# Plasticity in astrocyte subpopulations regulates heroin relapse

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**ABSTRACT** (133/150 words)

Opioid use disorder (OUD) produces detrimental personal and societal consequences. Astrocytes are a major cell group in the brain that receives little attention in mediating OUD. We determined how astrocytes and the astroglial glutamate transporter, GLT-1, in the nucleus accumbens core adapt and contribute to heroin seeking in rats. Seeking heroin, but not sucrose, produced two transient forms of plasticity in different astroglial subpopulations. Increased morphological proximity to synapses occurred in one subpopulation and increased extrasynaptic GLT-1 expression in another. Augmented synapse proximity by astroglia occurred selectively at D2-dopamine receptor expressing dendrites, while changes in GLT-1 were not neuron-subtype specific. Importantly, mRNA-antisense inhibition of either morphological or GLT-1 plasticity promoted cue-induced heroin seeking. We show that heroin cues induce two distinct forms of transient plasticity in separate astroglial subpopulations that dampen heroin relapse.

31 **TEASER** (94/125 characters w spaces)

32 Different subpopulations of astrocytes engage with accumbens synapses to dampen heroin

33 relapse.

34 **INTRODUCTION**

35 Relapse to opioid use is a leading cause of death in the United States. Decades of research
36 demonstrate the importance of glutamate dysregulation at nucleus accumbens core (NAcore)
37 synapses as a causative factor in relapse-like behavior in animal models (*1*). Glutamate
38 dysregulation in animal models of addiction is due in large part to changes in NAcore astroglia
39 that express the glutamate transporter GLT-1, which terminates the actions of the bulk of
40 synaptically-released glutamate (*2, 3*). Addictive substances, including alcohol, nicotine,
41 psychostimulants, and opioids induce an enduring downregulation of GLT-1 on astrocytes in the
42 NAcore (*4*). Furthermore, astroglial processes that insulate synapses and take up glutamate
43 during synaptic transmission retract from NAcore synapses after extinction from cocaine, heroin,
44 and methamphetamine use, but not following sucrose self-administration and extinction (*5-7*).
45 Synaptic retraction of astrocyte processes and downregulation of GLT-1 disrupt glutamate
46 homeostasis at NAcore synapses, permitting spillover of synaptic glutamate and postsynaptic
47 potentiation required for drug-associated cues to initiate drug seeking (*8*).

48

49 We previously found that synaptic retraction of NAcore astroglia after heroin withdrawal is partially
50 and transiently reversed during cue-induced heroin seeking (*6*). We hypothesize that heroin cue-
51 induced increased proximity of astroglia to synapses increases the synaptic adjacency of GLT-1
52 on perisynaptic astroglial processes, thereby serving to minimize spillover of synaptically-
53 released glutamate that mediates cue-induced drug seeking. We also sought to determine
54 whether synaptic reassociation of astroglial processes stimulated by heroin-associated cues was
55 selective for either of the two main neural subtypes in the NAcore, D1 or D2 receptor-expressing
56 medium spiny neurons (D1- or D2-MSNs) (*9*), which exert opposing control over drug-seeking
57 behaviors (*10-13*).

58

59 We used confocal microscopy to measure the synaptic co-registration of labeled astrocytes and
60 astroglial GLT-1 in the NAcore after extinction or reinstatement of heroin or sucrose seeking. We
61 found that cued reinstatement of heroin, but not sucrose, seeking produced two transient
62 adaptations that parsed into distinct astroglial subpopulations. In one subpopulation, heroin cues
63 increased synaptic adjacency of astroglial processes without increasing surface-proximal GLT-1,
64 and in the second subpopulation, cued reinstatement elevated surface GLT-1 on astroglia
65 situated more than 250 nm from the synaptic cleft. We independently disrupted the two forms
66 cue-induced plasticity in each subpopulation of NAcore astroglia by knocking down GLT-1 or by
67 reducing synthesis of ezrin, an astroglial selective actin binding protein that mediates
68 morphological plasticity in distal astroglial processes (*14-18*). Either knockdown augmented the
69 capacity of cues to induce heroin seeking. When we assessed whether astrocytes exhibited a
70 bias in their synaptic adjacency with D1- or D2-MSN synapses, we found that astrocytes
71 selectively associated with D1-MSN synapses after extinction of heroin seeking and retracted
72 from D2-MSNs, and that this pattern was reversed during cue-reinstated heroin seeking.
73 Moreover, the cue-induced increase in surface GLT-1 was not detected adjacent to either D1- or
74 D2-MSN dendrites during reinstated heroin seeking, supporting a functional role for GLT-1-
75 deficient astroglial processes in shaping synaptic activity. In keeping with abundant literature
76 demonstrating suppression of synaptic activity by perisynaptic astroglia (*19, 20*), we propose that
77 astrocyte insulation of D1-MSN synapses after extinction training suppresses D1-MSN activity to
78 reduce seeking, while retraction from D2-MSNs permits their potentiation. The reversal of this
79 pattern during cue presentation would instead permit D1-MSN potentiation, which is known to
80 drive cued drug seeking (*10*). In all, our data show that astrocyte morphological plasticity is neuron
81 subtype-selective and that two distinct forms of astroglial plasticity are transiently induced by
82 heroin cues in separate subpopulations of astrocytes to dampen cue-induced heroin seeking.

83 **RESULTS**

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

85 To examine whether enhanced synaptic proximity of NAcore astroglia suppressed cue-induced
86 heroin seeking through changes in synaptic adjacency of GLT-1, NAcore astroglia were
87 selectively labeled with a membrane-bound fluorescent reporter prior to operant training. Rats
88 were trained to self-administer heroin or sucrose and reward delivery was paired with light and
89 tone cues (Fig. 1A). Importantly, heroin intake was equivalent in rats placed in the extinction, 15-
90 min or 120-min reinstated groups (Fig. 1A inset). Animals that received yoked saline delivery and
91 cues served as controls for heroin-trained rats and animals that received yoked cues were
92 controls for sucrose-trained rats. Operant responding was extinguished by removing both the
93 cues and reinforcers, and a portion of rats were reinstated by restoring conditioned cues without
94 reinforcers to active lever pressing for 15 or 120 min (Fig. 1B). Rats used to generate the data in
95 Figs. 1A-B were included in a previous study (*6*) and in the present report mCherry transduced
96 astrocytes in tissue slices from these rats were immuno-labeled for both Synapsin I and GLT-1.
97 NAcore slices from each treatment group (yoked, extinguished, 15-min reinstated, 120-min
98 reinstated) were double-labeled for GLT-1 and the presynaptic marker Synapsin I and imaged
99 using confocal microscopy (Fig. 1C). Total GLT-1, co-registered Synapsin I, and co-registered
100 GLT-1 and Synapsin I were quantified and normalized to the volume of each mCherry-labeled
101 astrocyte (Fig. 1D-E). To estimate the proportion of surface-proximal GLT-1, we digitally isolated
102 GLT-1 within 250 nm of the astroglial membrane as a proportion of total GLT-1 in each astrocyte
103 (Fig. 1F-H).

104

105 Co-registration of the astroglial membrane with Synapsin I was reduced after withdrawal from
106 heroin (Fig. 2A), but not sucrose (Fig. S1A). We also found reductions in total astrocyte GLT-1
107 expression after extinction from heroin (Fig. 2B), but not sucrose self-administration (Fig. S1B).
108 Although total GLT-1 was reduced in heroin extinguished rats, the proportion of surface-proximal

109 GLT-1 was unaltered in extinguished rats (Fig. 2C). Moreover, 15 min of cue exposure transiently
110 increased the proportion of astroglia expressing the highest levels of surface-proximal GLT-1 (Fig.
111 2D). Both the increase in synapse-proximal astroglia and GLT-1 after 15-min of heroin cue were
112 transient and returned to extinction levels by 120-min after initiating cued reinstatement (Fig.
113 2A,C-D). Interestingly, the ratio of surface:total GLT-1 was reduced in the NAcore of rats
114 extinguished from sucrose (Fig. S1C), but not altered by cued reinstatement (Fig. S1C-D). The
115 co-registration of GLT-1 with Synapsin I was reduced after extinction from heroin self-
116 administration (Fig. 2E), but not sucrose self-administration (Fig. S1E). Despite increases in
117 surface GLT-1 and synaptic adjacency by NAcore astroglia, the proximity of GLT-1 to the
118 presynaptic marker Synapsin I was not restored by cued heroin seeking (Fig. 2E-F). Instead, the
119 increase in surface-proximal GLT-1 in reinstated animals was targeted extrasynaptically (>250
120 nm from the synapse) (Fig. 2G-H). In sucrose-trained rats, levels of synaptic and extrasynaptic
121 GLT-1 were not changed by extinction training or cued reinstatement of sucrose seeking (Fig.
122 S1F-H). Importantly, extinction from heroin self-administration did not change total Synapsin I
123 expression in the NAcore (Fig. S2).
124
125 **Astrocyte heterogeneity in heroin cue-induced plasticity**
126 The fact that astrocyte synaptic proximity was transiently increased by 15 min of cued heroin
127 reinstatement, but the increase in surface-proximal GLT-1 did not co-register with Synapsin I
128 indicates that these cue-induced astroglial adaptations may occur in distinct subpopulations of
129 astrocytes. Indeed, principal component analysis (PCA, Fig. S3) (*21*) of astroglia from saline-,
130 heroin- and sucrose-trained rats identified three distinct clusters of astroglia we arbitrarily refer to
131 as types 1-3: type 1, astrocytes with high synaptic co-registration; type 2, astrocytes with high
132 levels of extrasynaptic GLT-1, and type 3, astrocytes with low-to-moderate synaptic adjacency
133 and surface GLT-1 expression (Fig. 3A). A majority of NAcore astrocytes from yoked saline rats
134 were in the type 1 and 3 subpopulations (Fig. 3B, E). Following extinction from heroin self-

135 administration there was a loss of type 1 and an increase in type 2 astrocytes (Fig. 3C, E). Fifteen
136 min of cued heroin reinstatement resulted in a transient restoration of type 1 astrocytes, and an
137 increase in the proportion of type 2 astroglia (Fig. 3D, E). In contrast with heroin self-administration
138 and akin to saline rats, all three treatment groups of sucrose-trained rats contained predominately
139 type 1 and 3 astrocytes (Fig. S4A). Along with the lack of GLT-1 co-registration with Synapsin I
140 during cued reinstatement (Fig. 2E), these data are consistent with the presence of three distinct
141 astroglial populations in heroin-seeking animals, which is also indicated by the lack of GLT-1
142 immuno-staining in some NAcore astroglia (Fig. S5). Fig. 3F illustrates the distinct morphology
143 and GLT-1 localization characteristic of each astroglial type.
144
145 **$G_q$ signaling in NAcore astroglia increased synaptic adjacency, but not surface GLT-1**
146 $G_q$-coupled signaling through metabotropic glutamate receptor mGluR5 on cultured astroglia
147 promotes ezrin-dependent astrocyte fine process motility (*15*). Moreover, cue-induced heroin
148 seeking is associated with increased ezrin phosphorylation (*6*) and astrocyte proximity to
149 synapses (Fig. 2A), and increases in synaptic glutamate spillover in NAcore that occur during
150 cued reinstatement of drug seeking (*22*) could stimulate mGluR5 on astroglial processes (*23*). To
151 determine if $G_q$-type mGluR signaling triggers cue-induced astrocyte process motility, a $G_q$-
152 coupled designer receptor activated by designer drug (DREADD) was delivered selectively to
153 NAcore astroglia (Fig. 4A). Rats were trained to self-administer heroin before undergoing 10 days
154 of extinction training (Fig. 4B). In lieu of reinstatement, animals were given clozapine N-oxide
155 (CNO) or vehicle and their brains were removed after 30-min for analysis of astrocyte synaptic
156 adjacency and surface-proximal GLT-1 expression. CNO delivery increased the co-registration of
157 the astroglial membrane with Synapsin I-positive puncta (Fig. 4C), but did not impact surface-
158 proximal GLT-1 levels (Fig. 4D). These data indicate that $G_q$ signaling in NAcore astroglia triggers
159 morphological plasticity during reinstatement, while the increase in extrasynaptic GLT-1
160 expression (Fig. 2G-H) likely involves a different signaling cascade.

161

162 **Reducing cue-induced plasticity in astroglial subpopulations increased heroin seeking**

163 To determine whether cue-induced type 1 and type 2 astroglial plasticity (as shown in Fig. 3F)
164 impacted heroin seeking, we implanted cannulae above the NAcore and trained rats to self-
165 administer heroin (Fig. 5A). Rats were divided to maintain equal heroin intake across the
166 reinstated groups (Fig. 5B) and received bilateral infusions of an inert control vivo-morpholino
167 antisense oligomer, or an oligo targeted to GLT-1 (*24*) or ezrin (*6*) on days 6-8 of extinction
168 training. Rats were reinstated by exposure to heroin cues and either ezrin or GLT-1 knockdown
169 potentiated cued seeking (Fig. 5C). Thus, the presence of either type 1 or type 2 astroglial
170 plasticity during cued heroin seeking serves a compensatory function to suppress heroin seeking.

