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Astrocytes mediate oxytocin's effect on central amygdala circuitry that regulates emotional behavior in rodents

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69 SUMMARY

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71 Oxytocin (OT) orchestrates social and emotional behaviors through modulation of neural 72 circuits. In the central amygdala (CeA), the release of OT modulates inhibitory circuits and 73 thereby suppresses fear responses and decreases anxiety levels. Using astrocyte-specific 74 gain- and loss-of-function and pharmacological approaches, we demonstrate that a 75 morphologically distinct subpopulation of astrocytes express OT receptors and mediate 76 anxiolytic and positive reinforcement effects of OT in the CeA of mice and rats. The 77 involvement of astrocytes in OT signaling challenges the long-held dogma that OT acts 78 exclusively on neurons and highlight astrocytes as essential components for modulation of 79 emotional states under normal but also chronic pain conditions.

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82 INTRODUCTION

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84 Oxytocin (OT) is a neuropeptide that acts both as a peripheral neurohormone and a central 85 neuromodulator to modulate key physiological functions, from ion homeostasis to complex social behaviors^{1,2}. Early studies indicated that locally released OT induces morphological 86 87 and functional changes in both the astroglial and neuronal networks of the hypothalamic 88 nuclei producing the neuropeptide³. Activation of oxytocinergic hypothalamic magnocellular 89 neurons, that project further to virtually all forebrain regions, leads to both synaptic and extrasynaptic OT release in the extra-cellular fluid^{4,5}. This last mode of OT release has the 90 91 potential to activate virtually every cell type expressing OT receptors (OTR) located in close 92 proximity to the sites of axonal OT release⁵.

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A few studies using either autoradiography on cell cultures derived from rat tissue,
 immunohistochemistry on CNS sections or knock-in mice models found that not only
 neurons, but also astrocytes express OTRs^{6–9}. Astrocytes are part of the tripartite synapse

97 and capable to modulate neuronal activity as well as to sense the release of 98 neuromodulators into the neuropil¹⁰. There is now accumulating evidence which demonstrate 99 that astrocytes express receptors for various neuromodulators and actually mediate their 100 neurophysiological effects^{11,12}. Mapping of OTR expression in the rodent brain found it was 101 expressed at high levels in several brain regions⁸, and of interest in this study particularly in 102 the lateral and capsular part (CeL) of the central amygdala (CeA)¹³, albeit the types of cells 103 expressing the OTR in this brain region was never elucidated.

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105 Functionally, the release of OT in the CeL leads to increased firing of GABA-expressing interneurons^{4,13}. These interneurons inhibit projection neurons in the medial CeA (CeM), 106 107 which serve as CeA output. OT action in this circuit affects amygdala-related functions, 108 including activity of the autonomous nervous system, fear expression and anxious behaviors^{4,14,15}. Furthermore, the CeA is involved in the pathophysiology of a number of 109 110 neurological diseases, including neuropathic pain and anxiety^{16,17}, in which both astrocytes¹⁸ and the OT system¹⁹ are thought to play a significant role. Therefore, it is crucial to 111 112 understand how the OT system controls pain and its emotional comorbidities, and if 113 astrocytes are involved in these mechanisms.

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Indeed, a role for astrocytes in the regulation of CeA circuits has already been proven in the CeM²⁰, where astrocyte activity can reduce fear-expression in a fear-conditioning paradigm, a role that is surprisingly similar to the effect of OTR signaling in the CeL⁴. We therefore sought to investigate if CeL astrocytes could also play a role in the OT-mediated regulation of CeA circuits and some of their behavioral correlates.

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Our study shows the expression of oxytocin receptors (OTR) in CeL astrocytes and demonstrates that OT directly acts on CeL astrocytes to gate CeL neurons excitability through N-methyl-D-aspartate receptor (NMDAR) (co-)activation. Further, we found that the astrocyte-mediated OTR signaling underlies the anxiolytic and positive reinforcement effects of OT in the CeA. We thus provide the first evidence that OT drives astrocyte activity and that this effect is an essential part of the OTergic modulation of amygdala neuronal circuits and the behaviors they regulate.

130 **RESULTS**

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CeL astrocytes express functional OTRs in rats and mice

134 To investigate whether CeA astrocytes express OTRs, we performed fluorescent in situ 135 hybridization (FISH) combined with immunohistochemistry on rat CeA sections and found 136 some overlap between OTR mRNA signal and an astrocyte marker, glutamine synthase (GS) 137 (Fig. 1a-b; Extended Data Fig. 1a). Within the rat CeL, 18.6±1.8% of astrocytes and 138 67.8±3.1% of neurons expressed OTR mRNA (Fig. 1c), with similar results in mice 139 (Extended Data Fig. 1b). Using another astrocyte marker, the aldehyde dehydrogenase 1 140 family member L1 (ALDH1L1), we found a similar proportion of OTR mRNA positive (OTR+) 141 rat astrocytes (Extended Data Fig. 1c). Combining RNAscope for OTR mRNA with a third 142 astrocyte marker (glial fibrillary acidic protein, GFAP), we again found OTR mRNA to be 143 present in mouse astrocytes (Extended Data Fig. 1d). Next, to unequivocally demonstrate 144 that CeL astrocytes were positive for OTR, we employed IHC-based three-dimensional 145 reconstruction using Imaris technique and combining immunohistochemical staining for GS 146 and GFAP with FISH for OTR mRNA in the rat CeL. Employing glial morphometric profiler, 147 we performed three-dimensional reconstruction of cells through a semi-automated pipeline²¹ 148 (Fig. 1d-e). This analysis confirmed the presence of OTR mRNA in astrocytes and revealed 149 that OTR+ astrocytes have on average a significantly larger cell volume, surface area, a 150 higher number and an increased length of processes compared to OTR- astrocytes (Fig. 151 1e). Subsequent Sholl analysis revealed that OTR+ astrocytes bear more complex 152 morphological features than OTR- astrocytes (Fig. 1f), suggesting that OTR+ astrocytes 153 represent a distinct subpopulation of astrocytes within the CeL.

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155 To test whether CeL astrocytes respond to endogenous OT release, we expressed the 156 ChR1/VChR1 chimaera channel rhodopsin variant²² (referred here as C1V1) in OTergic neurons to optogenetically control CeA-innervating OT axons (Pot-C1V1-mCherry; referred 157 158 to as OxytOpto). To this end, we employed a previously characterized adeno-associated viral 159 vector (AAV) equipped with the OT promoter⁴, which was injected into the paraventricular 160 (PVN), supraoptic, and accessory nuclei of rat hypothalamus (Fig. 2a). First, we validated 161 that red shifted light (λ 542 nm light pulses of 10 ms width at 30 Hz) induced activation of 162 C1V1-expressing OT neurons in the PVN (Extended Data Fig. 2a-b). We then identified CeL 163 astrocytes through sulforhodamine 101 (SR101) labeling in acute brain slices and confirmed 164 astrocytes specificity of the labeling by measuring electrophysiological properties of SR101-165 labelled cells in CeL (Extended Data Fig. 2c-e). We then assessed SR101 labeled astrocytes 166 activity by measuring the relative changes in cytosolic calcium using the small organic dye 167 Oregon Green[®] 488 BAPTA-1 (OGB1). (Fig. 2b). We found that of recorded astrocytes, 168 61.9±8.7% responded to the optogenetic OT axons stimulation (Fig. 2c-d). To avoid the 169 possibility that astrocyte activity was increased due to the increased spiking of CeL 170 interneurons caused by optogenetically evoked OT release, tetrodotoxin (TTX) was added to 171 the bath prior the stimulation. Further calcium imaging experiments described below use 172 TTX incubation (if not stated otherwise) for the same reason,. We also compared the 173 frequency of calcium transients as well as the area under the curve before and after 174 optogenetic stimulation, expressing the results as a ratio between post-stimulation and baseline values (referred to as AUC and Ca²⁺ transient frequency normalized to baseline, 175

analysis method detailed in Extended Data Fig. 2f). As depicted in Fig. 2d, both parameters
were increased following stimulation of OT axons. Taken together, these results indicate that
optogenetically-evoked OT release from axons present in the CeL elicits an increase in
activity of CeL astrocytes.

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181 To ensure that the observed responses are exclusively due to OTR activation, we applied the 182 selective agonist of OTR ([Thr⁴Gly⁷]-oxytocin, TGOT), which increased calcium transients in 60.1±9.2% of the recorded astrocytes, a result unchanged when replicated with slices pre-183 184 incubated with TTX (Fig. 2e-f). Similar pre-incubation of the slices with the OTR antagonist 185 $[d(CH_2)^5, Tyr(Me)^2, Orn^3]$ -vasotocin (dOVT) reduced the proportion of TGOT-evoked calcium 186 responses in astrocytes and their properties (Fig. 2e-f), confirming that the activation of 187 astrocytes by TGOT is indeed mediated by OTR. However, it cannot be discarded that other 188 OTR-mediated and astrocyte-independent mechanisms may drive CeL astrocyte activity up.

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190 To exclude this possibility, we employed a transgenic mouse line with a LoxP sites flanking 191 the OTR gene²³ (OTR cKO mice). To specifically delete OTRs in astrocytes, we injected an AAV driving expression of Cre recombinase under the control of the GFAP promoter in the 192 193 CeL (PGFAP-Cre, Fig. 2g). This led to to expression of Cre in 56.0±4.9% of astrocytes within 194 the injection site, with a specificity reaching 96.2±2.1% (Extended Data Fig. 2g). PGFAP-Cre 195 AAV injection in OTR cKO mice resulted in a drastic decrease in OTR mRNA signal in 196 astrocytes (Fig 2h), but not in neurons (Extended Data Fig. 2h). Similarly to results obtained 197 in rats, TGOT application in TTX-treated acute slices of the CeL from control mice led to 198 responses in 43.9±7.2% of astrocytes (Fig. 2i), whereas these responses were largely 199 reduced in CeL astrocytes from PGFAP-Cre AAV injected OTR cKO mice (GFAP OTR KO, 200 Fig. 2i). These results confirm that astrocytes respond directly to OTR activation 201 independently from TGOT effects on CeL neurons.

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203 Activation of OTR+ astrocytes propagates through an astrocyte network

Since astrocytes are known to form extensive intercellular networks, we next studied the spatial connectivity of OTR+ astrocytes in the CeL. (Fig. 3a, Extended Data Fig. 3a). We found that the distance between OTR+ astrocyte pairs (95.8±4.0µm) was larger than the distance between OTR- astrocyte pairs (37.4±0.9µm, Fig. 3a). In addition, we found that OTR+ astrocytes had significantly more contacts with OTR-, than with OTR+ astrocytes (Fig. 3a), whereas OTR- regularly contact other OTR- astrocytes (3.7±1.3 contacts).

