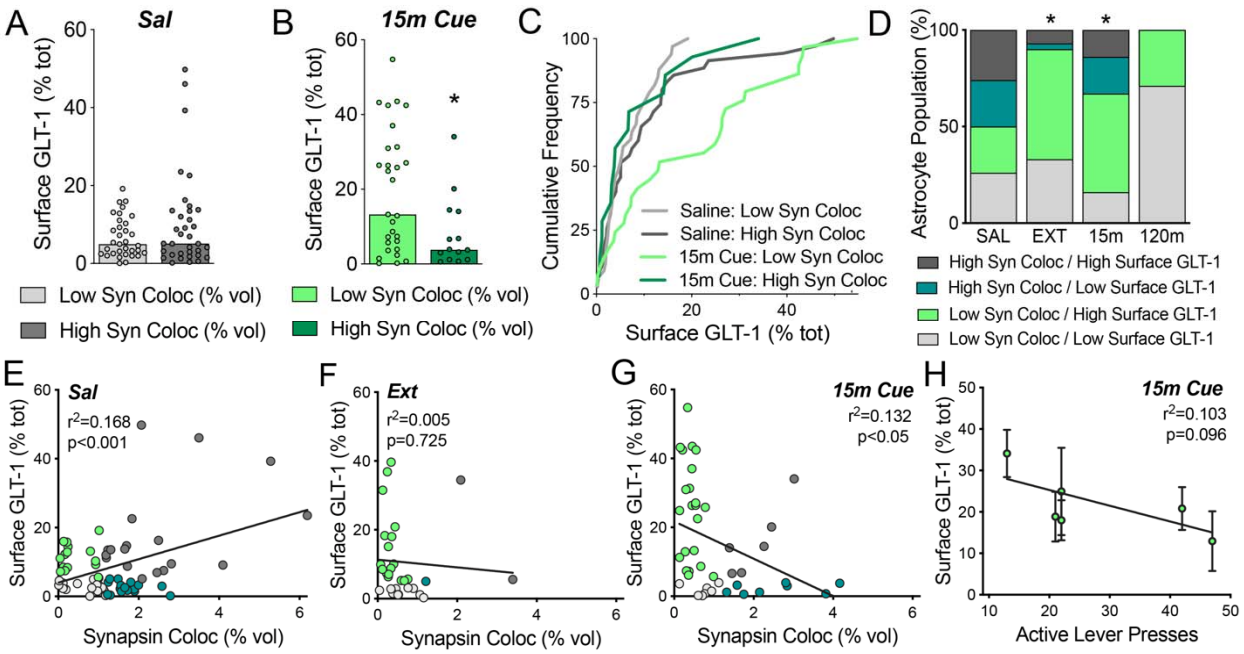

Figure 3. During reinstated seeking, astrocytes with high surface GLT-1 exhibited low synaptic proximity. (**A**) Splitting astrocytes from yoked saline animals according to the median of Synapsin I co-registration (1.18% astroglial volume) gives groups of astrocytes with equivalent levels of surface-proximal GLT-1 (Mann-Whitney=530, p=0.566). (**B**) Applying the same parameters to astrocytes from rats during 15-min of cued reinstatement revealed that NAcore astrocytes with high synaptic co-registration exhibited a significant reduction in surface-proximal GLT-1 relative to astroglia with low synaptic proximity from the same animals (Mann-Whitney=119, p<0.05). (**C**) Frequency distribution showing that astrocytes with the highest levels of surface GLT-1 were found in rats reinstated for 15-min and were retracted from Synapsin I (Kolmogorov-Smirnov=0.3699, p<0.05 Heroin Low vs. Saline Low). (**D**) NAcore astroglia were subdivided into 4 populations according to median values of Synapsin I co-registration and surface GLT-1 (5.11% total GLT-1) in yoked saline rats. Subpopulations were evenly distributed in yoked saline rats, but not in heroin-trained animals (Chi^2_6=20.34; p<0.01; lack of subgroups in 120m reinstated prevents statistical analyses using this group). The distribution of Synapsin I co-registration and surface GLT-1 measures across populations of astrocytes is shown in correlation plots in (**E**-**H**). Synaptic proximity and surface-proximal GLT-1

were positively correlated in NAcore astrocytes from yoked saline rats (**E**, r^2=0.168, p<0.001). During 15-min of cued reinstatement, however, the two measures were negatively correlated (**G**, r^2=0.132, p<0.05), and astrocytes with high synaptic proximity had low levels of GLT-1 surface expression, and vice versa. No correlation was observed in extinguished rats (**F**, r^2=0.005, p=0.725). (**H**) A negative correlation between cells with "high" surface GLT-1 for each animal and active lever pressing during a 15-min reinstatement session was nearly significant (r^2=0.103, p=0.096). *p<0.05 compared to yoked saline.