171

172 Confirming that cannulae placement and morpholino infusions did not impact cue-induced
173 astrocyte plasticity, both type 1 and type 2 adaptations were observed during 15 min of cue-
174 induced heroin seeking in rats treated with control oligo (compare Fig. S4B with Fig. 3D). Ezrin
175 knockdown reduced the synaptic proximity of astroglia in the NAcore (Fig. 5D) and GLT-1
176 knockdown reduced total and surface proximal GLT-1 (Fig. 5E-F). Validating the specificity of the
177 oligos and supporting the presence of distinct subpopulations of astrocytes, the ezrin-targeted
178 oligo selectively eliminated type 1 astrocytes while the GLT-1 targeted oligo selectively eliminated
179 type 2 astrocytes in rats reinstated by heroin cues for 15 min (Fig. S4B). Moreover, GLT-1
180 knockdown did not alter synaptic proximity of NAcore astroglia (Fig. 5D), and ezrin knockdown
181 did not impact levels of total GLT-1 or surface-proximal GLT-1 (Fig. 5E-F). As expected,
182 independent knockdown of either GLT-1 or ezrin reduced GLT-1 co-registration with Synapsin I
183 (Fig. 5G).

184

185 Taken together these data show that all three types of astroglia are present during cue-induced
186 heroin seeking (Fig. 3F). Akin to extinguished rats, heroin cues are associated with type 2

187 astroglia having increased surface-proximal expression of GLT-1, and akin to their presence in
188 control saline or sucrose rats, cues induce the presence of type 1 astroglia that have high synaptic
189 proximity. The subpopulations are distinct cells and are regulated by distinct signaling pathways.
190 Importantly, the presence of either type 1 or type 2 subpopulations serves to inhibit cue-induced
191 heroin seeking.
192
193 **NAcore astrocytes differentially associated with D1- and D2-MSNs at baseline and after**
194 **heroin**
195 Activity in the two main neuronal subtypes in the NAcore, D1- and D2-MSNs promotes drug
196 seeking and extinction of seeking, respectively (*25, 26*) and individual astrocytes in the dorsal
197 striatum signal uniquely with these two neuronal subclasses (*19, 27*). We hypothesized that the
198 different functional types of astroglia depicted in Fig. 3F would associate uniquely with D1- and
199 D2-MSNs, thereby contributing to their differential functional roles in heroin extinction and cue-
200 induced seeking. To test this hypothesis, we labeled astrocytes and neurons in male and female
201 D1- and D2-Cre transgenic rats before training animals to self-administer heroin for 10 days (Fig.
202 6A-B). Some rats were then reinstated for 15-min by exposure to heroin-associated cues (Fig.
203 6C). Isolated astroglia were imaged and their co-registration with virally-labeled D1- or D2-MSN
204 dendrites and the synaptic marker Synapsin I or GLT-1 was quantified (Fig. 6D-G). Pooling data
205 from D1- and D2-Cre rats revealed the same synaptic retraction by astroglia from NAcore
206 dendrites after extinction and re-insertion of astroglial processes toward synapses during cued
207 reinstatement (Fig. 6H) as was observed in Sprague-Dawley rats (Fig. 2A). Interestingly, when
208 astrocyte-synapse co-registration data were analyzed separately in D1- and D2-Cre rats, the
209 astrocyte association with D1-MSN synapses was lower in yoked saline animals compared with
210 D2-MSN synapses (compare saline-treated groups in Fig. 6I-J). After extinction training, astrocyte
211 co-registration with Synapsin I was increased at D1-MSN synapses (Fig. 6I) and decreased at

212 D2-MSN synapses (Fig. 6J). Fifteen minutes of exposure to heroin cues restored astrocyte
213 association with both synaptic types to control levels (Fig. 6I-J).
214
215 In contrast with the morphological adaptations, when data from D1- and D2-Cre rats were pooled
216 the surface-proximal GLT-1 that co-registered with NAcore dendrites did not fully parallel the
217 findings in Sprague-Dawley rats (Fig. 2C). Although GLT-1 was reduced in extinguished rats, the
218 increase in surface-proximal GLT-1 after 15-min cued heroin seeking (Fig. 2D) was replicated in
219 the pooled D1- and D2-Cre data (Fig. 6K). When dendritic adjacency of GLT-1 was examined
220 separately in D1- and D2-Cre rats, levels of GLT-1 adjacent to D2 dendrites was higher when
221 compared with D1 dendrites (compare saline-treated groups in Fig. 6L-M). The extinction-
222 associated reduction in the pooled data occurred at D2-, not D1-dendrites (Fig 6L-M). However,
223 the increase in surface-proximal GLT-1 after 15 min of cue exposure did not co-register with either
224 D1 or D2-dendrites (Fig. 6L-M). Both the morphological plasticity and GLT-1 dynamics in NAcore
225 astroglia from D1- and D2-Cre animals occurred in a manner that was not sex-specific (Fig. S6).
226
227 Taken together, these data show that the cue-induced increase in type 1 astroglia during heroin
228 seeking (Fig. 3D-E) can be linked to morphological plasticity of astroglia surrounding D2-dendrites
229 (Fig. 6H-J), but that the cue-induced increase in type 2 astroglia exhibiting high levels of
230 extrasynaptic GLT-1 (Fig. 3D-E) are not associated with either D1- or D2-dendrites (Fig. 6K-M).
231 A summary schematic illustrating subcircuit-selective astrocyte adaptations after extinction of
232 heroin seeking is shown in Fig. S7.

233 **DISCUSSION**

234 Synaptic glutamate spillover from prefrontal cortical synapses in NAcore is produced by drug
235 associated cues and is necessary for drug cues to induce synaptic adaptations that regulate the
236 intensity of drug seeking. Astroglia possess the capacity to strongly regulate glutamate spillover
237 from synapses by their morphological proximity to synapses (*28*) and their expression of GLT-1,
238 especially in perisynaptic astroglial processes (*29-32*). We show that during cue-induced heroin
239 seeking, astroglia employ both of these mechanisms to dampen the intensity of seeking.
240 Surprisingly, cue-induced increases in synaptic proximity and surface GLT-1 expression occurred
241 in separate astroglial subpopulations. Type 1 astroglia had greater proximity to synapses in
242 NAcore, while type 2 astroglia showed increased surface expression of GLT-1. The functional
243 relevance of cues transiently inducing these astroglial subpopulations was shown by selectively
244 eliminating either type 1 or type 2 astroglial plasticity and potentiating cue-induced heroin seeking.
245 Finally, we demonstrated that the type 1 adaptation was selective for D2-, not D1-MSNs during
246 seeking, while the cue-induced type 2 adaptation was not significantly associated with either D1-
247 or D2-MSNs. Together, our data reveal at least two mechanisms whereby astroglia negatively
248 regulate cue-induced motivation to seek heroin, but not sucrose. At least part of this action may
249 arise from $G_q$-coupled stimulation of astroglial processes toward D2-MSN synapses.

250

251 **Distinct subpopulations of astroglia during cue-induced heroin seeking**

252 During cue-induced heroin seeking three distinct subpopulations of astroglia were present, type
253 1 showing increased synaptic proximity, type 2 showing upregulated surface GLT-1 and type 3
254 having relatively lower levels of both synaptic adjacency and surface GLT-1. Unbiased PCA
255 revealed that type 1 and type 2 adaptations were in distinct subpopulations of astroglia. The
256 appearance of type 1 and type 2 subpopulations induced by cued seeking was transient and
257 absent after 120 min of unrewarded seeking. This is similar to the transient potentiation of D1-
258 MSNs triggered by drug-associated cues (*26, 33*).

259

260 Control saline or sucrose rats showed largely type 1 and 3 astroglia. Type 2 astroglia emerged
261 while type 1 were eliminated in heroin extinguished rats. Cue-induced heroin seeking was
262 accompanied by transient return of type 1 astroglia to levels present in control rats and a transient
263 elevation in the proportion of astroglia expressing the type 2 adaptation. In sum, our data clearly
264 show that the appearance and disappearance of different types of astroglial adaptations depends
265 on their stage in the addiction protocol (i.e. extinguished vs. relapsing) and reveals remarkable
266 morphological and signaling plasticity in astroglia to negatively regulate cue-induced heroin
267 seeking.

268

269 **Type 1 astroglia: Cue-induced morphological plasticity with no increase in surface GLT-1**

270 A portion of mature synapses throughout the brain are insulated by astroglia (*34*), and the
271 perisynaptic astroglial membrane expresses the highest density of GLT-1, glutamate receptors
272 and other proteins, including actin binding proteins like ezrin, which together contribute to
273 modulating glutamatergic synaptic activity. Perisynaptic astroglia modulate synaptic glutamate
274 homeostasis in part through dynamic morphological plasticity in response to synaptic
275 neurotransmission (*29, 34, 35*). In the hippocampus and cerebellum, the astroglial sheath is
276 biased toward the postsynapse (*36*), permitting access of synaptically released glutamate onto
277 presynaptic mGluR2/3 autoreceptors that negatively regulate release probability (*37*). Notably,
278 dense expression of GLT-1 proximal to the synapse may prevent recruitment of this autoinhibitory
279 mechanism, by retrieving glutamate from the synapse and thus reducing glutamate concentration
280 at presynaptic autoreceptors. In this way, the GLT-1-deficient astroglial processes present in the
281 type 1 subpopulation can promote autoinhibitory regulation of excitatory transmission in the
282 NAcore by sterically guiding synaptically released glutamate towards presynaptic mGluR2/3 in
283 the absence of glutamate uptake. The importance of guiding glutamate to presynaptic mGluR2/3

284 to negatively regulate relapse is supported by the fact that stimulating mGluR2/3 in the
285 accumbens inhibits drug seeking (*38, 39*).
286
287 Astrocyte fine process motility is increased by activating $G_q$-coupled mGluR5 (*15, 16*). Also,
288 stimulating $G_q$-DREADD in NAcore astroglia reduces reinstated cocaine seeking by promoting
289 astroglial glutamate release onto presynaptic mGluR2/3 (*40*). Our findings complement this work
290 by showing that activating astroglial $G_q$-DREADD in the NAcore increases the number of synaptic
291 puncta with near-adjacent astroglial processes, although the resolution limit of confocal
292 microscopy does not permit us to determine whether astroglial processes were oriented toward
293 the pre- or postsynapse after DREADD stimulation. Increasing $G_q$ signaling in NAcore astroglia
294 did not impact surface-proximal GLT-1 levels, supporting discrete signaling mechanisms for
295 heroin cue-induced formation of type 1 and type 2 astroglial plasticity.
296
297 In addition to potentially guiding synaptically released glutamate toward mGluR2/3 autoreceptors,
298 the synaptic re-association induced by heroin cues in type 1 astroglia may limit access of synaptic
299 glutamate (i.e. glutamate spillover) to perisynaptic NMDA glutamate receptors containing the 2B
300 subunit (NR2B) that must be stimulated for cues to initiate heroin seeking, and for the synaptic
301 potentiation that accompanies heroin seeking (*33*). Finally, not only would synaptic insulation by
302 astroglial processes be more likely to engage presynaptic autoinhibition and block access to
303 NR2B necessary for synaptic potentiation, but synaptic adjacency of astroglial processes reduces
304 the possibility of synaptic recruitment that occurs via transmitter spillover to neighboring
305 synapses. This is supported by the fact that synaptic retraction of astroglial processes after LTP
306 induction promotes synaptic crosstalk (*41*).
307
308 **Type 2 astroglia: Cue-induced increases in surface-proximal GLT-1 with no change in**
309 **synaptic proximity**