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212 These anatomical results suggest a specific distribution pattern of OTR+ astrocytes within 213 the CeL, where OTR+ astrocytes are found distant from their counterparts and exhibit 214 numerous connections with others, OTR- astrocytes. Interestingly, the proportion of rats and 215 mice astrocytes responsive to OTR activation always exceeded 40% of recorded cells (Fig. 216 2) despite our FISH/Imaris results indicating that less than 20% of astrocytes expressed 217 OTRs mRNA (Fig. 1). This apparent discrepancy led us to test whether the minor population 218 of OTR+ astrocytes could spread their activation following OTR signaling to a much larger 219 population of OTR- astrocytes.

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There are two common signaling pathways typical for astrocytes which could facilitate the spread of activation from OTR+ to OTR- astrocytes: (i) paracrine purinergic communication and (ii) gap-junctions mediated spread of activity. To decipher the underlying mechanism, we 224 first blocked purinergic receptors using pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid 225 (PPADS) and found that this blocker did not significantly affect the average proportion of 226 astrocytes activated by TGOT (Fig. 3b), nor the properties of these responses (Fig. 3b), a 227 result replicated using a set of purinergic receptors antagonists blocking the majority of 228 known purinergic receptors (Extended Data Fig. 3b-c). Yet, the incubation with the gap-229 junctions blocker carbenoxolone (CBX) significantly reduced the number of astrocytes 230 responsive to TGOT+TTX application (Fig. 3b). Given that astrocyte gap-junctions are 231 primarily composed of the connexins (Cx) 30 and 43, we further tested their involvement in 232 OTR-induced signal spreading by using a Cx30/Cx43 double KO mice line, previously shown to lack astrocyte gap junctional communication²⁴ (Fig. 3c). As found with CBX, the number of 233 234 astrocytes activated by TGOT was significantly reduced in these mice (Fig. 3c). These 235 results indicate that gap junctions play a role in the propagation of responses following OTR 236 activation in the CeL astrocytes network, possibly from OTR+ to OTR- astrocytes. This is 237 corroborated by the anatomical observations that biocytin infused in the cytosol of astrocytes 238 spread from one cell to another within the CeL network at least in part via gap-junctions (Fig. 239 4e).

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242 CeL astrocyte activity increases CeL neurons excitability

244 We next wanted to test if this direct astrocyte network response to OTR activation could be relevant to the known effect of OTR signaling on CeA neuronal networks 4,13,14. To 245 246 manipulate astrocytic activity, we expressed the opsin C1V1 under the control of the GFAP 247 promoter using rAAV injections (PGFAP-C1V1-mCherry; referred to as AstrOpto, Fig. 4a). 248 After confirmation of vector expression in 62.5±3.1% of all astrocytes in the CeL, with a 249 specificity reaching 98.8±0.7% (Extended Data Fig. 4a), we evaluated the effect of C1V1 250 stimulation on astrocyte calcium activity (Fig. 4b). We observed responses in 60.8±9.0% of 251 astrocytes (Fig. 4c), a proportion comparable to what we observed following OTR stimulation 252 by TGOT+TTX (AstrOpto vs TGOT+TTX: p = 0.8265, unpaired *t*-test). The removal of 253 extracellular calcium prevented the C1V1-driven astrocyte activity (Extended Data Fig. 4b). 254 This indicates that influx of extracellular calcium through the plasma membrane is required to 255 generate calcium transient activity in astrocytes following C1V1 activation (Extended Data 256 Fig. 4b).

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258 Next, we measured the effect of evoked astrocyte activity, using AstrOpto or direct OTR 259 stimulation through TGOT application, on CeL neurons excitability measured through patch-260 clamp techniques. Optical stimulation of C1V1 in astrocytes increased the frequency of 261 miniature excitatory post-synaptic currents (mEPSCs) in CeL neurons (Fig. 4d) while bath-262 applied TGOT had a similar effect (Fig. 4f). We next infused BAPTA specifically in the 263 astrocyte network (Fig. 4e) to block the increase in calcium transients evoked by TGOT, and 264 found it also blocked TGOT effect on CeL neurons mEPSCs (Fig. 4e-f). This indicates that 265 OTR activation and the ensuing increased in calcium transients in astrocytes is driving up 266 excitatory inputs to CeL neurons.

267

To identify the intercellular communication pathway involved in astrocytes-neurons
 communication following OTR-dependent activation of astrocytes, we studied whether N Methyl-D-Aspartate receptor (NMDAR), a well-known neuronal receptor that can be activated

by astrocytic gliotransmission^{12,25,26}, was involved. Thus, we applied a NMDAR antagonist 271 272 (2R)-amino-5-phosphonovaleric acid (AP5) prior to TGOT application and found it inhibited 273 TGOT effect on mEPSCs frequency (Fig. 4f). Importantly, AP5 had no effect on astrocyte 274 responses to TGOT, thereby excluding the involvement of NMDAR in CeL astrocytes 275 responses to TGOT (Extended Data Fig. 3d). Finally, to unambiguously demonstrate that 276 astrocytes underlie the effect of OTR signaling on CeL neuron excitatory synaptic 277 transmission, we repeated the mEPSCs measurements in control and GFAP OTR KO mice. 278 TGOT application increased mEPSCs frequency in control mice, but not in GFAP OTR KO 279 mice (Fig. 4g-h), confirming the role of astrocytic OTR in mediating the effects of OTR 280 signaling on synaptic inputs in CeL neurons.

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282 We then investigated whether the increase in mEPSCs frequency in CeL neurons following 283 OTR activation was able to increase their firing frequency. To address this question, we 284 applied the same stimulation protocols as before, and subsequently recorded spontaneous 285 action potential (AP) firing (Fig. 5a-b). First, AstrOpto increased firing of CeL neurons, an 286 effect that was blocked by previous infusion of BAPTA in the astroglial network, hence 287 proving its dependence on astrocyte calcium signaling (Fig. 5c). Similarly, and in agreement 288 with its effect on CeL neurons mEPSC, TGOT also increased neuronal spiking activity (Fig. 289 5d), an effect which was abolished following either BAPTA infusion in the astroglial network 290 or pre-incubation of AP5 in the recording bath (Fig. 5d). Crucially, TGOT application 291 increased the firing of CeL neurons in control but not in GFAP OTR KO mice (Fig. 5e-f). 292

These results unequivocally demonstrate that OTR signaling in the CeL requires the expression and activation of OTR and its associated calcium signaling in astrocytes, which further lead to an increase in the excitability of CeL interneurons through NMDAR-dependent mechanisms.

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298 CeL astrocytes activity increases inhibitory inputs onto CeM projection neurons

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Previous work showed that the activation of CeL OTRs leads to an increased activity of
 GABAergic CeL neurons, a result we replicated here (Fig. 5). A demonstrated direct
 consequence of this is an increased frequency of GABA_A-mediated inhibitory post-synaptic
 currents (IPSCs) in CeM projection neurons^{4,13,14}.

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305 In agreement with these findings, we found that optogenetic activation of CeL astrocytes 306 (through AstrOpto) evoked an increase in IPSC frequency in rat CeM neurons (Fig. 6a-c). 307 This effect was also dependent on CeL astrocytic calcium signaling, as BAPTA infusion in 308 CeL astrocytes abolished it (Fig. 6c). Furthermore, the effect of AstrOpto on CeM IPSCs 309 frequency was suppressed by application of AP5, but also by prior degradation of D-serine 310 (an NMDAR co-agonist) with D-amino acid oxidase (DAAO) (Extended Data Fig. 5a). D-311 Serine is a known gliotransmitters, whose levels have been found to be increased following astrocytic GPCR activation²⁷. Altogether, these results indicate that the effect of AstrOpto on 312 313 IPSCs in CeM neurons is dependent on the activation of NMDARs in CeL neurons. TGOT 314 application produced a similar effect on CeM neurons, also dependent on CeL astrocytic 315 calcium signaling (Fig. 6d) and on NMDARs (Fig. 6d). Initial incubation with DAAO similarly 316 blocked the effect of TGOT on IPSCs frequency (Extended Data Fig. 5b). Crucially, D-serine 317 supplementation in the DAAO-treated slices following the first, effectless, TGOT application 318 rescued the effect of a second TGOT application on IPSCs frequency (Extended Data Fig. 319 5b-d). This confirms the involvement of neuronal NMDARs and its co-agonist D-Serine in 320 OTR-mediated modulation of CeA neuronal network. To further complement these results, 321 we found that bath application of 5,7-dichlorokynurenic acid (DCKA), a potent antagonist of 322 the NMDAR glycine/D-Serine modulatory site, also abolished TGOT effects on CeM IPSC 323 frequency, while the AMPAR antagonist DNQX had no effect (Extended Data Fig. 5e). In 324 addition, pre-incubation with the purinergic receptors antagonists PPADS, CPT or SCH 325 58261 had no effects either (Extended Data Fig. 5f). This confirms that the primary mode of 326 astrocyte to neuron communication engaged following OTR signaling in CeA involves 327 NMDARs, but not purinergic signaling in neurons, and is mediated by an increase in D-serine 328 levels.

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As in rats, TGOT application also led to an increased IPSC frequency in CeM neurons in brain slices from control mice, but not in slices from GFAP OTR KO mice (Fig. 6e-f). These *ex vivo* results clearly demonstrate that direct of OTR-mediated CeL astrocytes activation leads to an NMDAR-dependent increase in CeL neurons excitability, which further increases inhibitory inputs into CeM projection neurons.

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Our data so far demonstrate that when OTR signaling is engaged in astrocytes, these cells increase the excitability of CeL neurons, directly leading to an increase in inhibitory inputs into CeM projection neurons. Altogether, these results demonstrate that the effect of OT on neuronal activity is abolished when astrocyte function is compromised, establishing for the first time that OT signaling in CeA circuitry is locally transduced by astrocytes.

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2 OTR signaling through astrocytes is crucial for emotional balance regulation.

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The CeA is a key nucleus for the processing of emotional information, and notably plays an important role in pain-associated disorders¹⁶, for which OT has been demonstrated as a crucial regulator¹⁹. We therefore chose to first test the involvement of OTR-mediated (or direct) astrocyte activation in modulating mechanical pain hypersensitivity (Fig. 7b,f), levels of anxiety (Fig. 7c,g), and reinforcement behavior (Fig. 7f,h) in rats and mice that developed neuropathic pain and the associated increased anxiety following a spared nerve injury (SNI) surgery²⁸ (Extended Data Fig. 6a).

351

352 Given that there is a proven link between chronic pain etiology and synaptic changes in the 353 CeA²⁹, we initially tested whether the effect of TGOT on astrocytes calcium signaling and 354 CeM neurons IPSCs frequency was altered in SNI rats, but did not find any differences 355 compared to sham-operated animals (Extended Data Fig. 6b-c). This indicates that OTR 356 signaling in CeA, at least at the levels of the circuit studied here, is unaltered in SNI animals. 357 We further confirmed that D-Serine was still required for TGOT effects in the CeA of SNI 358 animals using DAAO pre-incubation followed by exogenous D-Serine supplementation in the 359 ex vivo setting (Extended Data Fig. 6d).