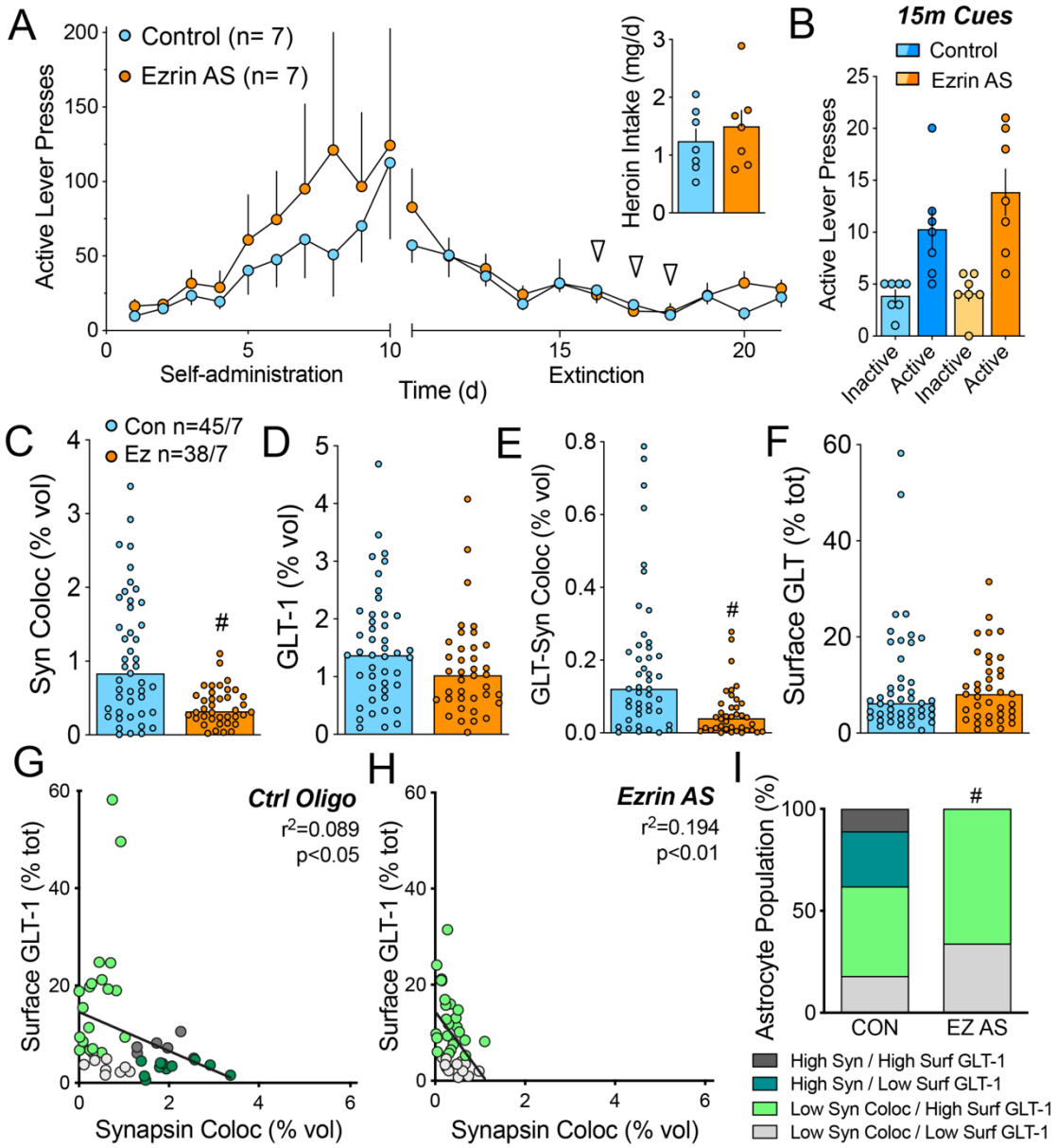

Figure 4. Impairing morphological plasticity in NAcore astroglia did not impact surface GLT-1 dynamics. (**A**) Rats were trained to self-administer heroin over 10 consecutive days. Starting on day 6 of extinction training prior to the session for 3 consecutive days (white arrowheads), animals received NAcore infusions of an ezrin antisense oligomer or a control oligomer. Inset shows that both groups received similar amounts of heroin during self-administration (unpaired Student's t_{12}=0.737, p=0.521). 24h after the final extinction session, animals were reinstated for 15-min by exposure to light and tone cues previously paired with