310 A portion of astroglia responded to heroin use and extinction by increasing surface-proximal GLT-
311 1 and this adaptation was transiently accentuated by heroin cues. However, the increase in GLT-
312 1 did not co-register with Synapsin I in either treatment group, indicating that surface GLT-1 in
313 extinguished and reinstated animals was distant (>250 nm) from the synapse (i.e. extrasynaptic).
314 Moreover, the increase in surface GLT-1 during seeking was not associated with either D1- or
315 D2-dendrites. We hypothesize that type 2 astroglia do not guide glutamate spillover to presynaptic
316 autoreceptors or sterically shield extrasynaptic high affinity glutamate receptors from synaptic
317 glutamate, as discussed above for type 1 astroglia. However, diffusion of glutamate to more distal
318 sites would be reduced by increased extrasynaptic GLT-1 (*22*). Thus, the primary impact of type
319 2 astroglia may be to deny access of synaptic glutamate to neighboring synapses thereby
320 preventing synaptic recruitment. Notably, by inhibiting glutamate access to neuronal nitric oxide
321 synthase (nNOS) interneurons where mGluR5 stimulation promotes NO-induced synaptic
322 potentiation in D1-MSNs (*42*) or reducing NR2B stimulation on adjacent synapses (*33*),
323 extrasynaptic GLT-1 could dampen cued heroin seeking and the associated synaptic potentiation
324 (*43*).
325
326 While some studies demonstrate that the bulk of GLT-1 is on the astroglial surface (*31*), there are
327 notable differences between hippocampal or cultured astrocytes and striatal astrocytes studied
328 here (*44*). Our data indicate that a proportion of NAcore astroglia express relatively high levels of
329 surface-proximal GLT-1 and that these cells target their GLT-1 extrasynaptically during relapse.
330 Heterogeneity in GLT-1 expression by NAcore astroglia is depicted in Fig. S5 and is further
331 supported by the non-Gaussian distribution of GLT-1 expression quantified on astrocytes from
332 yoked saline control animals (Fig. 2B-C). Notably, type 2 astrocytes that exhibit high levels of
333 surface-GLT-1, but low synaptic proximity were not abundant in control saline or sucrose groups,
334 but emerged after extinction from heroin self-administration. Whether this adaptation was a
335 consequence of heroin, heroin withdrawal or extinction training during withdrawal is a remaining

336 question. However, it is noteworthy that extinction from sucrose self-administration did not
337 increase the presence of type 2 astroglia, indicating that extinction train per se is not sufficient to
338 produce this adaptation.
339
340 That $G_q$ signaling did not stimulate plasticity in type 2 astroglia raises the possibility that levels
341 and/or patterns of $G_q$-coupled receptor expression may distinguish type 1 from type 2 astroglia.
342 In keeping with this hypothesis, astroglial mGluR5 signaling stimulates peripheral process motility
343 (*15*) and down-regulates GLT-1 (*45*). Such a dual role for mGluR5 signaling would contribute to
344 the relatively low levels of surface GLT-1 in type 1 astroglia. Alternatively, stimulation of astroglial
345 mGluR3 has been linked to increased GLT-1 expression (*45*), raising the possibility that cue-
346 induced glutamate spillover may act via receptors differentially expressed on type 1 and 2
347 astroglia.
348
349 **Astroglial adaptations are differentially regulated around D1- and D2-MSNs**
350 D1- and D2-MSNs together constitute 90-95% of all NAcore neurons (*9*) and different astroglial
351 subpopulations modulate synaptic transmission selectively on one or the other neuronal subtype
352 (*19, 27*). Consistent with astroglia associating selectively with one or the other, but not both types
353 of MSN, astroglia were more proximal to D2- than D1-MSN dendrites in control animals. The
354 finding that astroglia increased their proximity to D1-MSN synapses after extinction training, but
355 retracted from D2-MSNs, is consistent with the fact that synaptic adaptations associated with
356 extinction from addictive drug self-administration are found primarily on D2-MSNs, while
357 adaptations produced by drug cues are predominant on D1-MSNs (*26, 46-48*). We predict that
358 astrocyte insulation of D1-MSNs after extinction training would reduce synaptic glutamate
359 spillover, extrasynaptic receptor simulation and ultimately synaptic potentiation of D1-synapses
360 during extinction, relative to D2-MSNs (*26, 48*). Moreover since astrocytes can trigger long-term
361 depression at D1-MSN synapses via mGluR5-dependent ATP/adenosine signaling in the dorsal

362 striatum (*19*), an increase in astrocyte association with D1-MSN synapses after heroin extinction
363 may serve to suppress synaptic activity at D1-neurons and to thereby dampen seeking.
364 Conversely, retraction of astrocytes from D2-MSNs after extinction training is expected to be
365 permissive of synaptic potentiation at D2-MSN synapses via glutamate spillover. A number of
366 studies are consistent with this interpretation, including: extinction from cocaine is associated
367 with synaptic potentiation at D2- but not D1-MSN excitatory synapses (*26*), matrix
368 metalloprotease-2 (MMP-2) activity is elevated in heroin extinguished rats only around NAcore
369 D2-MSN dendritic spines (*46*), and chemogenetically activating D2-MSNs suppresses drug
370 seeking (*10*).
371
372 That astrocyte processes surrounding D1-MSNs retracted during heroin-associated cue exposure
373 is consistent with permitting glutamate spillover and potentiating synaptic activity at D1-synapses,
374 as has been reported for both glutamate transmission and spine morphology (i.e. increased spine
375 head diameter and/or spine density) during cued drug seeking (*26, 47*). Since astrocytes dampen
376 synaptic potentiation via a number of mechanisms (*20, 40*), we hypothesize that increased
377 astrocyte insulation of D2-MSNs during cue-reinstated heroin seeking may reduce D2-MSN
378 potentiation relative to the extinguished context (*26, 41*). However, a number of reports indicate
379 that subpopulations of D2-MSNs in the striatum promote, rather than inhibit motivated behaviors
380 (*49-52*). Moreover, cortical inputs linked to cued reinstatement (*53*) innervate D2-MSNs to a
381 greater extent than D1-MSNs (*54*). For this reason, the possibility remains that increased
382 astrocyte insulation on D2-MSNs during cued reinstatement may serve a compensatory function
383 by triggering autoinhibition at cortical terminals that release glutamate in response to heroin-
384 conditioned cues. In this way, astrocyte insertion onto D2-MSNs may contribute to reducing
385 glutamate spillover onto both D2-MSNs, as well as D1-MSNs, that receive less direct innervation
386 by prefrontal terminals (*54*), but are significantly engaged during cued reinstatement to drive
387 seeking (*26*).

388

389 Akin to astroglial synaptic proximity, surface GLT-1 was lower adjacent to D1- compared with D2-
390 dendrites in saline rats. However, the overall dynamic in type 2 astroglia after extinction and cued
391 reinstatement observed in wild-type rats was not recapitulated in either D1- or D2-MSNs. This
392 could result from increased surface-GLT-1 in type 2 astroglia targeting non-dendritic portions of
393 D1- and D2-MSNs or targeting subpopulations of interneurons or other astroglia that were not
394 labeled in D1- and D2-Cre rats.

395

396 **Astroglial plasticity during sucrose versus heroin seeking**

397 Sucrose training did not down-regulate GLT-1 nor did it produce changes in synaptic proximity by
398 NAcore astroglia in any treatment group (*6, 55*). The relative lack of changes in astroglial
399 subpopulation plasticity after extinction from sucrose self-administration and cued reinstatement
400 supports the likelihood that changes in the proportion of astroglial subtypes in heroin trained rats
401 may be selective for addictive drugs and not natural rewards. Interestingly, although we found no
402 change in co-registration of GLT-1 with Synapsin I in sucrose-trained or reinstated animals, we
403 observed reduced surface expression of GLT-1 after extinction from sucrose self-administration
404 compared to yoked controls, further reducing the proportion of type 2 astroglia in this group. As
405 described above, we predict that synaptic adjacency of astroglial processes deficient in GLT-1
406 would favor presynaptic autoinhibition upon transmitter release (*36*). Consistent with this
407 hypothesis, sucrose-trained rats exhibit more rapid extinction training and less perseverative
408 reward seeking compared with drug-trained animals (*56, 57*), even though during sucrose self-
409 administration produced more active lever presses than heroin self-administration.

410

411 **Implications for treating substance use disorders**

412 We found that astroglia in the NAcore exhibited two forms of plasticity capable of shaping cue-
413 induced heroin seeking that occurred in separate astroglial subpopulations and were regulated

414 by distinct signaling mechanisms. Moreover, both increasing astroglial proximity to synapses and
415 glutamate transport involve proteins selectively expressed in astroglia (ezrin and GLT-1,
416 respectively). The fact that none of the plasticity produced in NAcore astroglia from heroin-trained
417 rats was recapitulated by sucrose training supports the potential that selective interventions in
418 one or the other astroglial dynamic may be therapeutically beneficial in treating substance use
419 disorder. Indeed, pharmacological treatments such as ceftriaxone and N-acetylcysteine that
420 elevate GLT-1 are effective at reducing drug seeking in rodent models of relapse (*58-60*) and
421 reducing drug cue reactivity in humans (*61*). Unfortunately, while reducing craving induced by
422 drug cues, N-acetylcysteine has proven only marginally effective at reducing relapse in human
423 trials (*62-64*). Given that astroglia engage two distinct processes for dampening cue-induced drug
424 seeking, and that increasing synaptic adjacency of astroglial processes in the absence of GLT-1
425 reduces the intensity of relapse, the poor efficacy in relapse prevention by drugs restoring GLT-1
426 might be improved in combination with drugs that promote synaptic adjacency of NAcore
427 astroglia.

428 **MATERIALS AND METHODS**

429 Experimental design and statistical analyses

430 All experiments included groups with ≥ 4 animals each to minimize variability due to animal
431 behavior. All immunohistochemical and imaging procedures using tissue from experimental
432 groups (i.e. extinguished, reinstated) were conducted alongside yoked controls to minimize the
433 impact of experimental variability on independent groups. Data were analyzed using GraphPad
434 Prism and a D'Agostino-Pearson normality test followed by Kruskal-Wallis or Mann-Whitney tests
435 when one or more groups were not normally distributed. For non-Gaussian datasets, including all
436 astroglial measures, no outliers were removed and data were plotted with values for each cell
437 depicted individually and a bar to indicate the group median. Dunn's test was used for post hoc
438 comparisons. Normally distributed datasets, including all behavioral data, were analyzed using a
439 1- or 2-way ANOVA or a Student's t-test. To more clearly show median differences in Fig. 6, y-
440 axes show data ≤2.0% co-registration. Full datasets for Fig. 6 are reported in Table
441 S1. Cumulative distributions were analyzed using Kolmogorov-Smirnov or $Chi^2$ and a Bonferroni
442 correction was applied for multiple comparisons. In all cases, $p < 0.05$ was considered significant.

443

444 Self-administration

445 Experimental procedures involving animals were conducted in accordance with guidelines
446 established by the Institutional Animal Care and Use Committee at the Medical University of South
447 Carolina. Operant training was conducted as previously described (*6*). Briefly, male Sprague
448 Dawley rats or male and female Long Evans rats (200-250g) were anesthetized with i.m. ketamine
449 (100 mg/kg) and xylazine (7 mg/kg) and fitted with intrajugular catheters. Rats were trained to
450 self-administer heroin during 3h sessions for 10d and presses on an active lever were paired with
451 light and tone cues and i.v. heroin infusion. Animals trained to self-administer sucrose did not
452 undergo catheter implantation and received sucrose (45 mg, Bio-Serv) in place of heroin along
453 with cues during self-administration (2h/d). Yoked controls were played cues when a paired rat

454 received heroin or sucrose. Rats yoked to heroin self-administering animals also received i.v.
455 saline infusions. After self-administration, animals underwent 10-12d of extinction training (3h/d
456 after heroin, 2h/d after sucrose) where active lever presses yielded no reward or cues.
457 Extinguished rats and yoked controls were sacrificed 24h after the final extinction session.
458 Reinstated animals were placed in the operant chamber for 15 or 120m and cues were restored
459 to the active lever, but no reward was delivered.
460
461 Viral labeling
462 After catheter implantation or 5d before starting sucrose self-administration, rats received
463 microinjections (1 μL/hemisphere, 0.15 μL/minute, 5 min diffusion) of a virus driving expression
464 of membrane-targeted mCherry under control of the GFAP promoter (AAV5/GFAP-hM3dq-
465 mCherry, University of Zurich, Figs. 1-5 or AAV5/GfaABC1D-Lck-GFP, Addgene, Fig. 6) in the
466 NAcore (+1.5mm AP, ±1.8mm ML, -7.0mm DV). To label D1- and D2-MSN dendrites, AAV1/CAG-
467 Flex-Ruby2sm-Flag (Addgene) was co-injected in D1- or D2-Cre rats along with virus used to
468 label astroglia. Virus incubation occurred over the course of operant training.
469
470 Confocal imaging and image analysis
471 Animals were anesthetized with an overdose of pentobarbital (20 mg i.v. or 100 mg i.p.) and
472 perfused transcardially with 4% PFA. Brains were incubated overnight in 4% PFA and sliced at
473 100 μm using a vibratome (Thermo Fisher). Slices containing the NAcore were permeabilized in
474 PBS with 2% Triton X-100 for 1h at room temperature. Non-specific epitope binding was blocked
475 by incubation in PBS with 0.2% Triton X-100 (PBST) and 2% NGS (block) for 1h at room
476 temperature before incubation in primary antibodies (rabbit anti-Synapsin I, ab64581, Abcam;
477 guinea pig anti-GLT-1, ab1783, EMD Millipore; mouse anti-FLAG, F1804, Sigma-Aldrich) at
478 1:1000 in block for 48h. After washing in PBST, tissue was incubated overnight in biotinylated
479 anti-guinea pig antibody (BA-7000, Vector Laboratories) in PBST and then overnight in