360

Following our finding that SNI procedure did not alter the effect of TGOT at the circuit level, we next used a nociceptive assay to measure the mechanical threshold for paw withdrawal in both rats and mice. We found that SNI animals displayed a decreased mechanical threshold, but bilateral micro-injections of TGOT in the CeA had no clear effect on it, with only a mild anti-nociceptive effect only in rats (Fig. 7b,f). Moreover, bilateral optogenetic stimulation of rats CeL astrocytes (AstrOpto) had no noticeable effect on mechanical threshold in rats (Fig.7b; Extended Data Fig. 7b).

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369 Next we performed an elevated plus maze (EPM) test, and found that SNI animals spent 370 significantly more time in the closed arm compared to sham-operated ones, indicating the 371 SNI procedure induced a state of elevated anxiety (Fig. 7c,g) as expected. When TGOT was 372 injected in CeA, time spent in the closed arm was significantly reduced to levels comparable 373 to those observed in sham animals, indicating an anxiolytic effect of OTR signaling in both 374 rats and mice (Fig. 7c,g). Crucially, AstrOpto in rats had a comparable effect (Fig. 7c), while 375 the deletion of OTR from CeL astrocytes in mice abolished the anxiolytic effects of TGOT 376 (Fig. 7g), highlighting the involvement of astrocytic OTRs also at the behavioral level. 377 Interestingly, sham mice with deletion of OTR in CeL astrocytes displayed an anxious 378 behavior (Fig. 7g), which indicates OTR signaling through astrocytes is involved in emotional 379 balance, even under pain-free conditions.

380

381 In order to further investigate the role of the CeA in attributing emotional valence to stimuli, 382 we used the conditioned place preference (CPP) test in rats and mice that underwent an SNI 383 or sham procedures. Here, both SNI and sham animals exhibited a clear preference for the 384 chamber paired with TGOT infusion (Fig. 7d,h). The optogenetic activation of astrocytes in 385 rat CeL also led to a place preference, mimicking TGOT action (Fig. 7d), while TGOT lost its 386 positive conditioning effects in GFAP OTR KO mice (Fig. 7h). Importantly, neither the 387 treatments nor the SNI procedure significantly affected the motor activity of rats and mice in 388 both the EPM or CPP tests (Extended Data Fig. 7).

389

390 Collectively, our findings demonstrate from the local circuit to the behavioral levels that OTR 391 signaling through CeL astrocytes is a novel and important mechanism involved in emotional

392 states regulation, this under both normal and chronic pain conditions (Fig. 7i).

394395 **DISCUSSION**

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397 We here demonstrate that OTR signaling through astrocytes is crucial for the oxytocinergic 398 modulation of the local CeA microcircuit and its behavioral correlates. We further propose 399 that this effect relies on a morphologically defined subpopulation of OTR+ astrocytes (Fig. 1-400 2), that convey their activation by OT to other astrocytes through gap-junctional 401 communication (Fig. 3), leading to an increase in activity in the majority of CeL astrocytes 402 (Fig. 2-3). In turn, astrocytes increase the excitability of CeL interneurons by gating activation 403 of NMDA receptors (Fig. 4-5), leading to an increase in GABAergic inhibitory inputs in post-404 synaptic neurons located in the CeM (Fig. 6). At the behavioral level, this OTR-mediated 405 modulation of CeA astro-neuronal network promotes a positive emotional state, measured as 406 clear anxiolytic and positive reinforcement effects (Fig. 7).

407 Mechanistically, our results reinforce previous demonstrations of a role of astrocytes in 408 transforming neuromodulators signaling into a change in the gain of neuronal circuits, notably by (co-)activating neuronal NMDAR^{12,25,26}. Despite the high proportion of OTR+ neurons (up 409 410 to 70%) compared to the limited number of OTR+ astrocytes (~18%) in the CeL, the 411 activation of OTRs in astrocytes was required to gate CeL neurons responses to OTR 412 activation by allowing a sustained (co)activation of NMDARs. Other mechanisms are 413 probably at play: OTR activation in CeL neurons might inhibit K+ leak current, as has been shown in olfactory neuronal cells³⁰ and in spinal cord³¹. These mechanisms might act in 414 415 synergy to induce an elevation in firing rates of CeL neurons by increasing the gain of 416 excitatory inputs in these cells. Furthermore, similar mechanisms of astrocytes to neuron 417 communication through neuronal NMDARs (co)activation have been proposed by other studies to favor a synchronous increase in excitability across an ensemble of neurons^{32,33}. 418 419 This would allow a synchronized and long-lasting switch in the gain of the CeA neuronal 420 circuits, thereby amplifying the effect of OT on CeA outputs in both the spatial and temporal 421 domains. In light of the predominantly non-synaptic mode of OT release from axons en 422 passant, which could lead to CeL-restricted micro-volume transmission of the neuropeptide⁵, 423 it seems then plausible that astrocytes are required to relay and amplify OT signaling to CeL 424 neurons. Another mechanism that was repeatedly found to be involved in neuromodulators signaling through astrocytes is the activation of purinergic signaling in neurons^{34,35}. However, 425 426 in the case of OT signaling within the CeA, purinergic signaling was not involved (Extended 427 Data Fig. 5). It would then be interesting to test if astrocytes are important to OT signaling in 428 other brain regions, and if they are, if the same mechanisms of astrocytes to neurons 429 communication are at stake.

430 We found that CeA astrocytes can be divided into two defined populations: OTR+ and OTR-431 astrocytes. OTR+ astrocytes are morphologically more complex and have more close 432 contacts with neighboring, OTR- astrocytes, while being guite distant from their OTR+ 433 counterparts with whom they have almost no contacts (Fig. 1, 2). Thus, we hypothesize that 434 these morphological peculiarities allow the propagation of OTR evoked calcium transients 435 (Fig. 2) to a much larger number of OTR- astrocytes through, at least partially, gap junctions 436 (Fig. 3b-c). Therefore only a few, strategically positioned OTR+ astrocytes are sufficient to 437 result in a network wide effect of OT release in the CeL, despite the relatively moderate 438 number of OT fibers found in this nucleus⁴. By describing a new population of astrocytes, our work add to the recent advances in describing molecular, morphological and functional
 heterogeneity in astrocytes population^{36,37} and opens up new perspectives into
 understanding how astrocytes subpopulations are functionally organized and communicate
 inside CNS circuits.

443 OT and its effects on brain circuits, and ultimately behavior, are under intense scrutiny, from 444 fundamental research in animal models to behavioral and physiological studies in humans^{38,39}. Among other roles, OT is considered a powerful anxiolytic peptide through its 445 446 action in the human amygdala⁴⁰. Yet, all studies so far considered as an accomplished fact 447 that OT was acting directly on neurons, despite the controversies regarding the cellular 448 substrates of BOLD fMRI signals often used in human studies, with some results arguing for a major contribution of astrocyte activity to these signals^{41,42}. Furthermore, a significant 449 number of studies demonstrated that astrocytes or their invertebrates counterparts are key, if 450 not primary, targets of neuromodulators^{12,25,26,34,35,43}, and that astrocytes might be the causal 451 elements behind shifts in brain states^{11,43,44}, a function usually attributed mainly to 452 neuromodulators direct action on neurons⁴⁵. Thus, further research focused on astrocytes-453 454 mediated modulation of human brain circuits' activities is particularly promising to develop 455 refined strategies for future therapeutic approaches. Indeed, neuromodulators are the targets 456 of numerous, already available, treatments of psychiatric diseases using either small molecule drugs or electrical stimulation protocols, such as deep brain⁴⁶ or transcranial 457 stimulations⁴⁷, for which astrocytes also seem particularly involved^{46,47}. Taken together, this 458 459 indicates a need for a more global and systematic consideration of astrocytes roles in brain 460 circuits, notably regarding the effect of neuromodulators, and in particular OT. We believe 461 this is especially relevant for the development of better therapeutics in the field of chronic pain, which imposes a massive burden to society, impacting ~20% of the global population⁴⁸. 462

463 Indeed, considering that general anxiety and depression are frequent comorbidity of chronic 464 pain⁴⁹, our finding that the activation of OT signaling in CeL astrocytes promotes a form of 465 emotional comfort by alleviating anxious behavior and has positive reinforcement properties 466 is promising: it demonstrates that activation of a particular, astrocyte-mediated, OTR 467 pathways affect one of the most impactful aspect of the chronic pain sphere, the emotional 468 one. This further highlights astrocytes as important cellular substrates of emotional 469 regulation, which several studies have also argued for (see⁵⁰ and references therein). In this 470 context, targeting the OT system and/or astrocytes specifically, in the amygdala or other 471 CNS regions, might lead to the development of new therapeutic avenues to improve patient's 472 well-being.

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506 AUTHOR CONTRIBUTIONS

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519	DECLARATION OF INTERESTS
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521	The authors declare no competing interest
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690 FIGURE TITLES AND LEGENDS

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692 Figure 1. Specific CeL astrocytes express oxytocin receptors. (a) Overview of CeA 693 fluorescent in situ hybridization of OTR mRNA (red) and glutamine synthase immunostaining 694 (GS, green). (b) High magnification images of cells positive for OTR mRNA and/or GS 695 (double arrows); green arrows point GS positive cells; red arrows point OTR mRNA-positive 696 cells. Scale bars are 100 (a) and 10µm (b). (c) Proportion of CeL astrocytes (GS positive 697 cells, left) and neurons (NeuN positive cells, right) positive for OTR mRNA (red) (n astrocytes = 698 1185, n neurons = 1254, n rats = 4). (d) Cells were reconstructed in 3D using Imaris and 699 morphological parameters were evaluated. Scale bar = $20\mu m$ (e) OTR-expressing 700 astrocytes are bigger and more complex as indicated by several morphological parameters 701 (cell volume, surface, number of processes, process length and domain volume). (f) OTR-702 expressing astrocytes display a more complex morphology as revealed by Sholl analysis. All 703 data are expressed in mean ± SEM except for violin plots where hatched line represent the 704 median and the dot lines are the first and third quartiles. $\frac{1}{p} < 0.0001$, student's t-test or two-705 way ANOVA followed by Tukey post-hoc test. (Statistics in Extended Data Table 1).