heroin delivery. (**B**) Rats that underwent ezrin knockdown pressed higher on the active lever during a 15-min reinstatement session (1-way ANOVA $F_{3,24}$=3.663, p<0.05 control active lever vs. ezrin AS active lever). (**C**) Astrocytes from rats treated with the ezrin antisense oligo exhibited a significant reduction in synaptic co-registration, consistent with knockdown of astrocyte peripheral process motility (Mann-Whitney=461, p<0.001). (**D**) GLT-1 levels were unchanged by ezrin antisense oligo delivery (Mann-Whitney=652, p=0.064). (**E**) The co-registration between GLT-1 and Synapsin I on astroglia was significantly reduced after ezrin knockdown (Mann-Whitney=424, p<0.0001), but the proportion of surface-proximal GLT-1 was unchanged (**F**, Mann-Whitney=806.5, p=0.661). (**G**) Subpopulations from control oligo-treated rats were similar to previously quantified subpopulations during 15-min of reinstated seeking (Ctrl Oligo, r^2=0.0875, p<0.05). (**H**) The negative correlation between Synapsin I co-registration and surface GLT-1 was maintained after ezrin knockdown, despite loss of subpopulations characterized by a high degree of synaptic co-registration (r^2=0.194, p<0.01). Proportion of subpopulations in the two treatment groups is shown in (**I**, Chi^2_3=18.29, p<0.001). #p<0.05 compared to control oligo treatment.

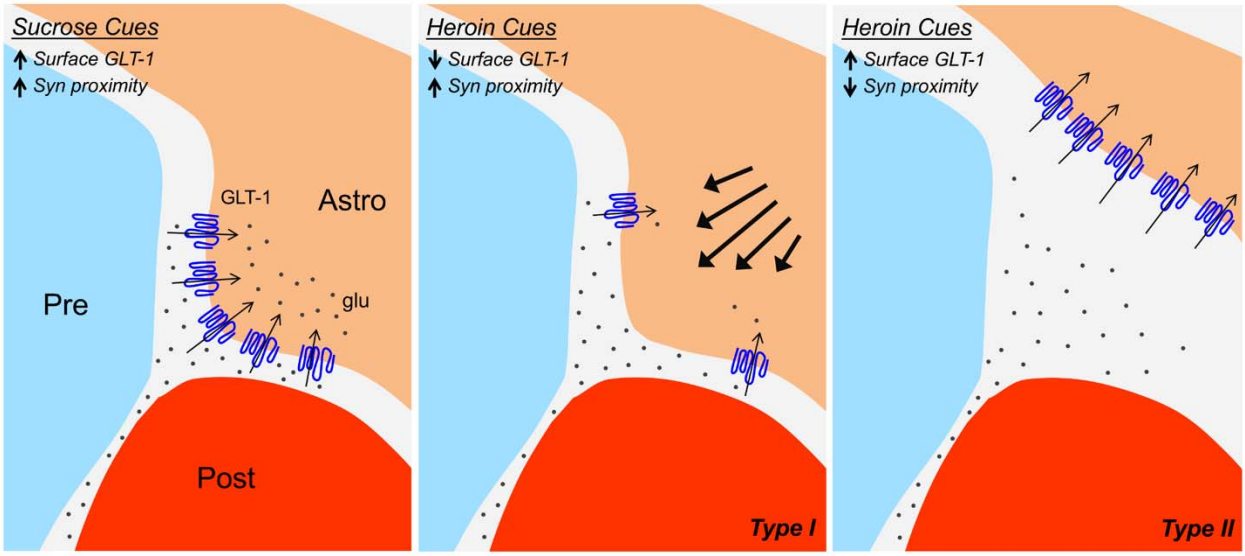

Figure 5. Heroin cues reveal two types of astroglial plasticity that regulate cue-induced heroin seeking. Following operant training with a natural reinforcer like sucrose, synaptic glutamate release is rapidly recovered by high levels of GLT-1 on the astroglial surface adjacent to NAcore synapses (left). Perisynaptic proximity of astroglial processes facilitates glutamate uptake and autoinhibition of glutamate release through stimulation of presynaptic autoreceptors, such as mGluR2/3. During heroin withdrawal, astrocyte processes retract from NAcore synapses and downregulate GLT-1, permitting glutamate spillover and disrupting autoinhibitory mechanisms that regulate glutamate release. During reinstated heroin seeking, NAcore astroglia adapt in one of two discrete ways to partly suppress reinstated heroin seeking. In Type I astroglia (middle), morphological plasticity restores synaptic proximity of astroglial processes, presumably at the postsynapse, biasing synaptic glutamate spillover towards autoinhibitory mGluR2/3 and away from postsynaptic NR2B that potentiates seeking. In Type II astroglia, GLT-1 is increased on the astroglial surface at extrasynaptic domains (right). We hypothesize that this process may prevent recruitment of nNOS interneurons that trigger post-synaptic plasticity and enhance reinstated seeking through release of NO and activation of matrix metalloproteases.