480 fluorescently-labeled antibodies (Thermo Fisher) in PBST after washing. Tissue was mounted
481 onto glass slides before imaging with a Leica SP5 laser scanning confocal microscope. All images
482 were acquired at 63x using an oil immersion objective lens, 1024 x 1024 frame size, 12-bit
483 resolution, 4-frame averaging and a 1-μm step size. Z-stacks were iteratively deconvolved 10
484 times (Autoquant) and digital analysis of astroglial mCherry or GFP signal intensity relative to
485 background was used to generate a digital model of each astrocyte (Bitplane Imaris). Rendered
486 astrocytes were used to mask Synapsin I and GLT-1 or Flag signal that was not co-registered
487 with the astroglial volume. Co-registration (astrocyte with Synapsin I, astrocyte with GLT-1,
488 astrocyte with GLT-1 and Synapsin I, astrocyte with Flag and Synapsin I, astrocyte with Flag and
489 GLT-1) was determined based on thresholded signal intensity in each channel. Voxels containing
490 signal intensity greater than noise in each channel were determined empirically using the
491 colocalization module and were used to build a colocalization channel. The surface module was
492 then used to determine the net volume of co-registered signal. All measures, with the exception
493 of surface-proximal GLT-1 (Fig. 2C-D, Fig. S1C-D), and synaptic and extrasynaptic GLT-1 (Fig.
494 2F-G, Fig. SF-G), were normalized to the volume of the astrocyte from which they were generated
495 to control for changes in astroglial volume (*6*) and to permit inclusion of astrocytes that were
496 cropped along the z-axis during imaging. Surface-proximal GLT-1 was determined by excluding
497 co-registered signal that was within the astrocyte volume, but >250nm (a distance roughly
498 equivalent to the lateral resolution of the microscope used for imaging) from the membrane and
499 was normalized to total GLT-1 from the astroglial volume. Synaptic and extrasynaptic GLT-1
500 values were normalized to surface GLT-1 levels from each astrocyte. In all case, imaging and
501 analyses were conducted blind to animal treatment.
502
503 <u>Principal component analysis and hierarchical clustering</u>
504 To identify astroglial clusters according to synaptic co-registration as percent of astrocyte volume,
505 and surface-proximal GLT-1 expression as percent of total GLT-1 expression, hierarchical

506 clustering on principal components was computed using R software (R Core Team, 2020)
507 according to (*21*). Principal components were computed along the two specified dimensions and
508 Ward's criterion was applied on selected principal components (Fig. S3). The output dendogram
509 was separated along 2 main breaks in the data to form 3 clusters (Fig. S3).
510
511 $G_q$-DREADD stimulation
512 AAV5/GFAP-hM3dq-mCherry (University of Zurich) was diluted 1:10 in sterile saline and delivered
513 to the NAcore (+1.5mm AP, ±1.8mm ML, -7.0mm DV) while rats were under anesthesia for jugular
514 catheter placement as in (*40*). This dilution was used, because it produced sufficient astroglial
515 labeling with little off-target labeling in neurons (Fig. 4A). Viral incubation occurred over the course
516 of operant training. Twenty-four hours after the last extinction session, rats received CNO (3mg/kg
517 i.p., Abcam) or vehicle (5% DMSO in sterile saline) 30 minutes prior to perfusion and brain
518 extraction.
519
520 Vivo-morpholino knockdown
521 After catheter placement, animals were fitted with bilateral cannulae above the NAcore (+1.5mm
522 AP, ±1.8mm ML, -5.5mm DV). Starting on day 6 of extinction training, animals received infusions
523 of an ezrin antisense vivo-morpholino oligo (*6*), a GLT-1 antisense vivo-morpholino oligo (*24*), or
524 a control oligo (Gene Tools, Inc.) 1.5mm beyond the base of the guide cannulae (50μmol/L,
525 1μL/hemisphere, 0.5μL/min) for 3 consecutive days. After 3 additional days of extinction training,
526 rats underwent cued reinstatement for 15-min prior to sacrifice.

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**Competing interests:** The authors declare no competing financial interests.

**Data and materials availability:** All data are available within the main text or the supplementary material.

## FIGURES AND LEGENDS

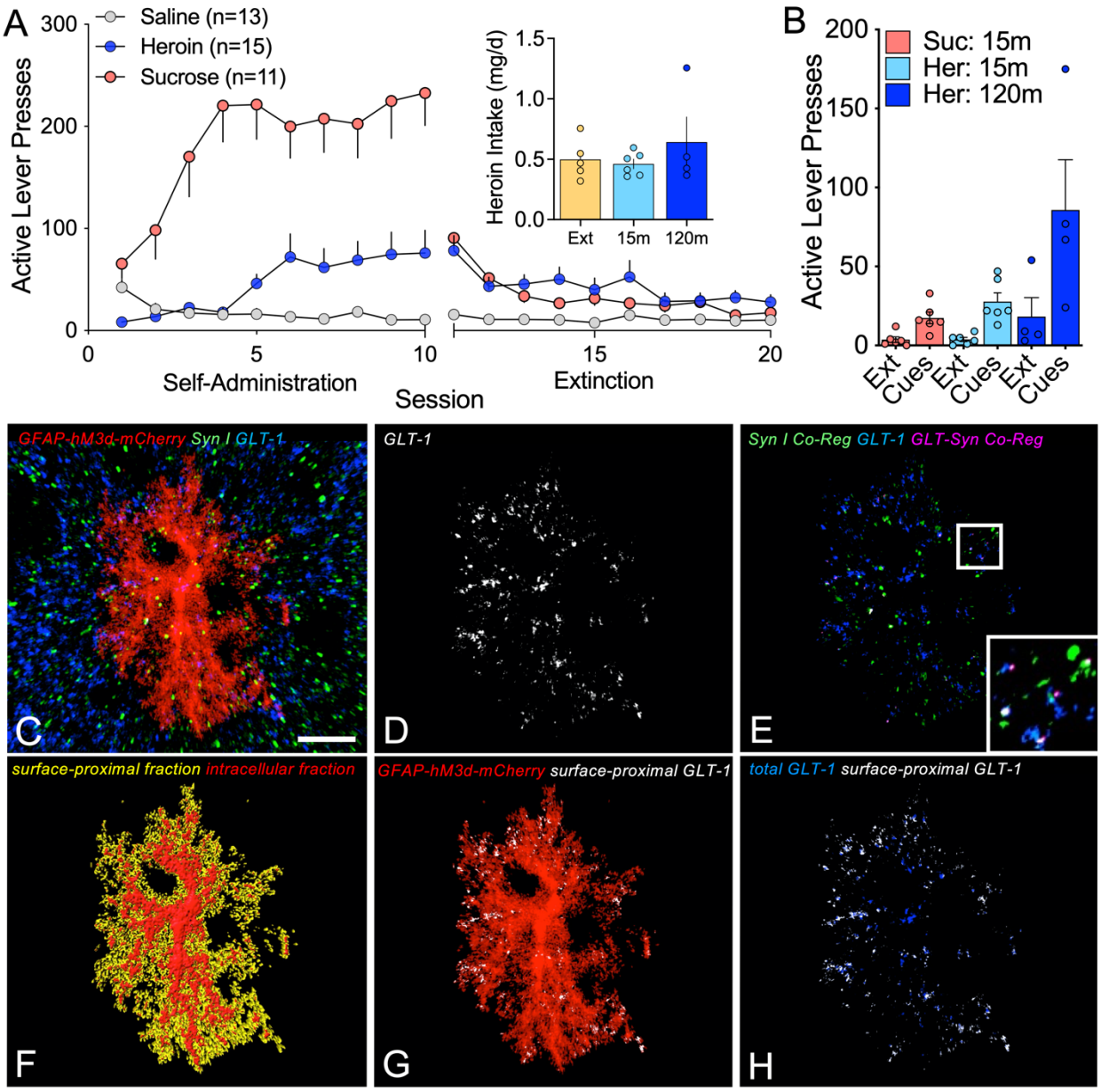



**Fig. 1. Workflow used for confocal analysis of astroglial morphology and surface-proximal GLT-1.** (**A**) Rats were trained to self-administer heroin or sucrose over 10 consecutive days of operant training and reward delivery was paired with light and tone cues. During extinction training, heroin or sucrose and cues were not delivered in response to active lever pressing and operant responding gradually decreased. Heroin- and sucrose-trained rats differed in active lever pressing during self-administration (2-way ANOVA, $F_{1,124}$=33.44, p<0.0001). Inset shows that

706 groups of heroin-trained animals took similar amounts of heroin (1-way ANOVA $F_{2,12}$=0.9187,
707 p=0.4984). (**B**) 24h after the last extinction session, reinstated animals were placed in the operant
708 chamber and cues were restored to the active lever for 15- or 120-min to reinstate seeking (2-
709 way ANOVA Session $F_{1,26}$=16.07, p=0.0005). (**C**) Z-series depicting an NAcore astrocyte
710 transfected with AAV5/GFAP-hM3d-mCherry (red) and immuno-labeled for Synapsin I (green)
711 and GLT-1 (blue). (**D**) GLT-1 immunoreactivity co-registered with mCherry in (**C**) is shown in
712 white. (**E**) Co-registration of GLT-1 (blue) with Synapsin I (green) from the region occupied by the
713 astrocyte in (**C**) is shown in pink. (**F**) Digital rendering of the astroglial surface (yellow) was used
714 to identify GLT-1 signal within 250 nm of the cell membrane (**G**-**H**, white) relative to total GLT-1
715 from the same astrocyte (**H**, blue). Bar in (**C**)= 10 µm. In (**A**, inset), Ext, extinguished; 15m, 15-
716 min cued reinstatement; 120m, 120-min cued reinstatement. In (**B**), Suc, sucrose; Her, heroin;
717 Ext, extinction.

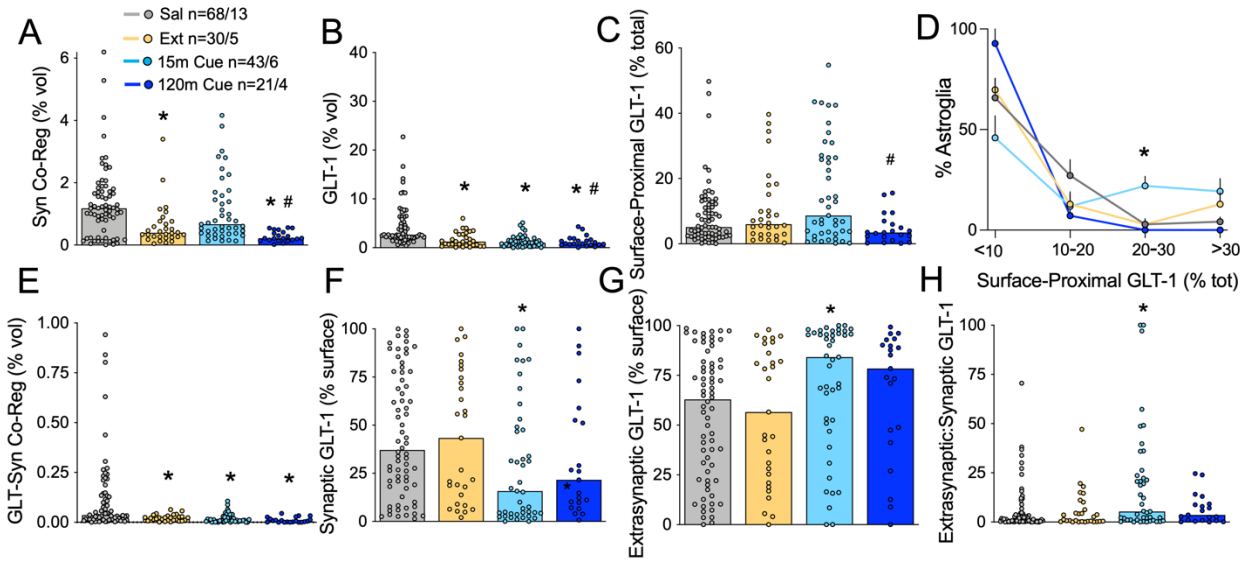



**Fig. 2. Extrasynaptic GLT-1 was transiently elevated during heroin seeking.** We previously found that co-registration of labeled NAcore astroglia with Synapsin I is reduced during extinction from heroin (**A**, Kruskal-Wallis=30.17, p<0.0001) and that 15-min of cued heroin seeking restores synaptic insulation by astrocytes. (**B**) Withdrawal from heroin self-administration produced a downregulation of GLT-1 on NAcore astroglia, whether or not rats were reinstated (Kruskal-Wallis=51.77, p<0.0001). (**C**) Surface-proximal GLT-1, shown here as percent of total GLT-1 from each astrocyte, was increased during active seeking (15m Cue) when compared to a timepoint at which cues no longer evoke seeking (i.e. cue extinction; 120m Cue, Kruskal-Wallis=9.848, 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 (**D**, 2-way ANOVA, $F_{6,48}$=2.904, p<0.05). Co-registration of GLT-1 with the presynaptic marker Synapsin I was found to be reduced in heroin-trained rats, and was not restored during cued reinstatement (**E**, Kruskal-Wallis=35.48, p<0.0001). When synaptic and extrasynaptic fractions of surface GLT-1 were analyzed separately, we found that 15-min of cued heroin seeking decreased synaptic GLT-1 (**F**, Kruskal-Wallis=9.493, p<0.05) and increased extrasynaptic GLT-1 (**G**, Kruskal-Wallis=9.493, p<0.05). The ratio of extrasynaptic and synaptic GLT-1 illustrates the robust increase in extrasynaptic GLT-1 during cued heroin seeking (**H**, Kruskal-Wallis=9.476). N shown in (**A**) as cells/animals. *p<0.05

compared to yoked control, #p<0.05 compared to 15-min reinstated using Dunn's test. Sal, yoked saline; Ext, extinguished; 15m Cue, 15-min cued reinstatement; 120m Cue, 120-min cued reinstatement.