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707 Figure 2. Astrocytic OTR activation evokes calcium transients in CeL astrocytes of 708 rats and mice. (a) Experimental strategy to express the ChR1/VChR1 chimaera channel 709 rhodopsin variant C1V1 in OTergic neurons (OxytOpto). (b) Experimental scheme of the 710 horizontal CeA slice preparation used, showing C1V1 expressing OT axons (yellow) arising 711 from PVN and projecting to the CeL. (c) Typical traces of relative changes in intracellular 712 calcium in astrocytes (ΔF) induced by the activation of C1V1 in OT axons located in the CeL 713 through λ 542 nm light pulses (10 ms width, 30 Hz, duration 20 s). (d) Proportion of responding astrocytes (left), AUC of ΔF traces (middle) and Ca²⁺ transients frequency (right) 714 715 normalized to baseline values following C1V1 activation in CeL OT axons, n slice $(n_s) = 7$, n 716 astrocytes (n_a) = 36. (e) (left) Images of CeL astrocytes identified through SR101 (red, top) 717 and corresponding pseudo-color images of OGB1 fluorescence during baseline and after 718 drug application (middle, bottom, stacks of 50 images over 25s of recording). Scale bar = 719 10μm. (right) Typical ΔF traces following TGOT+TTX (Ctrl) application (f) Proportion of responding astrocytes (left), AUC of ΔF traces (middle) and Ca²⁺ transients frequency (right) 720 721 normalized to baseline values following application of TGOT (0.4 µM) with TTX (1 µM) (Ctrl, 722 $n_s = 18$, $n_a = 136$), without TTX (No TTX, $n_s = 7$, $n_a = 43$), and with an OTR antagonist (dOVT, 723 1 μ M; n_s = 3, n_a = 24). Data are expressed as means across slices plus SEM (g) 724 Experimental strategy for the specific deletion of OTRs in mice CeL astrocytes. (h) (left) 725 Example pictures of OTR mRNA (red) and GS (green) labelling in mice injected with PGFAP-726 GFP-IRES-Cre (top) or PGFAP-GFP rAAV vector (bottom) ; (right) Proportion of CeL 727 astrocytes (GS positive cells) also positive for OTR mRNA (left, blue, PGFAP-GFP: n astrocytes = 728 1340, n mice = 3 ; right, red PGFAP-GFP-IRES-Cre: n astrocytes = 1561, n mice = 4). Scale bar = 729 50µm. (i) Proportion of responding astrocytes (left), AUC of ΔF traces (middle) and Ca²⁺ 730 transients frequency (right) normalized to baseline values following application of 731 TGOT+TTX in control (blue, $n_s = 12$, $n_a = 237$) or GFAP OTR KO mice (red, $n_s = 5$, $n_a = 47$; 732 red) acute brain slices. Calcium imaging data are expressed as means across slices plus 733 SEM and white circles indicate averages across astrocytes per slices. p < 0.05, p < 0.01, 734 ***p<0.001, Unpaired *t*-test or Mann-Whitney U test. (Statistics in Table Extended Data Table 735 2). 736

737 Figure 3. OTR-expressing CeL astrocytes are positioned to recruit a CeL astrocyte 738 network through gap junction. (a) (left) The distance between two OTR+ astrocytes is 739 larger than the distance between two OTR- astrocytes. (right) OTR+ astrocytes 740 predominantly form contacts with OTR- astrocytes, (n astrocytes = 1270, n OTR+ astrocytes = 236, n 741 rats = 4). Contacts are defined by a proximity of less than 1µm between GFAP-positive 742 fibers/endfeet from two different astrocytes. (b) (left) Typical ΔF traces following TGOT+TTX application in presence of CBX (100 µM, top) or PPADS (50 µM, bottom). (right) Proportion 743 of responding astrocytes, AUC of ΔF traces and Ca²⁺ transients frequency normalized to 744 745 baseline values following application of TGOT+TTX (0.4 μ M) in presence of CBX (n_s = 9, n_a = 746 75) or PPADS ($n_s = 7$, $n_a = 53$). (c) (left) Typical ΔF traces following TGOT+TTX application in 747 Cx30/Cx43 KO mice brain slices. (right) Proportion of responding astrocytes, AUC of ΔF traces and Ca²⁺ transients frequency normalized to baseline values following TGOT+TTX 748 749 application in WT mice ($n_s = 19$, $n_a = 78$) and in Cx30/Cx43 KO mice acute brain slices ($n_s = 19$) 750 17, $n_a = 100$). Calcium imaging data are expressed as means across slices plus SEM and 751 white circles indicate averages across astrocytes per slices. p < 0.05, p < 0.01, p752 0.001, unpaired *t*-test or Mann-Whitney U test. (Statistics in Table Extended Data Table 3).

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754 Figure 4. CeL astrocyte activity promotes excitatory transmission into CeL neurons.

(a) Experimental strategy for the specific expression of C1V1 in mice CeL astrocytes 755 756 (AstrOpto). (b) Experimental scheme of the horizontal CeA slice preparation used, showing a 757 C1V1 expressing astrocytes and a patched CeL neuron, recorded in whole-cell voltage-758 clamp configuration with bath-applied TTX and bicuculline to isolate mEPSC. (c) (left) 759 Proportion of responding astrocytes, AUC of ΔF traces and Ca²⁺ transients frequency 760 following AstrOpto activation ($n_s = 12$, $n_a = 49$) (right) Typical ΔF traces following AstrOpto 761 activation. (d) Frequency of mEPSCs in CeL neurons before (basal), during (AstrOpto) and 762 after (Wash) AstrOpto activation (λ 542 nm, 3 min long, 1 s width pulse at 0,5 Hz; n = 17). (e) 763 Example of an astrocyte in CeL patched with a BAPTA-Biocytin filled capillary (white arrow). 764 Biocytin can be seen diffusing in neighboring astrocytes. f) Frequency of mEPSCs in CeL 765 neurons before (basal), during (TGOT, 0.4 µM) and after (Wash) TGOT application (left, 766 n=16). This effect can be blocked by loading BAPTA in the CeL astrocyte network (middle, 767 BAPTAstro, n= 11) and by prior incubation of an NMDAR antagonist (right, AP5, 50 μ M, n = 768 9). (g) Experimental strategy for the specific deletion of OTRs in mice CeL astrocytes. (h) 769 Frequency of mEPSCs in CeL neurons before (basal), during (TGOT, 0.4 µM) and after 770 (Wash) TGOT application in acute brain slice from WT (left, blue, n = 10) and from GFAP 771 OTR KO mice (right, red, n= 8).). Calcium imaging data are expressed as means across 772 slices plus SEM and white circles indicate averages across astrocytes per slices. Patch-773 clamp data are expressed as averaged frequency plus SEM across cells, linked white circles 774 indicate individual cell values, example traces of the various conditions are displayed on the 775 right. $p^{*} < 0.05$, $p^{*} < 0.001$, Friedman and Dunn's Multiple comparisons, $p^{*} < 0.05$, $p^{*} < 0.$ 776 0.01, Unpaired *t*-test or Mann-Whitney U test. (Statistics in Table Extended Data Table 4).

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Figure 5. CeL astrocyte activity promotes CeL neuron firing. (a) Experimental strategy for the specific expression of C1V1 in mice CeL astrocytes (AstrOpto) (b) Experimental scheme of the horizontal CeA slice preparation used, showing a C1V1 expressing astrocytes and a patched CeL neuron, recorded in whole-cell current-clamp configuration to record action potentials (APs). (c) Frequency of APs in CeL neurons before (basal), during (AstrOpto) and after (Wash) AstrOpto activation (λ 542 nm, 3 min long, 1 s width pulse at 0,5 Hz) in control condition (left, n = 10) or following BAPTA loading in the CeL astrocyte network 785 (right, BAPTAstro, n = 12). (d) Frequency of APs in CeL neurons before (basal), during 786 (TGOT, 0.4 μM) and after (Wash) TGOT application (left, n = 9). This effect can be blocked 787 by loading BAPTA in the CeL astrocyte network (middle, BAPTAstro, n = 9) and by prior 788 incubation of the NMDAR antagonist AP5 (right, AP5, 50 µM, n = 7). (e) Experimental 789 strategy for the specific deletion of OTRs in mice CeL astrocytes (GFAP OTR KO). (f) 790 Frequency of APs in CeL neurons before (basal), during (TGOT, 0.4 µM) and after (Wash) 791 TGOT application in acute brain slices from WT (left, blue, n = 7) and GFAP OTR KO mice 792 (right, red, n = 11). Example traces of the various conditions are displayed on the right. 793 Patch-clamp data are expressed as averaged frequency plus SEM across cells; linked white circles indicate individual cell values. $p^{*} < 0.05$, $p^{*} < 0.01$, Friedman and Dunn's Multiple 794 comparisons, *p < 0.05, ***p < 0.001, Unpaired *t*-test or Mann-Whitney U test. (Statistics in 795 796 Table Extended Data Table 5).

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798 Figure 6. Astrocyte-driven CeL neuron activity modifies amygdala output. (a) 799 Experimental strategy for the specific expression of C1V1 in mice CeL astrocytes (AstrOpto) 800 (b) Experimental scheme of the horizontal CeA slice preparation used, showing a C1V1 801 expressing astrocytes and a patched CeM neuron, recorded in whole-cell voltage-clamp 802 configuration to record IPSCs. (c) Frequency of IPSCs in CeL neurons before (basal), during 803 (AstrOpto) and after (Wash) AstrOpto activation (λ 542 nm, 3 min long, 1 s width pulse at 0,5 804 Hz) in control condition (left, n = 19) or following BAPTA loading in the CeL astrocyte network 805 (right, BAPTAstro, n = 9). (d) Frequency of IPSCs in CeL neurons before (basal), during 806 (TGOT, 0.4 μ M) and after (Wash) TGOT application (left, n = 17). This effect can be blocked 807 by loading BAPTA in the CeL astrocyte network (middle, BAPTAstro, n = 17) and by prior incubation of the NMDAR antagonist AP5 (right, AP5, 50 µM, n = 5). (e) Experimental 808 809 strategy for the specific deletion of OTRs in mice CeL astrocytes (GFAP OTR KO). (f) 810 Frequency of APs in CeL neurons before (basal), during (TGOT, 0.4 µM) and after (Wash) 811 TGOT application in acute brain slices from WT (left, blue, n = 27) and GFAP OTR KO mice 812 (right, red, n = 15). Example traces of the various conditions are displayed on the right. 813 Patch-clamp data are expressed as averaged frequency plus SEM across cells; linked white circles indicate individual cell values. $p^{*} < 0.05$, $p^{***} < 0.001$, Friedman and Dunn's Multiple 814 815 comparisons, *p < 0.05, **p < 0.01, ***p < 0.001, Unpaired *t*-test or Mann-Whitney U test. 816 (Statistics in Table Extended Data Table 6).