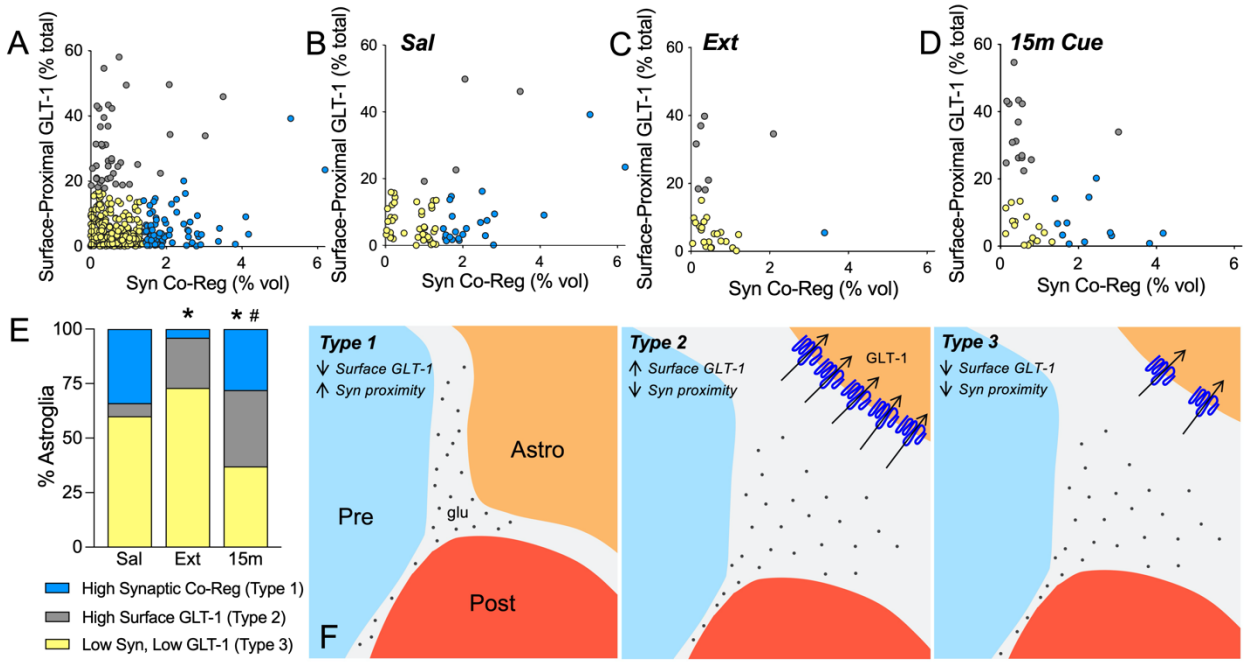



**Fig. 3. Cue-induced increases in astrocyte motility and GLT-1 surface expression occurred in different astroglial subpopulations.** (**A**) Principal component analysis was used to identify subpopulations of NAcore astroglia according to their synaptic adjacency and levels of surface-proximal GLT-1. Astrocytes with high synaptic adjacency (blue) or high levels of surface-proximal GLT-1 (gray) were identified as separate cell clusters. Distribution of clusters in tissue from yoked saline, extinguished or cue-reinstated rats is shown in (**B**-**D**). Astrocytes with high surface-proximal GLT-1, but low synaptic co-registration were largely absent from yoked control rats, but emerged after operant heroin training (**E**, $Chi^2$=14.10 *p=0.0018 Ext. vs. Sal). Half of astroglia in cue-reinstated rats exhibited one or the other type of transient plasticity (**E**, $Chi^2$=15.97, *p=0.0006 vs. Sal; $Chi^2$=11.20, #p=0.0074 vs. Ext). (**F**) Schematic illustrating 3 astroglial subtypes identified by PCA. Type 1 astroglia expressed low levels of GLT-1, but exhibited high measures of synaptic-adjacency (**F**, left panel). Type 2 astroglia expressed high levels of largely extrasynaptic GLT-1 (blue) and were observed heroin training, but were not abundant in control animals (**F**, middle panel). Type 3 astroglia were predominant in control animals and had low-to-moderate GLT-1 expression and synaptic adjacency (**F**, right panel).

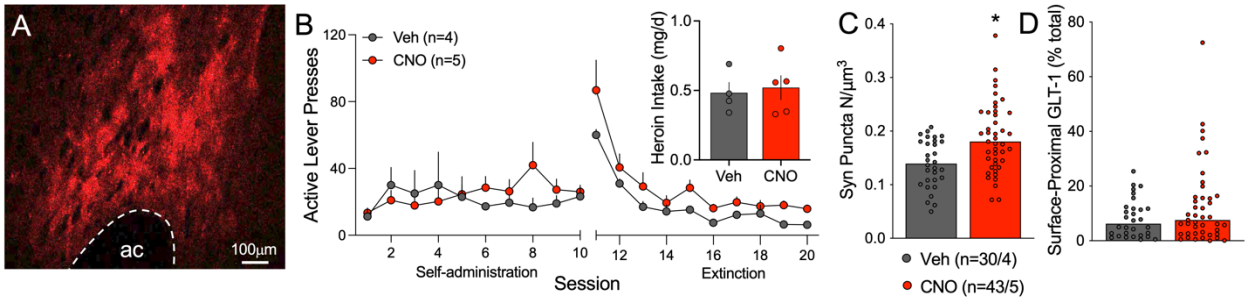



**Fig. 4. $G_q$-signaling increased synaptic adjacency in NAcore astroglia from heroin-trained rats.** After viral delivery of $G_q$-DREADD in NAcore astroglia (**A**, red), rats were trained to self-administer heroin before undergoing extinction training (**B**). The following day, rats received i.p. injections of vehicle or CNO, but did not undergo cued reinstatement. Heroin intake did not differ between vehicle- or CNO-treated rats (**B**, inset, $t_7$=0.173, p=0.7602). (**C**) $G_q$ signaling increased co-registration of NAcore astroglia with near-adjacent immunolabeled Synapsin I puncta (Mann-Whitney U=363, p=0.0013), but did not impact levels of surface-proximal GLT-1 (**D**, Mann-Whitney U=568, p=0.3934). In (**A**), ac, anterior commissure. In (**B**-**C**), Veh, Vehicle. In (**C**), N shown as cells/animals.

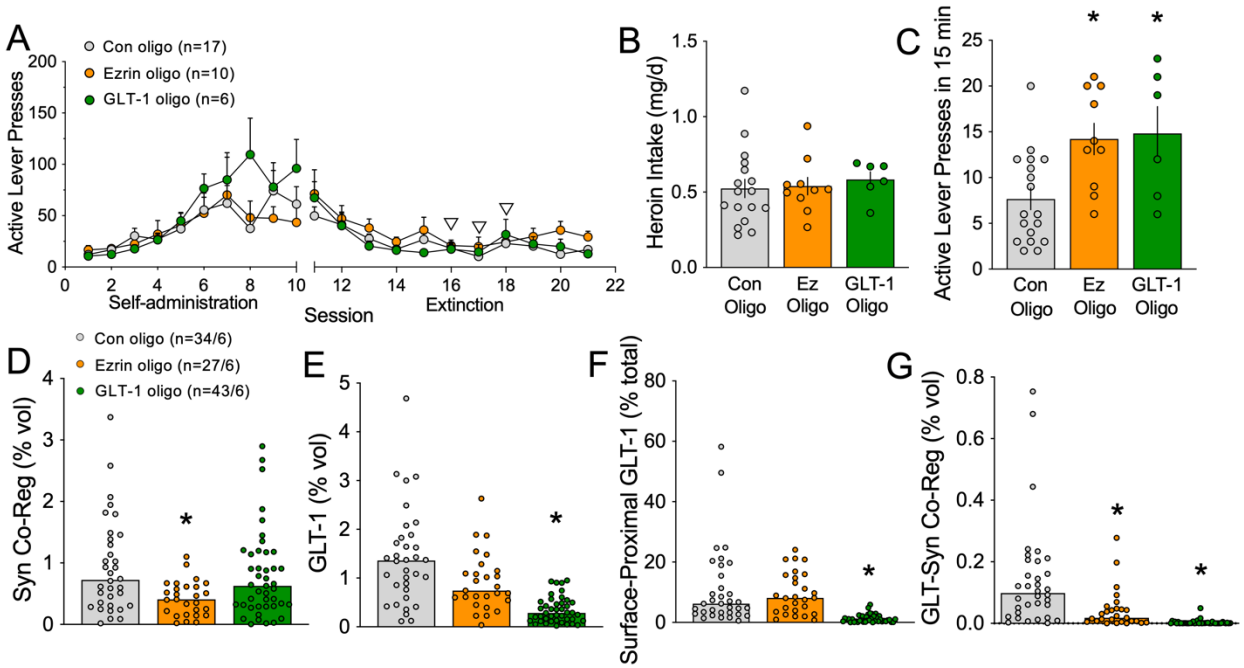



**Fig. 5. Impairing cue-induced astrocyte motility or surface GLT-1 elevated heroin seeking.** (**A**) Rats were trained to self-administer heroin over 10 consecutive days. Starting on day 6 of extinction training for 3 consecutive days (white arrowheads), animals received NAcore infusions of an ezrin antisense oligomer, a GLT-1 antisense oligomer or a control oligomer. Animals in each treatment group did not differ in total heroin intake during self-administration. (**B**) Rats receiving different oligo treatments did not differ in heroin intake (1-way ANOVA $F_{2,30}$=0.1739, p=0.8412). 24h after the final extinction session, animals were reinstated for 15-min by exposure to light and tone cues previously paired with heroin delivery. (**C**) Rats that underwent ezrin or GLT-1 knockdown pressed higher on the active lever during the 15-min reinstatement session (1-way ANOVA $F_{2,30}$=6.230, p=0.0055, *p=0.0058 Con vs. Ezrin Oligo, *p=0.0104 Con vs. GLT-1 Oligo using Fisher's test). (**D**) Astrocytes from rats treated with the ezrin oligo exhibited a significant reduction in synaptic co-registration, consistent with knockdown of astrocyte peripheral process motility (Kruskal-Wallis=15.85, p=0.0004, *p=0.0002 vs. Con). The GLT-1 oligo did not impact synaptic co-registration by NAcore astroglia (p=0.4676 vs. Con). (**E**) GLT-1 levels were unchanged by ezrin antisense oligo delivery (p=0.3888), but were reduced by treatment with the

781 GLT-1 oligo (Kruskal-Wallis=54.85, $p<0.0001$, *$p<0.0001$). (**F**) Likewise, surface-proximal GLT-1
782 was reduced after application of the GLT-1 oligo (Kruskal-Wallis=80.22, $p<0.0001$, *$p<0.0001$ vs.
783 Con), but not the ezrin oligo ($p>0.9999$ vs. Con). (**G**) The co-registration between GLT-1 and
784 Synapsin I on astroglia was significantly reduced after ezrin (Kruskal-Wallis=87.13, $p<0.0001$,
785 *$p=0.0147$) or GLT-1 knockdown (*$p<0.0001$). In (**A**-**D**), Con, control oligo; Ez, ezrin oligo; GLT-
786 1, GLT-1 oligo. In (**D**), N shown as cells/animals.
787