817

818 Figure 7. CeL astrocytes modulate CeA behavioral correlates of comfort and are 819 required for their OTR-mediated modulation. (a, e) Experimental strategy for the specific 820 expression of C1V1 in mice CeL astrocytes (a, AstrOpto) or the specific deletion of OTRs in 821 mice CeL astrocytes (e, GFAP OTR KO). The treatments applied are color coded as the 822 legend key indicate. Control indicate a vehicle injection. (b, f) 4 weeks after the SNI surgery. 823 mechanical pain threshold was assessed on the neuropathic paw before (Ctrl) and at 824 different time points after either TGOT injection or C1V1 activation of CeL astrocytes (gray 825 arrow) for sham (top) and SNI (bottom, gray box) animals (c, g) Anxiety levels were 826 assessed through measurements of the time spent in the closed arms of the elevated plus 827 maze after drugs injections or C1V1 light-driven activation of CeL astrocytes for sham (top) 828 and SNI (bottom, gray box). (d, h) Conditioned place preference (CPP) was assessed 829 through measurements of the Δ time spent in the paired chamber before and after pairing. 830 Pairing was realized through drugs injections or C1V1 light-evoked activation of CeL 831 astrocytes for sham (top) and SNI (bottom, gray box). Data are expressed as averages 832 across rats or mice plus SEM. n = 4-18 per group (details and statistics in Extended Data

Table 7). # P < 0.001, ## P < 0.01 Wilcoxon signed rank test; P < 0.05, ** P < 0.01, *** P < 0.01, 833 0.001; ANOVA or mixed-design ANOVA followed by posthoc Bonferroni test (Statistics in 834 835 Table S7). (i) Oxytocin-dependent cellular interactions in the CeA. We hypothesize that OT 836 released from axons of PVN neurons within the CeL activates OTR+ astrocytes (red) which 837 consequently spread their activation to neighboring OTR- astrocytes (green) through, at least 838 partly, gap junctions. Subsequently, the CeL astrocytes release D-serine which gates the 839 activation of NMDAR on CeL interneurons, ultimately increasing their firing rate. This in turn 840 inhibits CeM output projection neurons, resulting in anxiolysis and the promotion of a positive 841 emotional state.

- 843 METHODS
- 844

845 Animals 846

847 Animals were housed under standard conditions with food and water available ad libitum and 848 maintained on a 12-hour light/dark cycle and all experiments were conducted in accordance 849 with EU rules and approbation from French Ministry of Research (01597.05). For ex vivo and 850 in vivo experiments, Wistar rats or C5BL/6 mice were used. Ex vivo experiments used 851 animals between 18 and 25 days old, except in experiments where rAAVs were injected, in 852 which case animals were between 2 and 6 months old at the time of sacrifice. In vivo 853 experiments used 2-month-old animals at the time of the first surgery.

854

855 Specific deletion of OTRs in CeL astrocytes. To specifically ablate OTRs in CeA astrocytes, transgenic cKO mice, in which loxP sites flank the OTR coding sequence²³, 856 857 received bilateral injections (280 nl) of rAAV-GFAP-GFP-IRES-Cre. Following four weeks of 858 expression of the viral proteins, mice were intracardially perfused with 1x PBS and 4% PFA. 859 Brain sections were used for FISH (OTR mRNA) and IHC (GS) to verify the validity of the 860 approach. Representative images and quantifications are provided in Figure 2H, Extended 861 Data Fig. 2g-h.

862

863 Specific deletion of Cx30 and Cx43 in astrocytes. To specifically impaire gap-junctions 864 coupling, we used Cx30-/-Cx43fl/fl:hGFAP-Cre mice (Cx30/Cx43 double KO), which were previously characterized^{24,51,52}, with conditional deletion of Cx43 in astrocytes⁵³ and 865 additional deletion of Cx30⁵⁴. 866

- 868 **Cloning and Production of rAAV Vectors:**
- 869

867

870 The generation of rAAVs allowing for the specific expression of the protein of interest in OT-871 cells is described in our previous work⁴. Briefly, the conserved promoter region of 2.6 kb was 872 chosen using the software BLAT from UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat), was 873 amplified from BAC clone RP24-388N9 (RPCI-24 Mouse, BACPAC Resources, CHORI, 874 California, USA) and was subcloned into a rAAV2 backbone carrying an Ampicillin-875 resistance.

876

877 To construct the OTp-C1V1(t/t)-TS-mCherry AAV vector we used previously cloned OTp-DIO-GFP-WRE plasmid² equipped with the characterized 2.6 kb OT promoter⁴. In this 878 879 plasmid the DIO-GFP sequence was replaced by C1V1(t/t)-TS-mCherry from the rAAV 880 CaMKIIa-C1V1(t/t)-TS-mCherry (Addgene, plasmid #35500).

881

882 To generate GFAP-C1V1(t/t)-TS-mCherry AAV vector, we replaced the CamKIIa promoter 883 from the rAAV CaMKIIa-C1V1(t/t)-TS-mCherry by the Gfa promoter from the pZac2.1 884 gfaABC1D-tdTomato (Addgene, plasmid: 44332). The cell type specificity of the rAAV 885 carrying the Gfa promoter was confirmed⁵⁵. In analogy, the generation of the GFAP-GFP-886 IRES-Cre vector was achieved using pZac2.1 gfaABC1D-tdTomato (Addgene, plasmid: 887 44332). First, the promoter was cloned into a rAAV2 backbone and sticky ends were blunted 888 with EcoR1 and Basrg1. Next, pAAV-CamKIIa-C1V1(t/t)-TS-mCherry was blunted using 889 BamHI and BsrGI. Finally, the pBS-ires cre construct was used and IRES-Cre was inserted 890 into the GFAP-driven vector resulting in the GFAP-GFP-IRES-Cre construct.

Production of chimeric virions (recombinant Adeno-associated virus 1/2; rAAV 1/2) was described in⁴. Briefly, human embryonic kidney cells 293 (HEK293; Agilent #240073) were calcium phosphate-transfected with the recombinant AAV2 plasmid and a 3-helper system. rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc., San Diego, California, USA) and are ~10¹³ genomic copies per ml for all rAAV vectors used in this study.

- 898 899 Surgeries
- 900

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901 Neuropathic Pain Model: Spared Nerve Injury (SNI) Procedure

903 Animals were randomly separated in two groups to undergo either posterior left hindpaw SNI 904 or sham procedure, with right hindpaw untouched. Animals were anaesthetized using 905 isoflurane at 1.5–2.5%. Incision was made at mid-thigh level using the femur as a landmark 906 and a section was made through the biceps femoris. The three peripheral branches (sural, 907 common peroneal and tibial nerves) of the sciatic nerve were exposed. Both tibial and 908 common peroneal nerves were ligated using a 5.0 silk suture and transected. The sural nerve was carefully preserved by avoiding any nerve stretch or nerve contact²⁸. For animals 909 910 undergoing sham surgery, same procedure was performed but nerves remained untouched. 911 Animals were routinely observed daily for 7 days after surgery and daily tested by the 912 experimenter (Extended Data Fig. 6). Besides observing weight, social and individual 913 behavior, the operated hindpaw was examined for signs of injury or autotomy. In case of 914 autotomy or suffering, the animal was euthanized in respect of the ethical recommendations 915 of the EU. No analgesia was provided after the surgery in order to avoid interference with 916 chronic pain mechanisms and this is in accordance with our veterinary authorization. 917 Suffering was minimized by careful handling and increased bedding.

918

919 Stereotaxic Surgery: Injections of rAAV Vectors

920

921 Stereotaxic surgery was performed under deep ketamine-xylazine anesthesia, using the 922 KOPF (model 955) stereotaxic system. For specific control of rats CeA astroglial cells, 200 nl 923 of rAAV serotype 1/2 (GFAPp-C1V1(t/t)-mCherry, cloned from plasmids #35500 and 44332, 924 Addgene) were injected bilaterally at the coordinates corresponding to CeL: rostro-caudal: -925 2.7mm, medio-lateral: 4.2mm, dorso-ventral: -8.0mm (From Paxinos and Watson Atlas). For 926 specific control of OT neurons, 200 nl of rAAV serotype 1/2 (OTp-C1V1(t/t)-mCherry or OTp-927 ChR2-mCherry) were injected bilaterally at the coordinates corresponding to each 928 hypothalamic OT nuclei. PVN: rostro-caudal: -1.8mm; medio-lateral: +/-0.4mm; dorso-ventral: 929 -8.0mm; SON: rostro-caudal: -1.4mm; medio-lateral: +/-1.6mm; dorso-ventral: -9.0mm; AN: 930 rostro-caudal: -2mm; medio-lateral: +/-1.2mm; dorso-ventral: -8.5mm (From Paxinos and 931 Watson Atlas). For specific deletion of OTR in mice CeL astrocytes, 280 nl of rAAV serotype 932 1/2 (GFAPp-GFP-IRES-Cre) were injected bilaterally at the coordinates corresponding to 933 CeL: rostro-caudal: -1.4mm, medio-lateral: +/-2.6mm, dorso-ventral: -4.3mm (From Paxinos 934 and Watson Atlas) in OTR cKO mice.

935

936 Stereotaxic Surgery: intra-CeL Cannulae

938 **Cannulae Implantation.** Animals were bilaterally implanted with guide cannulae for direct 939 intra-CeL infusions. As guide cannulae we used C313G/Spc guide metallic cannulae 940 (Plastics one, VA, USA) cut 5.8 mm below the pedestal. For this purpose, animals were 941 deeply anesthetized with 4% isoflurane and their heads were fixed in a stereotaxic frame. 942 The skull was exposed and two holes were drilled according to coordinates that were 943 adapted from brain atlas (rat, 2.3 mm rostro-caudal; 4 mm lateral; 7.5 mm dorso-ventral 944 relative to bregma; mice, 1.4 mm rostro-caudal; 2.6 mm lateral; 4.3 mm dorso-ventral relative 945 to bregma) by comparing the typical bregma-lambda distance with the one measured in the 946 experimental animal. Two screws were fixed to the caudal part of the skull in order to have 947 an anchor point for the dental cement. Acrylic dental cement was finally used to fix the 948 cannulae and the skin was sutured. In case of long lasting experiments (neuropathy-induced 949 anxiety) with a cannula implantation at distance of the behavioral assay (> 4 weeks), 950 cannulae were sometimes lost or cloaked, and concerned animals therefore excluded from 951 testing.

952

953 Drugs Infusions. We used bilateral injections of 0.5 µl containing either vehicle (NaCl 0.9%) 954 or oxytocin receptor agonist TGOT (1 µM) dissolved in NaCl 0.9%. For this procedure two 955 injectors (cut to fit 5.8 mm guide cannulae protruding 2 to 2.5 mm beyond the lower end of 956 the cannula in older animals and 1.8 mm in 3-4 week old rats) were bilaterally lowered into 957 the guide cannula, connected via polythene tubing to two Hamilton syringes that were placed 958 in an infusion pump and 0.5 µl of liquid was injected in each hemisphere over a 2-minute 959 period. After the injection procedure, the injectors were kept in place for an additional minute 960 in order to allow a complete diffusion of liquid throughout the tissue. Rats were subsequently 961 left in the home cage for 15 minutes to recover from the stress of the injection and then 962 handled for mechanical pain threshold or anxiety assessment. Animals that received TGOT 963 injections for the first experiment (mechanical sensitivity assessment) were switched to the 964 vehicle injected groups for the elevated plus maze experiment.

965

967

966 Stereotaxic Surgery: intra-CeL Optical Fiber

968 **Optical Fiber Implantation.** Sham and rAAVs injected animals both underwent a single 969 surgical procedure in which after vector injection or no injection for sham, optical fibers 970 designed to target the CeL were implanted and firmly maintained on the skull using dental 971 cement. See "*cannulae implantation*" for the surgical procedure. Implantable optical fibers 972 were homemade using optical fiber cut at appropriate length (FT200EMT, Thorlabs, NJ, 973 USA) inserted and glued using epoxy based glue in ferrules (CFLC230-10, Thorlabs, NJ, 974 USA).

975

976 Horizontal and Coronal Slices

977

978 Slices Preparations. In all cases, animals were anaesthetized using ketamine (Imalgene 90 979 mg/kg) and xylazine (Rompun, 10 mg/kg) administered intraperitoneally. Intracardiac 980 perfusion was then performed using one of the following artificial cerebrospinal fluids 981 (aCSFs) dissection solutions. For animals between 18 and 25 days old, an ice-cold sucrose 982 based dissection aCSF was used containing (in mM): Sucrose (170), KCI (2.5), NaH₂PO₄ 983 (1.25), NaHCO₃ (15), MgSO₄ (10), CaCl₂ (0.5), HEPES (20), D-Glucose (20), L-Ascorbic acid 984 (5), Thiourea (2), Sodium pyruvate (3), N-Acetyl-L-cysteine (5), Kynurenic acid (2). For 985 animals between 2 and 6 months old, an ice-cold NMDG based aCSF was used containing

986 (in mM): NMDG (93), KCI (2.5), NaH₂PO₄ (1.25), NaHCO₃ (30), MgSO₄ (10), CaCl₂ (0.5), 987 HEPES (20), D-Glucose (25), L-Ascorbic acid (5), Thiourea (2), Sodium pyruvate (3), N-988 Acetyl-L-cysteine (10), Kynurenic acid (2). In both cases, pH was adjusted to 7.4 using either 989 NaOH or HCl, this after bubbling in 95% O₂-5% CO₂ gas, bubbling which was maintained 990 throughout the duration of use of the various aCSFs. Those aCSFs formulae were based on the work of⁵⁶. Following decapitation, brain was swiftly removed in the same ice-cold 991 992 dissection aCSFs as for intracardiac perfusion, and 350 µm thick horizontal slices containing 993 the CeA was obtained using a Leica VT1000s vibratome. For experiments in Extended Data 994 Fig. 2a-b, coronal slices of the same thickness containing the PVN were used. Upon slicing, 995 brain slices were hemisected and placed, for 1 hour minimum before any experiments were 996 conducted, in a room tempered holding chamber, containing normal aCSFs. For 2 to 6 month 997 old animals, slices were first let for 10 minutes in 35°C NMDG aCSF before placing them in 998 the holding chamber at room temperature. Normal aCSF, also used during all ex vivo 999 experiments, is composed of (in mM): NaCl (124), KCl (2.5), NaH₂PO₄ (1.25), NaHCO₃ (26), 1000 MgSO₄ (2), CaCl₂ (2), D-Glucose (15), adjusted for pH values of 7.4 with HCL or NaOH and 1001 continuously bubbled in 95% O₂-5% CO₂ gas. All aCSFs were checked for osmolality and 1002 kept for values between 305-310 mOsm/L. In electrophysiology or calcium imaging 1003 experiments, slices were transferred from the holding chamber to an immersion recording 1004 chamber and superfused at a rate of 2 ml/min with normal aCSFs unless indicated otherwise. 1005

1006 Drug Application. OTR agonists were bath applied through a 20s long pumping of agonist 1007 solution, corresponding to several times the volume of the recording chamber. Other drugs 1008 (antagonists, TTX, etc.) were applied for at least 20 minutes in the bath before performing 1009 any experiments. BAPTA (or BAPTA-free solution for controls) loading of CeL astrocytes was 1010 realized following⁵⁷ protocol. Two distant CeL astrocytes per slice (label with SR101, 1 μ M) 1011 were patched in whole cell configuration and voltage steps were applied (2 Hz, Δ 40 mV) to 1012 help loading the BAPTA contained in the patch pipette (in mM): MgCl₂ (1), NaCl (8), ATPNa₂ 1013 (2) GTPNa₂ (0.4) HEPES (10), BAPTA (40) and osmolality checked to be between 275-285 1014 mOsm/I. The whole cell configuration was maintained during 45 min to allow BAPTA diffusion into the astrocyte network⁵⁸. 1015

1016

1017 Calcium Imaging and Identification of Astrocytes

1018 To identify astrocytes, SR101 (1 µM) was added to aCSF in a culture well and slices were 1019 incubated for 20 minutes at 35°C. The specificity of SR101 labelling to astrocytes of the CeL 1020 was verified through patch-clamp experiments, the results of which can be found in Extended 1021 Data Fig. 2c-d. The synthetics calcium indicators OGB1 or Rhod-2 was bulk loaded following 1022 an adapted version of the method described previously⁵⁹ reaching final concentrations of 1023 0.0025 % (~20 µM) for calcium indicators, 0.002% Cremophor EL, 0.01 % Pluronic F- 127 1024 and 0.5% DMSO in aCSF, and incubated for 45 to 60 minutes at 38°C. Upon incubation time, 1025 slices were washed in aCSF for at least an hour before any recording was performed. 1026 Astrocytes recorded for this study were those co-labeled, in rats for SR101 and OGB1 and in 1027 mice for GFP and Rhod2. The spinning disk confocal microscope used to perform astrocyte 1028 calcium imaging was composed of a Zeiss Axio examiner microscope with a 40x water 1029 immersion objective (numerical aperture of 1.0), mounted with a X-Light Confocal unit -1030 CRESTOPT spinning disk. Images were acquired at 2Hz with either a Rolera em-c² emCCD 1031 or an optiMOS sCMOS camera (Qimaging, BC, Canada). Cells within a confocal plane were 1032 illuminated for 100 to 150 ms for each wavelength (SR101 and Rhod-2: 575 nm, OGB1 and 1033 GFP: 475 nm) using a Spectra 7 LUMENCOR. The different hardware elements were 1034 synchronized through the MetaFluor software (Molecular Devices, LLC, Ca, USA) which was 1035 also used for online. Astrocytic calcium levels were measured in hand drawn ROIs 1036 comprising the cell body plus, when visible, proximal processes. In all recordings, the Fiji 1037 rolling ball algorithm was used to increase signal/noise ratio. Further offline data analysis 1038 was performed using a custom written python-based script available on editorial website. 1039 Intracellular calcium variation was estimated as changes in fluorescence signals. To take into 1040 account micro-movements of the specimen on long duration recordings, the fluorescence 1041 values were also calculated for SR101 (or GFP) and subsequently subtracted to the ones of 1042 OGB1 (or Rhod2), except in the case of Figure 2a-d, where astrocytes were identified 1043 through SR101 fluorescence after the recordings, to avoid unwanted stimulation of the C1V1 1044 opsin. On this last case, recordings in which movements / drifts were visible were discarded. 1045 Then, a linear regression and a median filter was applied to each trace. Calcium transients 1046 was detected using the find peaks function of the SciPy library. More precisely, fluorescence 1047 variation was identified as a calcium peak if its prominence exceeds the standard deviation 1048 (or two times the standard deviation for recordings acquired with the sCMOS camera) and if 1049 the maximum peak value surpasses 50 fluorescence units (or 3 units for sCMOS recordings). 1050 ROI with zero calcium variations were excluded from the analysis. The remaining ROI were 1051 considered as living astrocytes and the number of peaks was quantified before and after the 1052 drug application. All data were normalized according to the duration of the recording and 1053 astrocytes was labelled as "responsive" when their AUC or their calcium transient frequency 1054 was increased by at least 20% after drug application. Because the time post-stimulation is 1055 longer than the baseline (10 min vs 5 min), the probability of observing a spontaneous 1056 calcium peak is stronger post-stimulation. To avoid this bias, astrocytes with only one 1057 calcium peak during the whole recording were not considered as responsive. Finally, all data 1058 were averaged across astrocytes per slice, and this results was used as statistical unit. All 1059 data were expressed as ratio (baseline/drug effect), a ratio of 1 meaning neither an increase 1060 nor a decrease of the measured parameter. For inter-ratio comparison, parametric or non-1061 parametric (depending on data distribution) unpaired statistical tests were used. Fiji software 1062 was also used on SR101 / OGB1 pictures to produce illustrative pictures. All calcium imaging 1063 experiments was conducted at controlled room temperature (26°C).

1064

1065 Electrophysiology

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1067 Whole cell patch-clamp recordings of CeL neurons, CeL astrocytes and CeM neurons were 1068 visually guided by infrared oblique light visualization of neurons and completed by SR101 1069 fluorescence observation for astrocytes. Patch-clamp recordings were obtained with an Axon 1070 MultiClamp 700B amplifier coupled to a Digidata 1440A Digitizer (Molecular Devices, CA, 1071 USA). Borosilicate glass electrodes (R = $3.5 - 7 M\Omega$) with inner filament (OD 1.5 mm, ID 0.86) 1072 mm; Sutter Instrument, CA USA) were pulled using a horizontal flaming/brown micropipette 1073 puller (P97; Sutter Instrument, CA, USA). Recordings were filtered at 2 kHz, digitized at 40 1074 kHz and stored with the pClamp 10 software suite (Molecular Devices; CA, USA). Analysis of 1075 patch-clamp data were performed using Clampfit 10.7 (Molecular Devices; CA, USA) and 1076 Mini analysis 6 software (Synaptosoft, NJ, USA) in a semi-automated fashion (automatic 1077 detection of events with chosen parameters followed by a visual validation).

1078

1079 Whole-Cell Recording of CeL Neurons. Recording pipettes were filled with an intracellular
 1080 solution containing (in mM): KMeSO₄ (125), CaCL₂ (2), EGTA (1), HEPES (10), ATPNa₂ (2),
 1081 GTPNa₂ (0.3). The pH was adjusted to 7.3 with KOH and osmolality checked to be between

1082 290-295 mOsm/l, adjusted with sucrose if needed. For miniature excitatory post synaptic 1083 currents (mEPSCs) recordings, neurons were recorded in voltage clamp and hold at a 1084 membrane potential of -65 mV. For action potentials (APs) recordings, neurons were 1085 recorded in current clamp and hold at I = 0. Series capacitances and resistances were 1086 compensated electronically throughout the experiments using the main amplifier. For 1087 mEPSCs measurements in CeL neurons (Figure 4), whole cell recordings were conducted in a Mg²⁺ free aCSF, also containing biccuculin (10 μ M) and TTX (1 μ M) as in⁶⁰. Average 1088 1089 events frequencies per cell were calculated on 20s windows, chosen for TGOT or 1090 photostimulation during maximal effect, as determined by the visually identified maximal 1091 slope of the cumulative plot of the number of events. CeM neurons were classified as TGOT-1092 responsive when the average event frequency was increased by at least 20% during 20s 1093 after TGOT application when compared to baseline average frequency. Baseline and 1094 recovery frequencies were measured respectively at the beginning and end of each 1095 recording. All patch-clamp experiments were conducted at room temperature.