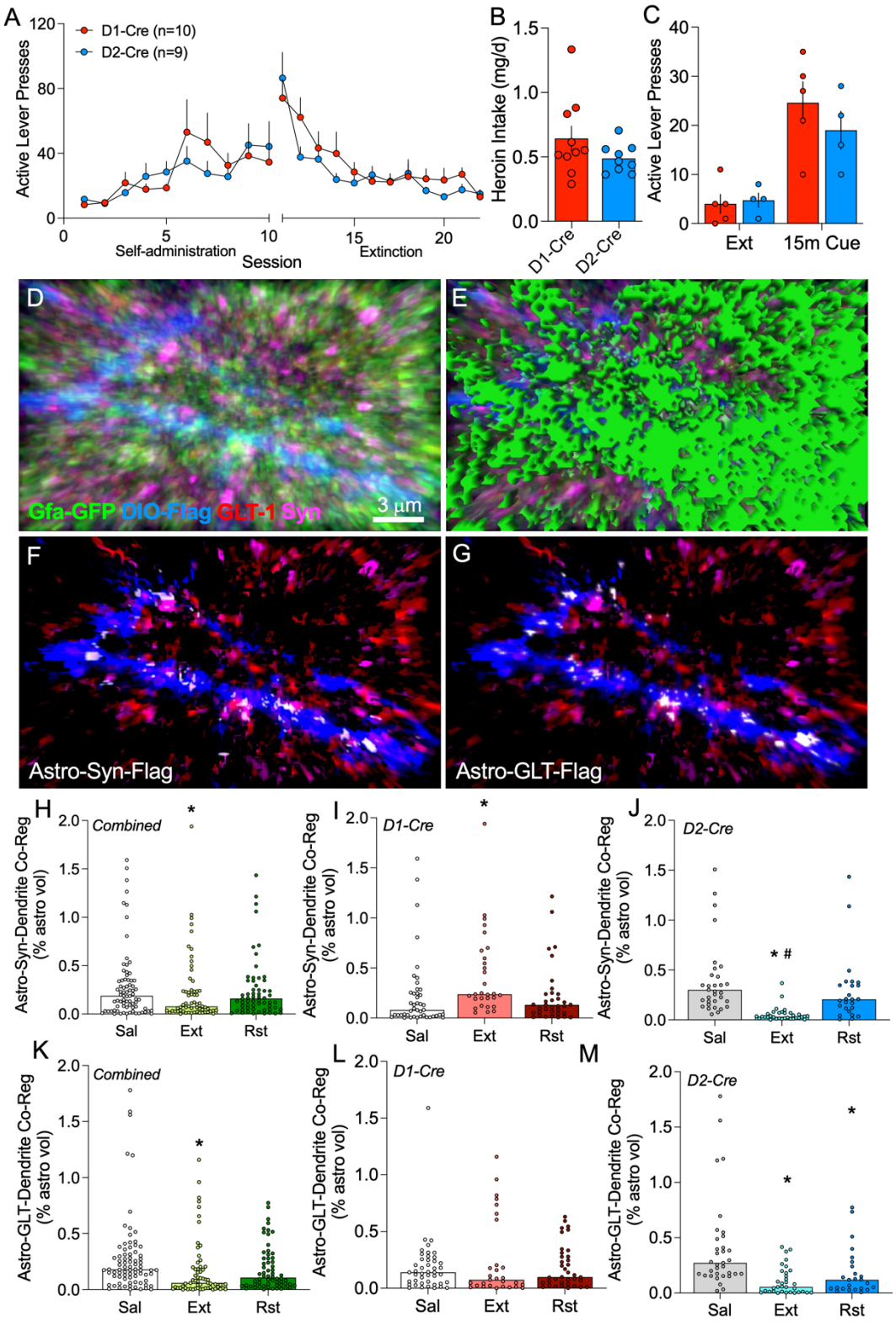
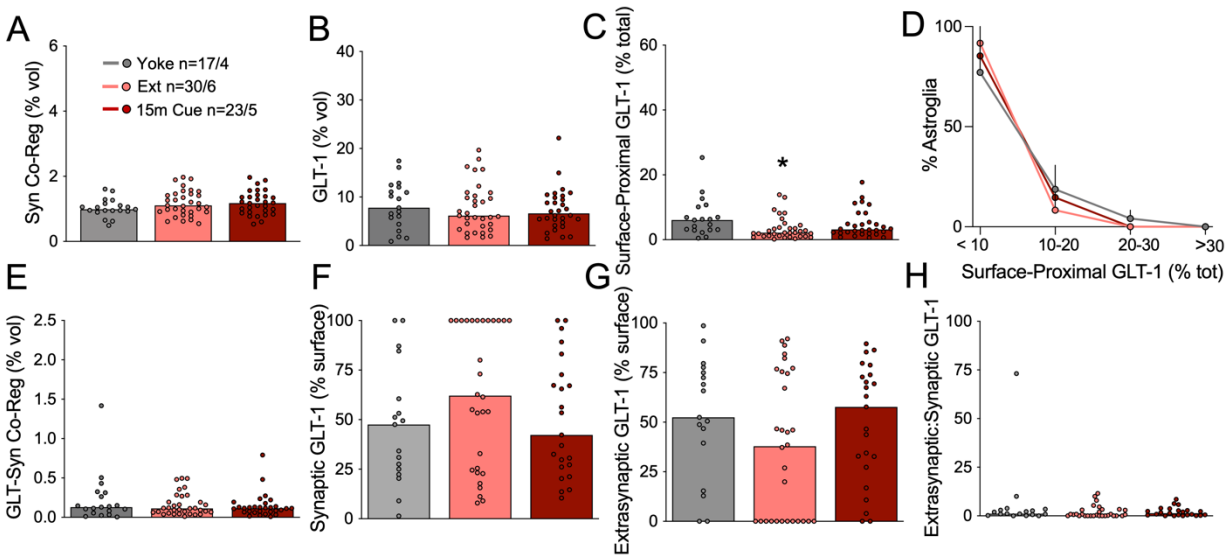



**Fig. 6. Astrocytes increased their adjacency to D1-MSN synapses but retracted from D2-MSN synapses after extinction training.** (**A**) Male and female D1- and D2-Cre rats were trained

791 to self-administer heroin over 10 days before undergoing extinction training. (**B**) Heroin intake did
792 not differ between the strains ($t_{17}$=1.449, p=0.1656). (**C**) 24h after the last extinction session, lever
793 pressing was reinstated by exposure to heroin-conditioned cues for 15-min (2-way ANOVA Time
794 $F_{1,7}$=20.36 p=0.0028, Genotype $F_{1,7}$=0.9944 p=0.3519). (**D**) NAcore tissue from these animals
795 was immunolabeled to identify D1- or D2-MSN dendrites (blue), GLT-1 (red), and Synapsin I
796 (pink). Signal associated with NAcore astroglia (green, **D**-**E**) was isolated to quantify the degree
797 of triple co-registration between astroglia and D1- or D2-MSN synapses (**F**, white), or astroglial
798 GLT-1 and D1- or D2-MSN dendrites (**G**, white). Synaptic association by astroglia was reduced
799 by extinction training after heroin self-administration when quantified in a cell-nonspecific manner
800 (**H**, Kruskal-Wallis=4.617 p=0.0994, *p=0.0380 vs. Sal). When analyzed separately, astrocyte
801 association with D1-MSN synapses was low at baseline and was increased by extinction training
802 (**I**, Kruskal-Wallis=12.35 p=0.0021, *p=0.0014 vs. Sal). Instead, D2-MSN synapses had high
803 astrocyte insulation at baseline (p<0.0001 vs. D1-Cre, Sal using Dunn's test) that was reduced
804 by extinction training and recovered during cued reinstatement (**J**, Kruskal-Wallis=45.84
805 p<0.0001, *p<0.0001 vs. Sal, #p<0.0001 vs. Rst). Surface-proximal, dendrite-associated GLT-1
806 was decreased overall after extinction training in NAcore astrocytes, and was restored during 15-
807 min cued reinstatement (**K**, Kruskal-Wallis=15.23 p=0.0005, *p<0.05 vs. Sal). However, the
808 increase in surface-proximal GLT-1 during cued reinstatement was not associated with dendrites
809 from D1- (**L**, Kruskal-Wallis=0.7498 p=0.6874), or D2-MSNs (**M**, Kruskal-Wallis=25.49 p<0.0001,
810 *p<0.05 vs. Sal). Like astrocyte-synaptic adjacency, GLT-1 co-registration with D2-dendrites was
811 higher at baseline compared with D1-dendrites (p=0.0043 using Dunn's test). In (**I**-**J**, **L**-**M**), N=25-
812 44/4-5 cells/animals per group and full data spread is reported in Table S2. Sal, yoked saline; Ext,
813 extinguished; Rst, 15-min cued reinstatement.

1 **SUPPLEMENTARY MATERIAL**




2

3 **Fig. S1. Astrocyte motility and GLT-1 expression were unchanged during reinstated**
4 **sucrose seeking.** Co-registration of labeled NAcore astroglia with Synapsin I was not changed
5 after extinction of sucrose self-administration or during 15-min of cue-reinstated sucrose seeking
6 (**A**, Kruskal-Wallis=2.153, p=0.341). (**B**) GLT-1 expression was unchanged after operant training
7 with sucrose (Kruskal-Wallis=0.7336, p=0.693). (**C**) Surface-proximal GLT-1, shown as percent
8 of total GLT-1 from each astrocyte, was reduced after extinction from sucrose self-administration
9 (Kruskal-Wallis=8.056, p<0.05). (**D**) Cued reinstatement of sucrose seeking did not change the
10 proportion of astroglia expressing high levels of surface-proximal GLT-1 (2-way ANOVA,
11 $F_{6,36}$=0.469, p=0.827). (**E**) Co-registration of GLT-1 with the presynaptic marker Synapsin I was
12 not changed by sucrose training (Kruskal-Wallis=0.3724, p=0.830). When synaptic (**F**, Kruskal-
13 Wallis=2.950) and extrasynaptic (**G**, Kruskal-Wallis=2.950) fractions of surface GLT-1 were
14 analyzed separately, we found no change in sucrose-trained rats compared with yoked controls.
15 (**H**) shows ratio of extrasynaptic:synaptic GLT-1 (Kruskal-Wallis=2.950). N shown in (**A**) as
16 cells/animals. *p<0.05 compared to yoked control using Dunn's test. Yoked, yoked cues; Ext,
17 extinguished; 15m Cue, 15-min cued reinstatement.

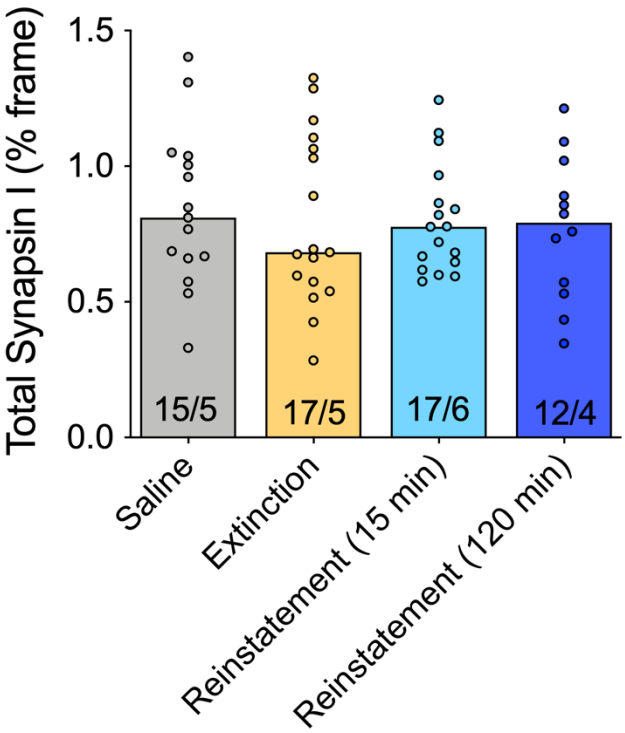



**Fig. S2. Heroin self-administration and extinction training did not impact Synapsin I immunoreactivity in the NAcore.** Kruskal-Wallis=0.4046, p=0.9393. N shown in bars as frames/animal.