1096

1097 Biocytin Filling of CeL Astrocytes. In the lateral part of the central amygdala slices 1098 visualized with infrared-differential contrast optics, astrocytes were identified by their 1099 morphological appearance revealed by SR101 and the absence of action potentials in 1100 response to depolarizing current injections. Cells were patched with pipettes filled with (in 1101 mM) 110 K-Gluconate, 30 KCl, 4 ATPNa₂, 10 phosphocreatine, 0.3 GTPNa₂, 10 HEPES and 1102 5 biocytin (pH: 7.3; 310 mOsm). After obtaining whole-cell configuration astrocytes were hold 1103 at -80 mV and typical filling time was 45 minutes. Then the pipettes were carefully retracted 1104 and slices were incubated for additional 20 minutes in the oxygenated aCSF before fixation. 1105 Only one cell was filled per slice. Slices with filled cells were immersion-fixed at 4°C for 5 1106 days in 4% PFA-PBS solution. Next, the slices were flat-embedded in 6% Agar-PBS, areas 1107 of interest were cut out of, re-embedded onto the Agar block and Vibratome-cut into 80 µm 1108 thick free-floating sections. The sections then were incubated with Avidin conjugated to Alexa 1109 Fluor488 (1:1000) (Thermo Fisher) in 1% Triton-PBS at 4°C, washed in PBS, mounted and 1110 cover-slipped. The tissue was analyzed and images taken at Leica TCS SP5 Confocal 1111 Microscope.

1112

1113 Whole-cell Recording of CeM Neurons. Pipettes were filled with an intracellular solution 1114 containing (in mM): KCI (150), HEPES (10), MgCl₂ (4), CaCl₂ (0.1), BAPTA (0.1), ATPNa₂ 1115 (2), GTPNa₂ (0.3). pH was adjusted to 7.3 with KOH and osmolality checked to be between 1116 290-295 mOsm/L, adjusted with sucrose if needed. All cells were hold at a membrane 1117 potential of -70 mV. Series capacitances and resistances were compensated electronically 1118 throughout the experiments using the main amplifier. Average events frequencies per cell 1119 were calculated on 20s windows, chosen for TGOT or photostimulation during maximal 1120 effect, as determined by the visually identified maximal slope of the cumulative plot of the 1121 number of events. CeM neurons were classified as TGOT-responsive when the average 1122 IPSCs frequency was increased by at least 20% during 20s after TGOT application when 1123 compared to baseline average frequency. Baseline and recovery frequencies were measured 1124 respectively at the beginning and end of each recording. All patch-clamp experiments were 1125 conducted at room temperature.

- 1126
- 1127 Immunohistochemistry and in situ Hybridization
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1129 In situ Hybridization for OTR mRNA in Rat CeL. The probe for OTR mRNA was in vitro 1130 transcripted from a 902-bp fragment containing 133-1034 bases of the rat OTR cDNA (NCBI 1131 Reference Sequence: NM 012871.3) subcloned into pSP73 Vector (Promega). The 1132 digoxigenin (DIG)-labeled antisense and sense RNA probe from the linearized oxtr cDNA 1133 template was synthesized using DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics). 1134 Sections containing 2 consecutive sections of the CeL (corresponding to Bregma: 2.5) were 1135 processed for fluorescent in situ hybridization (FISH). Rats were transcardially perfused with 1136 PBS followed by 4% PFA. Brains were dissected out and post fixed overnight in 4% PFA at 1137 4°C with gentle agitation. 50 µm vibratome sections were cut, collected and fixed in 4% PFA 1138 at 4°C overnight. The free-floating sections were washed in RNase-free PBS, immersed in 1139 0.75% glycine in PBS, treated with 0.5 µg/ml proteinase K for 30 min at 37 °C, acetylated 1140 with 0.25% acetic anhydride in 0.1 M triethanolamine, and then hybridized with DIG-labeled 1141 RNA probe overnight at 65 °C. After RNase treatment and following intensive wash, the 1142 hybridized DIG-labeled probe was detected by incubation with Anti-Digoxigenin-POD (1:200; 1143 11207733910; Roche Diagnostics) for 3 days at 4 °C. Signals were developed with tyramid 1144 signal amplification method. Rhodamine-conjugated tyramide was synthesized by coupling 1145 NHS-Rhodamine (Pierce Biotechnology, Thermo Fisher Scientific) to Tyramine-HCI (Sigma-1146 Aldrich) in dimethylformamide with triethylamine. For the quantification of OTR mRNA-1147 positive astrocytes, all confocal images were obtained using the same laser intensities and 1148 processed with the same brightness / contrast settings in Adobe Photoshop. Since the in situ 1149 signal for the OTR mRNA in astrocytes was weak, we first calculated the average intensity 1150 (signal intensity of all pixels divided by the total number of pixels) of the rhodamine-stained 1151 OTR mRNA signal for each individual section containing the CeL. Next, we calculated the 1152 standard deviation for each individual confocal image based on the intensity of all pixels 1153 comprising the image. We defined the threshold for OTR mRNA-positive astrocytes: If more 1154 than 1/4 of all pixels comprising an astrocyte soma displayed a signal intensity exceeding the 1155 average background intensity by more than 4-times the standard deviation, the astrocytes 1156 were considered as OTR mRNA-positive.

1157

1158 Astrocytes Markers. The aldehyde dehydrogenase 1 antibody is a commonly used marker 1159 Therefore, we used the ALDH1L1 for glial cells, including astrocytes. for 1160 immunohistochemistry in our initial experimental studies (Extended Data Fig. 1c, S4a). 1161 However, due to inconsistencies in staining quality as a result of batch-dependent antibody 1162 properties, especially in combination with the OTR mRNA FISH, we decided to employ Glutamine Synthetase (GS, Figure 1). GS is a commonly used glial marker⁶¹, which stains 1163 1164 astrocyte cell bodies, faint processes and even astrocytes not expressing GFAP. Using GS, 1165 we achieved consistent results in combination with our OTR mRNA FISH.

1166

1167 Glutamine Synthase, ALDH1L1 Colocalization with OTR mRNA in Rat CeL. After 1168 development and washing steps the sections were stained with antibodies against GS 1169 (mouse monoclonal, 1:500, ref: MAB302, MerckMilipore), ALDH1L1 (rabbit polyclonal, 1:500, 1170 ref: ab87117, abcam), in PBS and kept at 4°C on a shaker in a dark room overnight. After 1171 intensive washing with PBS, sections were stained with the respective secondary antibodies 1172 AlexaFluor488 (goat anti-mouse, 1:1000, ref: A11001, life technologies) and AlexaFluor680 1173 (goat anti-mouse, 1:1000, ref: A27042, ThermoFischer Scientific) for 2 hours at RT. 1174 Following intensive washing with PBS, sections were mounted using Mowiol.

1176 Double in situ Hybridizations for OTR mRNA and GFAP mRNA in Mice CeL. Fluorescent 1177 in situ hybridization (FISH) in Extended Data Fig. 1d was performed on 25-µm cryostat-cut 1178 coronal sections prepared from fresh-frozen mouse brain (male C57BL/6J, P22). After 1179 extraction, brains were immediately frozen in Tissue-Tek O.C.T. compound and stored at -80 1180 degrees Celsius. ISH was performed according to the manufacturer's instructions (Advanced 1181 Cell Diagnostics) for Fresh Frozen RNAscope Multiplex Fluorescent Assay. Treatment of 1182 amygdala containing sections were adjusted with the 3-plex negative control and then 1183 coexpression of OTR and GS examined using ACD designed target probes as well as the 1184 nuclear stain DAPI. Single plan images were collected with an upright laser scanning 1185 microscope (LSM-710, Carl Zeiss) using a 40x-objective with keeping acquisition parameters 1186 constant between control and probe treated sections.

1187

1188 AAV-GFAP-C1V1(t/t)-mCherry Specificity. After 3 weeks of vector expression in the brain, 1189 rats were transcardially perfused with 4% paraformaldehyde solution. Tissue blocks, 1190 containing CeA were dissected from the fixed brain and Vibratome-cut into 50 µm thick free-1191 floating sections. After several rinse steps sampled sections were blocked with 5% NGS in 1192 PBS and incubated for 48 h at 4°C with polyclonal rabbit anti-ALDH1L1 antibody (1:500, 1193 Abcam) in 1% Triton-PBS buffer, containing 0,1 % NGS. Appropriate secondary antibody 1194 (AlexaFluor488 conjugated goat anti-rabbit (1:1000, LifeTechnologies) was used for further 1195 antigen detection. Intrinsic mCherry fluorescence of vector-expressing cells was strong 1196 enough to detect them in the tissue without any additional antibody enhancement. The 1197 immunolabeled sections were mounted onto Superfrost slides, cover-slipped with Mowiol, 1198 analyzed and documented using LEICA SP5 confocal microscope.