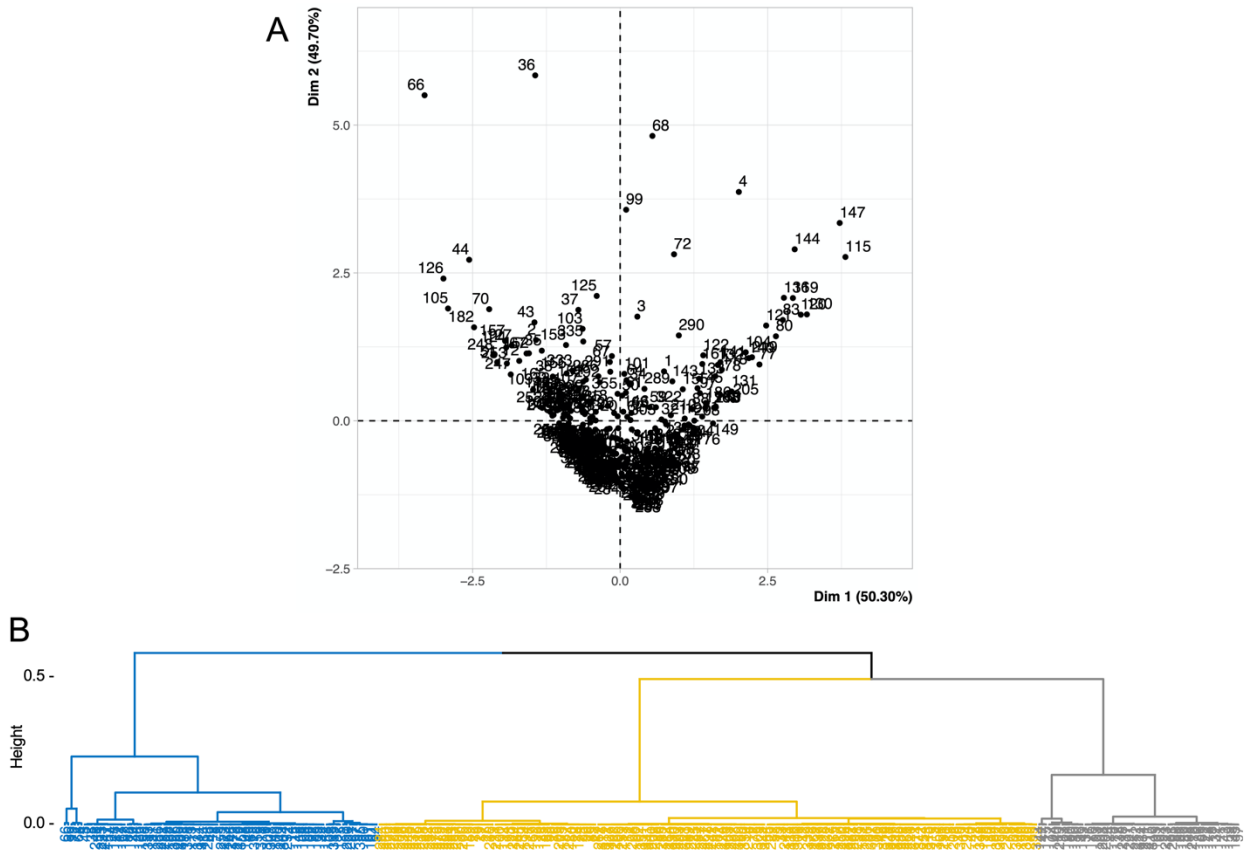



**Fig. S3.** (**A**) Principal component analysis showing individual data points organized according to dimensions 1 and 2, which account for 50.3% and 49.7% of the data variance, respectively. (**B**) Dendrogram shows three clusters representing type 1 (blue), type 2 (gray), and type 3 (yellow) astroglia.

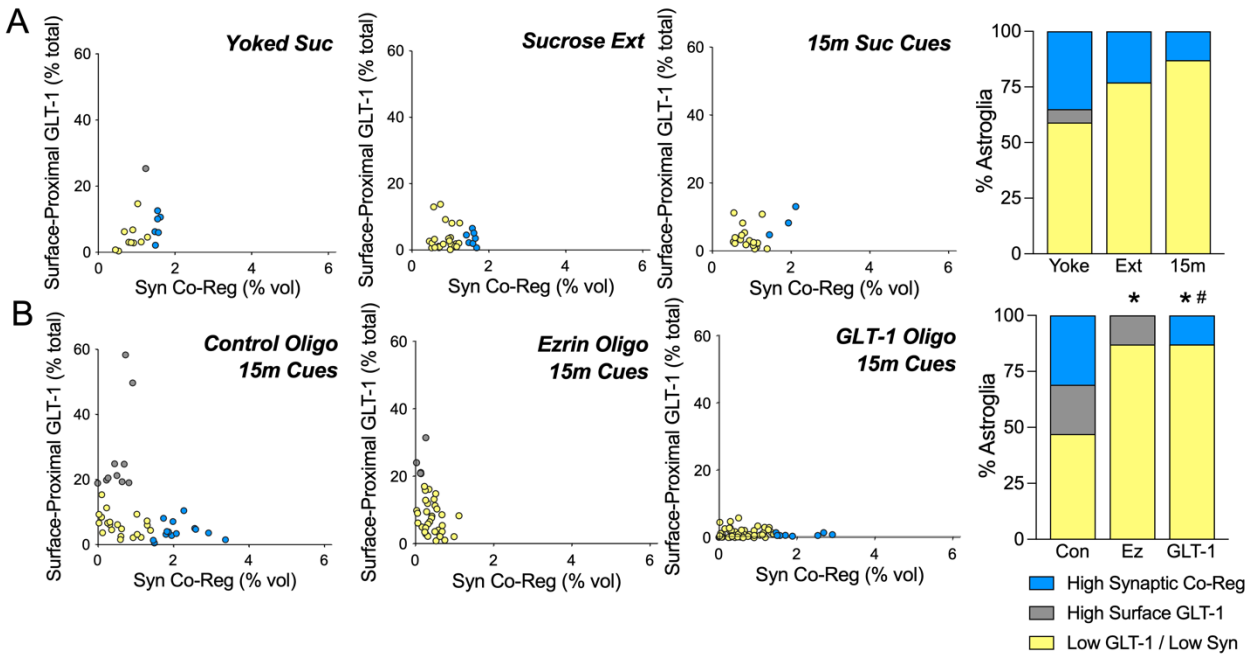



**Fig. S4. Astroglial subpopulations are not altered by operant training with sucrose, but are abolished by ezrin or GLT-1 oligo treatment.** (**A**) Astroglial clusters are not altered by operant training with sucrose ($Chi^2$=2.818 p=0.2444 Yoke vs. Ext, $Chi^2$=4.535 p=0.1036 Yoke vs. 15m). (**B**) Compared with astroglial subpopulations during reinstatement after control oligo treatment (left), ezrin or GLT-1 oligo treatment abolished subpopulations characterized by high synaptic adjacency (blue) or high surface-proximal GLT-1 (gray), respectively ($Chi^2$=17.87 *p=0.0002 Ez vs. Con, $Chi^2$=23.60 *p<0.001 GLT-1 vs. Con, $Chi^2$=13.01 #p=0.003 GLT-1 vs. Ez). In (**A**, right panel), Yoke, yoked cues; Ext, extinguished; 15m, 15-min cued reinstatement. In (**B**, right panel), Con, control oligo; Ez, ezrin oligo; GLT-1, GLT-1 oligo.

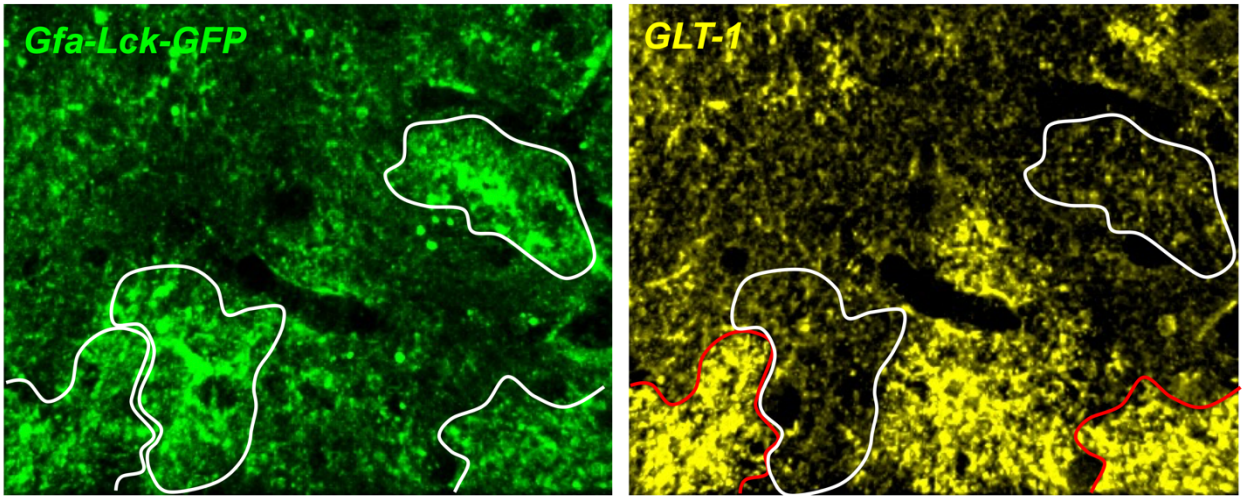



**Fig. S5.** Astroglial transduction with AAV5/GfaABC1D-Lck-GFAP labels the astroglial membrane (green). Astrocytes identified using this marker are outlined in white (left panel). (Right panel) In the same frame, immunolabeling for GLT-1 (yellow) shows astrocytes with high (red outline) and low levels of GLT-1 expression (white outline).

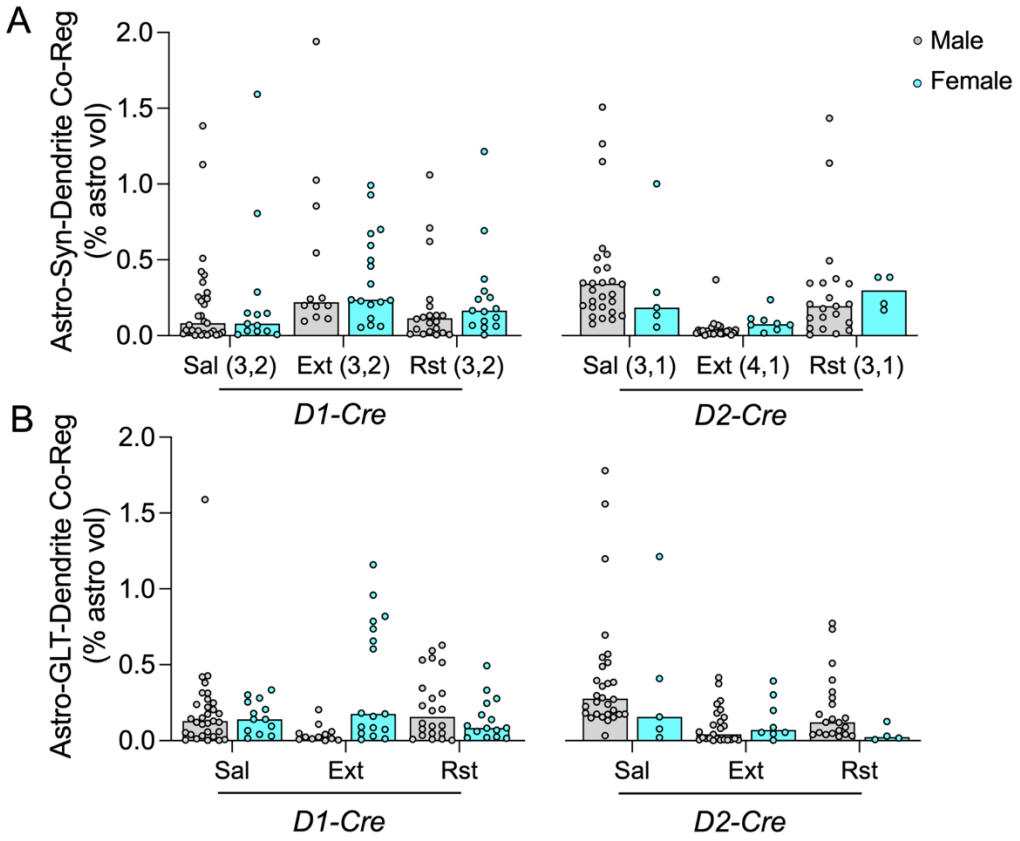



**Fig. S6. Cell-type specific dynamics in astrocyte-synapse and GLT-dendrite association are not sex-dependent.** (**A**) No sex differences were detected in D1- or D2-synaptic co-registration by NAcore astroglia after operant training with heroin (2-way ANOVA $F_{1,196}$=0.7529 p=0.3866). (**B**) Dendritic association of GLT-1 did not differ by sex (2-way ANOVA $F_{1,196}$=0.05632 p=0.8127). Sal, yoked saline; Ext, extinguished; Rst, 15-min cued reinstatement. Animal N shown below bars in (**A**) as (male, female).

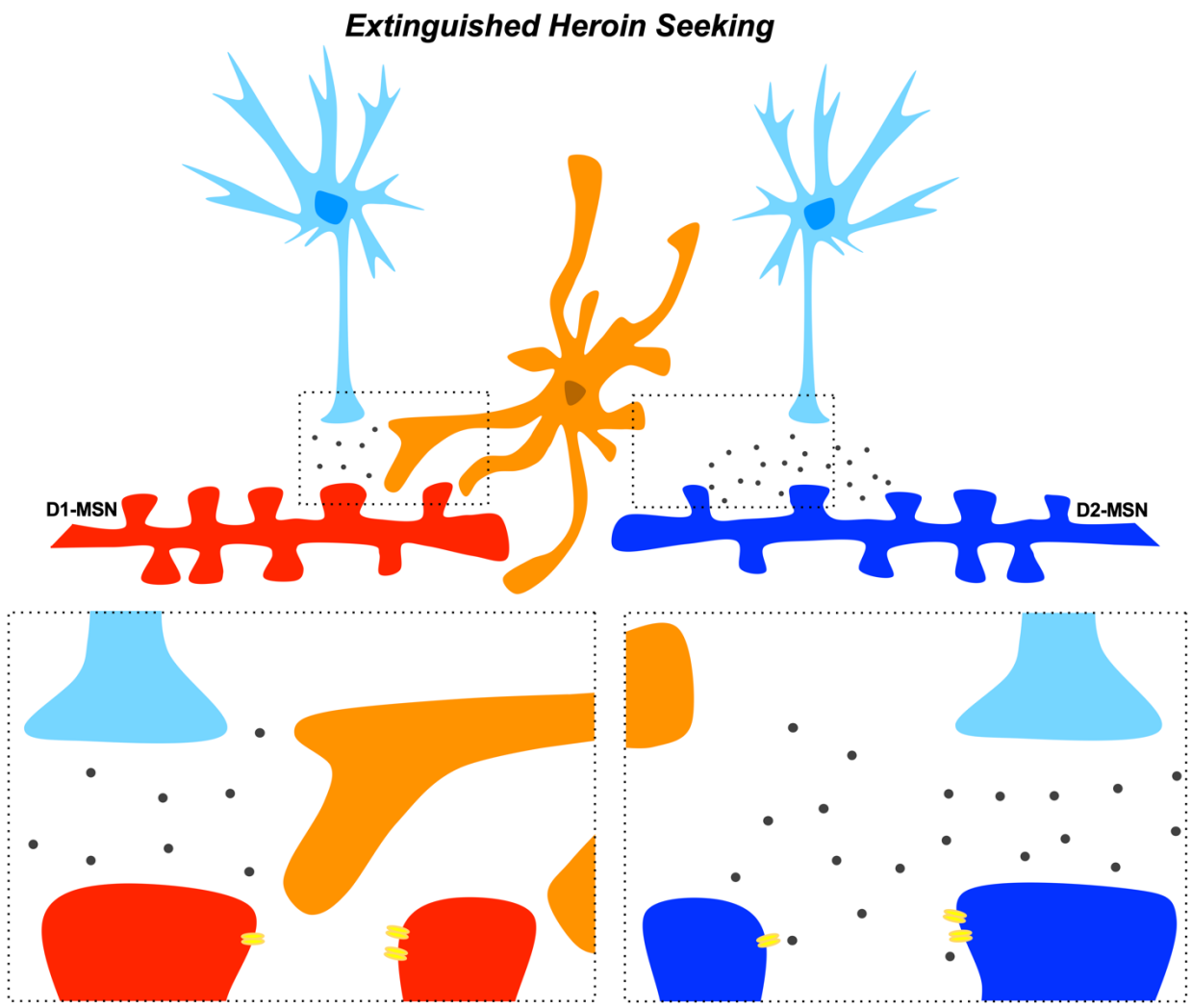



**Fig. S7. Synapse-selective proximity of astrocytes regulates seeking.** Extinguished heroin seeking is characterized by astrocytes exhibiting a high degree of association with synapses from D1-MSNs (left), but retraction from synapses from D2-MSNs (right). High synaptic co-registration by astroglial processes low in GLT-1 (orange) is predicted to induce autoinhibition through spatial buffering of glutamate toward presynaptic inhibitory autoreceptors (*36*). The high co-registration of astroglia with D1-MSN synapses during extinction training is also predicted to shield post-synaptic NR2B receptors (yellow) that produce postsynaptic potentiation when stimulated by glutamate (*33*). These two functions would suppress D1-MSN potentiation during extinction training. Instead, retraction from D2-MSNs after extinction of heroin seeking engages post-synaptic potentiation of D2-MSNs through stimulation of postsynaptic NR2B (*33*), synaptic

recruitment (*41*), and loss of autoinhibitory mechanisms at terminals synapsing onto D2-MSNs (*36*). These hypotheses are supported by data showing potentiation of D2-MSNs, but not D2-MSNs during extinction training (*26, 46*). Reversal of this pattern during cued reinstatement would permit potentiation of D1-MSNs and block signaling through D2-MSNs, permitting seeking behavior (*10*).

| D1-Cre | | | | | | D2-Cre | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Saline* | | *Extinguished* | | *Reinstated* | | *Saline* | | *Extinguished* | | *Reinstated* | |
| Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) | Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) | Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) | Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) | Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) | Astro-Syn-Dendrite Co-Reg (% vol) | Astro-GLT-Dendrite Co-Reg (% vol) |
| 0.0300 | 0.0076 | 0.7001 | 0.7365 | 0.1932 | 0.2788 | 0.1307 | 0.2724 | 0.0287 | 0.3753 | 0.1782 | 0.1425 |
| 0.2456 | 0.1780 | 0.0605 | 0.1774 | 0.1327 | 0.5443 | 0.1984 | 0.3842 | 0.0155 | 0.1248 | 0.1163 | 0.7739 |
| 0.5098 | 0.2482 | 0.3413 | 1.1587 | 0.1322 | 0.0502 | 0.1127 | 0.1744 | 0.0455 | 0.2622 | 0.3429 | 0.3991 |
| 0.0305 | 0.0043 | 0.6727 | 0.7868 | 0.0913 | 0.2309 | 0.3452 | 0.4887 | 0.3680 | 0.0975 | 0.3757 | 0.1197 |
| 0.2540 | 0.2729 | 0.2288 | 0.6566 | 0.1210 | 0.0947 | 0.2270 | 0.1807 | 0.0200 | 0.1809 | 0.2075 | 0.3220 |
| 0.0708 | 0.0527 | 0.2227 | 0.9594 | 0.1350 | 0.5158 | 0.2557 | 0.2551 | 0.0304 | 0.2420 | 1.4346 | 0.7354 |
| 0.0123 | 0.0277 | 0.0679 | 0.8191 | 0.6213 | 0.3455 | 0.3475 | 0.2966 | 0.0368 | 0.0078 | 0.0115 | 0.5105 |
| 0.0155 | 0.0398 | 0.5954 | 0.6045 | 0.7099 | 0.6298 | 0.1069 | 0.0336 | 0.0127 | 0.1029 | 0.0477 | 0.0883 |
| 0.0144 | 0.0020 | 1.0266 | 0.1080 | 0.0482 | 0.3122 | 0.3589 | 0.1326 | 0.0151 | 0.0145 | 0.3453 | 0.2403 |
| 0.2441 | 0.1499 | 0.1108 | 0.0421 | 0.0080 | 0.0475 | 0.0768 | 0.1742 | 0.0294 | 0.2039 | 1.1386 | 0.1740 |
| 0.2063 | 0.1212 | 1.9399 | 0.0129 | 2.4966 | 0.5931 | 0.1889 | 0.2818 | 0.0124 | 0.4163 | 0.4940 | 0.0402 |
| 0.0212 | 0.0581 | 0.1929 | 0.2035 | 0.0837 | 0.1153 | 1.5075 | 2.9899 | 0.0388 | 0.1537 | 0.0353 | 0.0314 |
| 0.1315 | 0.1291 | 0.2483 | 0.0004 | 0.0262 | 0.0093 | 5.6845 | 1.5592 | 0.0611 | 0.0035 | 0.3478 | 0.0415 |
| 0.0793 | 0.0665 | 0.1987 | 0.0241 | 0.0108 | 0.0082 | 2.0164 | 0.2007 | 0.0687 | 0.0054 | 0.1945 | 0.2818 |
| 0.1483 | 0.1803 | 0.1978 | 0.0131 | 0.0059 | 0.0021 | 0.3009 | 0.1548 | 0.0110 | 0.0584 | 0.0424 | 0.1492 |
| 0.0067 | 0.0151 | 0.2338 | 0.1819 | 0.0194 | 0.0102 | 1.1487 | 0.3968 | 0.0391 | 0.0448 | 0.0847 | 0.0475 |
| 0.0293 | 0.0416 | 0.1124 | 0.1596 | 0.0432 | 0.0984 | 0.4489 | 0.5708 | 0.0705 | 0.0551 | 0.2491 | 0.0681 |
| 0.1392 | 0.1404 | 0.9283 | 0.0909 | 0.0108 | 0.0757 | 0.3398 | 0.1734 | 0.1116 | 0.3930 | 0.2068 | 0.0527 |
| 0.0670 | 0.0305 | 0.0538 | 0.0847 | 0.1098 | 0.1977 | 0.5152 | 1.1983 | 0.0172 | 0.0019 | 0.1428 | 0.0393 |
| 0.4016 | 0.2204 | 0.2374 | 0.0477 | 3.9462 | 0.2067 | 0.2718 | 0.3590 | 0.0710 | 0.1988 | 0.1213 | 0.1213 |
| 0.2838 | 0.4279 | 0.9920 | 0.0307 | 0.2386 | 0.0353 | 0.2250 | 0.1535 | 0.1009 | 0.0544 | 0.0052 | 0.0276 |
| 1.1285 | 0.3126 | 0.4975 | 0.0118 | 1.0601 | 0.5309 | 1.2654 | 0.5146 | 0.2368 | 0.0877 | 0.3853 | 0.0165 |
| 0.3527 | 0.2036 | 0.2042 | 0.0075 | 0.0956 | 0.1388 | 2.2984 | 1.7794 | 0.0798 | 0.3014 | 0.3851 | 0.1269 |
| 0.0352 | 0.1150 | 0.4574 | 0.0740 | 0.3731 | 0.2772 | 0.5759 | 0.1510 | 0.0544 | 0.0044 | 0.1684 | 0.0301 |
| 0.0048 | 0.1748 | 0.5457 | 0.0033 | 0.2509 | 0.2463 | 0.1536 | 0.5491 | 0.0236 | 0.0132 | 0.2111 | 0.0072 |
| 0.0941 | 0.3145 | 0.2411 | 0.0600 | 0.2393 | 0.0616 | 0.1875 | 0.2406 | 0.0797 | 0.0416 | | |
| 0.2877 | 0.2814 | 0.1222 | 0.0089 | 0.1581 | 0.0183 | 0.3488 | 0.2252 | 0.0117 | 0.0136 | | |
| 0.0284 | 0.2051 | 0.8550 | 0.0477 | 0.0052 | 0.0247 | 0.4335 | 0.3767 | 0.0328 | 0.0070 | | |
| 1.5922 | 0.3352 | 0.0949 | 0.0229 | 0.6927 | 0.4945 | 0.5357 | 0.6957 | 0.0184 | 0.0157 | | |
| 0.8063 | 0.2548 | | | 0.1775 | 0.1694 | 0.1224 | 0.1730 | 0.0357 | 0.0801 | | |
| 0.1473 | 0.3016 | | | 1.2145 | 0.3333 | 0.2860 | 1.2131 | 0.0293 | 0.0202 | | |
| 0.0076 | 0.0951 | | | 0.0673 | 0.0841 | 0.1845 | 0.4097 | 0.0047 | 0.0005 | | |
| 0.0311 | 0.1405 | | | 0.1652 | 0.0209 | 0.0565 | 0.0780 | 0.0023 | 0.0039 | | |
| 0.4217 | 1.5885 | | | 0.0536 | 0.0818 | 0.1340 | 0.0199 | | | | |
| 1.3833 | 0.3813 | | | 0.1204 | 0.0696 | 1.0013 | 0.1578 | | | | |
| 0.2289 | 0.4215 | | | 0.0628 | 0.0153 | | | | | | |
| 0.1299 | 0.1208 | | | 0.2941 | 0.0977 | | | | | | |
| 0.0039 | 0.1085 | | | | | | | | | | |
| 0.0373 | 0.1434 | | | | | | | | | | |
| 0.0312 | 0.0317 | | | | | | | | | | |
| 0.0822 | 0.2388 | | | | | | | | | | |
| 0.0097 | 0.0025 | | | | | | | | | | |
| 0.0040 | 0.0594 | | | | | | | | | | |
| 0.0021 | 0.0123 | | | | | | | | | | |

**Table S1. Dendrite-specific co-registration values.** Triple co-registration of astroglia with Synapsin I and labeled dendrites, as well as triple co-registration of astroglia with GLT-1 and labeled dendrites from D1- and D2-Cre rats are presented as % astrocyte volume.