1199

1200 Three-dimensional assessment of astrocyte complexity and interaction analysis using 1201 Imaris. For the 3D reconstruction of astrocytes, we took Z-stack images (50 µm depth, 1µm 1202 steps, 40x magnification) CeL using a Zeiss LSM 780 confocal microscope (1024x1024 pixel, 1203 16-bit depth, pixel size 0.63-micron, zoom 0.7). Raw czi files were used for further analysis 1204 using Imaris software (Version 9.31, Oxford Instruments). First, Imaris was used to 1205 reconstruct the astrocyte surface using the following custom settings: surfaces Detail 0.700 1206 µm (smooth); thresholding Background subtraction (Local Contrast), diameter of largest 1207 Sphere, which fits into the object: 2.00; Color: base, diffusion transparency: 65%. After 1208 surface reconstruction, we used the filter function to remove unspecific background signals: 1209 Filter: Volume max – 400 µm 3. It is important to note that these settings have to be adjusted 1210 for every new batch / IHC staining to guarantee a reliable reconstruction. All astrocytes with 1211 incomplete somata (cut by either the x, y or z plane) were manually removed and not 1212 included in further analysis Fused astrocytes that were falsely recognized as one entity by 1213 the software were manually separated using the cut function, or entirely removed from the 1214 sample if a separation was not feasible. The 'filter/area function' was used to remove small 1215 astrocytic segments that occurred during manual deletion. After deletion of all background 1216 signals the 'mask all' function was used to create the final surface reconstruction. Next, the 1217 surface reconstruction was used as the template for the filament reconstruction using the 1218 following custom settings: detect new starting points: largest Diameter 7.00 µm, seed points 1219 0.300 µm; remove seed points around starting points: diameter of sphere regions: 15 µm. 1220 Seed points were corrected for (either placed in or removed from the center of the somata) 1221 manually if the Imaris algorithm placed them incorrectly. All surface and filament parameters 1222 were exported into separate Excel files and used for data analysis. All images used for 1223 analysis were taken with the same confocal settings (pinhole, laser intensity, digital gain and 1224 digital offset). Sholl analysis was performed using Imaris in the filament reconstruction mode 1225 and individual data sets were exported into separate Excel files for further analysis. each 1226 individual sphere) per individual astrocyte. For the nearest neighbor and interaction analysis 1227 we used the 'Native Distance Measurements' function as depicted in this video: 1228 https://imaris.oxinst.com/learning/view/article/imaris-9-5-native-distance-measurements. In 1229 brief, we reconstructed astrocytic surfaces based on the GFAP fluorescence and OTR 1230 mRNA signal. Next, we manually labelled OTR+ and OTR- astrocytes and performed the 1231 native distance measurement allowing us to assess the shortest distance between GFAP-1232 positive processes of different astrocytes. We defined 'astrocytic interaction' when GFAP-1233 positive processes of two different astrocytes were no further than 1µm apart. It is important 1234 to note that this method does not allow the discrimination of different astrocytic entities so 1235 that several close contacts (contacts being defined as a distance of less than 1µm between 1236 GFAP-positive processes or endfeets from two different astrocytes) originating from the 1237 same astrocyte result in a higher number of total interactions. For the nearest neighbor 1238 analysis, we calculated the distance from the center of the soma to the nearest astrocyte 1239 neighbor using GS fluorescence and an artificially created sphere that was placed within the 1240 soma and measured the distance accordingly. Distribution plots and correlations as well as 1241 all statistics were performed using GraphPad Prism 8.0.

1242

1243 Optogenetics1244

1245 **Ex vivo.** We opted for a ChR1/VChR1 chimaera channel rhodopsin displaying a red-shifted 1246 absorption spectrum, referred here as $C1V1^{22}$. This choice was made over a classical 1247 channelrhodopsin-2 to avoid unwanted stimulation of OT axons while imaging our 488nm 1248 light sensitive calcium indicator (OGB1). Optogenetic green light stimulation of C1V1 in *ex* 1249 *vivo* experiments was performed using either the Spectra 7 LUMENCOR (λ 542 nm) or light 1250 source X-Cite® 110LED from Excelitas Technologies through a Cy3 filter, controlled via 1251 MetaFluor or Clampex driven TTL pulses, respectively.

1252

1253 In vivo. Animals were habituated to the fixation of an optical fiber on the ferrule without light 1254 stimulation for one week before the experiment. In all cases, optical fibers were attached to 1255 the ferrules using an adapter (ADAF2, Thorlabs, NJ, USA) and animals let free to move in a 1256 typical home cage for the duration of the stimulation. Implanted optical fibers were connected 1257 to two lasers (LRS-0532-GFM-00100-03 LaserGlow 532nm DPSS Laser System) and the 1258 output power adjusted to correspond to 20 to 30 mW measured at the tip of 200 µm diameter 1259 fibers similar to the one implanted. Stimulation of 500 ms duration at a frequency of 0.5Hz 1260 were given for 3 min.

- 1261
- 1262 Behavior
- 1263

1264 Mechanical Sensitivity Assessment. In experiments with rats, we used a calibrated 1265 forceps (Bioseb, Chaville, France) previously developed in our laboratory to test the animal 1266 mechanical sensitivity⁶². Briefly, the habituated rat was loosely restrained with a towel 1267 masking the eyes in order to limit stress by environmental stimulations. The tips of the 1268 forceps were placed at each side of the paw and a graduate force applied. The pressure 1269 producing a withdrawal of the paw, or in some rare cases vocalization, was considered as 1270 the nociceptive threshold value. This manipulation was performed three times for each hind 1271 paw and the values were averaged as being the final nociceptive threshold value. In 1272 experiments with mice, we used von Frey filaments tests. Mechanical allodynia (a symptom 1273 of neuropathic pain) was tested using von Frey hairs and results were expressed in grams. 1274 Tests were performed during the morning starting at least 2 h after lights on. Mice were 1275 placed in clear Plexiglas boxes (7 cm x 9 cm x 7 cm) on an elevated mesh floor. Calibrated 1276 von Frey filaments (Bioseb) were applied to the plantar surface of each hindpaw until they 1277 just bent in a series of ascending forces up to the mechanical threshold. Filaments were 1278 tested five times per paw and the paw withdrawal threshold (PWT) was defined as the lower 1279 of two consecutive filaments for which three or more withdrawals out of the five trials were 1280 observed.

1281

1282 *Elevated Plus Maze.* Following protocol from⁶³, the arena is composed of four arms, two 1283 open (without walls) and two closed (with walls; rats 30 cm high; mice 15 cm high). Arms are 1284 10 cm wide, 50 cm long and elevated 50 cm off the ground for rats and 5 cm wide, 30 cm 1285 long and elevated 40 cm of the ground for mice. Two lamps with intensity adjustable up to 50 watts were positioned on the top of the maze, uniformly illuminating it. Animals were video 1286 1287 tracked using a video-tracking systems (Ethovision Pro 3.16 Noldus, Wageningen, 1288 Netherlands and Anymaze, Stoelting Europe, Ireland). After each trial, the maze was cleaned 1289 with 70% ethanol and dry with paper towel. Twenty minutes after intracerebral injections or 1290 directly after optical stimulation, the animal was let free at the center of the plus maze, facing 1291 the open arm opposite to where the experimenter is, and was able to freely explore the entire 1292 apparatus for six minutes. Total time and time spend in closed and open arms were recorded 1293 in seconds and the percentage of time spent in closed arms was calculated as a measure of 1294 anxiety-like behavior. As internal control, the total distance travelled during the test period 1295 was guantified and compared between all different groups (Extended Data Fig. 7). Animals 1296 falling from the apparatus during the test, freezing more than 50% of the total time, or with 1297 cannulae/optic fiber issues, were removed from the analysis.

1298

1299 **Conditioned Place Preference.** The device is composed of two opague conditioning boxes 1300 (rats: 30x32 cm; mice: 22x22 cm) and one clear neutral box (30x20 cm) Animals were video 1301 tracked using a video-tracking system (Anymaze, Stoelting Europe, Irland). After each trial, 1302 the device was cleaned with a disinfectant (Surfa'Safe, Anios laboratory). Based on⁶⁴, all rats 1303 underwent a 3 days habituation period during which they were able to freely explore the 1304 entire apparatus for 30 min. On the day 3, behavior was record for 15 min to verify the 1305 absence of pre-conditioning chamber preference. The time spend in the different 1306 compartment were measured and paired compartment was chosen as the compartment in 1307 which rat spent the less time during the 3rd day of habituation. On day 4, animals were 1308 placed the morning in one compartment for 15 min with no stimulation (unpaired box). Four 1309 hours after, the animal were placed 15 min in the opposite box (paired box) and CeL 1310 astrocyte expressing C1V1 vector were optogenetically stimulated (3 min – 1 s light pulse at 1311 0.5 Hz - λ 542 nm) or TGOT micro-infused through intracerebral cannulae. On day 5, the 1312 animals were place in the CPP box and allowed to freely explore the entire apparatus during 1313 15 min. As internal control, the total distance traveled during the test period was guantified 1314 and compared between all different groups (Extended Data Fig. 7). Rats falling spending 1315 more than 80% of the total time in a single chamber before the conditioning, or with 1316 cannulae/optic fiber issues, were removed from the analysis.

1317

1318 QUANTIFICATION AND STATISTICAL ANALYSIS

All parametrical statistical tests presented in figure captions or manuscript were performed following correct verification of the assumptions on the distribution of data, and if not nonparametric tests were used. Statistical test displayed in the text are not shown on figures. Tests were performed using either GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) or the SciPy Python-based library⁶⁵. All values, group compositions and statistical tests for each experiment and figure panel are detailed in Extended Data Tables 1-7.

1327 1328 SUPPLEMENTARY DISCUSSION – TECHNICAL LIMITATIONS

1329

1330 We acknowledged a number of technical limitations in our study, which are discussed in 1331 details here:

1332 mRNA detection. It is surprising to find that the loss of OTR expression in astrocytes results 1333 in a loss of function of OTR signaling in the CeA-CeM projection, especially considering the 1334 high proportion of OTR+ neurons (up to 70%) compared to the lower fraction of OTR+ 1335 astrocytes (18%) in the CeL. One methodological limitation here is that we remained limited 1336 to measuring mRNA levels through FISH, and could not measure OTR expression at the 1337 protein level, notably due to the poor availability of reliable OTR antibodies. It is also possible 1338 that a comparison between the astrocytes' and neurons' contents of OTR mRNA is biased, 1339 indeed astrocytes have a lower total mRNA contents than neurons, as publicly available 1340 databases of single cell RNA sequencing indicate^{66,67}. Interestingly, a similar discrepancy 1341 between low receptor levels observed in astrocytes compared to neurons and yet a crucial 1342 functional relevance of astrocytes' receptors in neuromodulation is also observed for another 1343 GPCR, the CB1 receptor⁶⁸ (and references therein).

1345 C1V1 red-shifted opsin to activate astrocytes. It has been recently demonstrated that the 1346 activation of such depolarizing channel rhodopsins in astrocytes or neurons can lead to a 1347 significant leak of potassium ions in the extracellular space, resulting in increased neuronal 1348 excitability⁶⁹. Yet, we provide results in which we buffered the astrocytes intracellular calcium 1349 using BAPTA infusion before activating astrocytic C1V1, and found that it abolished its 1350 effects on CeA neurons (Fig. 4-6). Furthermore, we demonstrate that the AstrOpto effect on 1351 CeA neuronal circuit is dependent on NMDARs (Extended Data Fig. 5a). This indicates that 1352 consequences of astrocytic C1V1 activation are dependent on the evoked astrocyte calcium 1353 signaling rather than potassium leakage.

1354

1344

Bulk loading of organic calcium indicator dyes. It should also be noted that we used bulk loading of organic calcium indicator dyes. This means we could not precisely measure cytosolic calcium changes in fine astrocyte processes⁷⁰, which may have revealed more complex activities of astrocytes. We note it has been proven that endogenous or exogenous (designer receptor exclusively activated by designer drugs receptors, DREADD) GPCR signaling in astrocytes produces a global increase in calcium in both the cell body and processes(⁶⁸ and references therein).



Figure 1



f













Figure 2











AUC (norm. to baseline) 2 0

WT

4





С Cx30/Cx43 KO mouse





Cx30/Cx43 KO



0 0 6 Ca²⁺ transient freq. (norm. to baseline) 3 0





Figure 5



Figure 6



















rAAV-GFAPp-C1V1(t/t)-mCherry (AstrOpto)







 $0 \ Ca^{2+}$















b













e







