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Morphine withdrawal recruits habenular cytokine signaling to reduce synaptic excitation and sociability

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35 **Abstract**

36 **Opiate withdrawal promotes a wealth of negative emotional states**
37 **including low sociability. The lateral habenula (LHb) encodes aversive**
38 **stimuli and contributes to negative symptoms of drug withdrawal.**
39 **However, whether adaptations at precise habenular circuits are**
40 **instrumental for the opiate withdrawal state remains unknown. We**
41 **report that, in mice, morphine withdrawal (MORwd) diminishes**
42 **glutamatergic transmission onto raphe-projecting lateral habenula (LHb)**
43 **neurons. This MORwd-driven synaptic plasticity occurs along with**
44 **microglia adaptations and increased cytokine levels. Specifically, it**
45 **requires TNF α release and subsequent activation of neuronal TNF-**
46 **Receptor-1, to ultimately gate sociability deficits. Hence, drug-driven**
47 **modulation of cytokines controls synaptic efficacy and opiate**
48 **withdrawal-evoked behavioral adaptations.**

49

50 **Introduction**

51 Opiate withdrawal produces negative emotional states including low mood
52 and reduced sociability, contributing to relapse during drug abstinence^{1,2}.
53 Dysfunction of the lateral habenula (LHb) – a nucleus controlling
54 monoaminergic systems and processing aversive stimuli – underlies
55 depressive symptoms typical of mood disorders and drug withdrawal³.
56 Opiates also affect LHb function^{4,5}. However whether morphine withdrawal
57 reorganizes LHb circuits to underlie specific aspects of the withdrawal state
58 remains unknown.
59 Using mice, we found that morphine withdrawal emerging after naloxone
60 precipitation or a prolonged abstinence period i. reduces glutamatergic
61 transmission onto LHb neurons projecting to the raphe nucleus and ii.
62 remodels microglia and cytokine signaling. Finally, the withdrawal-driven
63 cytokine adaptations are instrumental for the diminished synaptic strength and
64 opiate-withdrawal sociability deficits.

65

66

67 Results

68 Morphine withdrawal-driven synaptic plasticity in the LHb

69 We subjected mice to naloxone-precipitated MORwd to examine its
70 repercussions on glutamatergic synapses onto LHb neurons¹. Indeed,
71 aberrant LHb excitatory transmission underlies negative symptoms in rodent
72 models of depression and addiction³. Spontaneous excitatory postsynaptic
73 current (sEPSC) amplitudes, but not frequencies, were reduced only in LHb
74 neurons located in the medial aspect (^{Med}LHb; lateral LHb, ^{Lat}LHb;
75 Supplementary Fig. 1a and 1b). Accordingly, MORwd diminished
76 AMPAR:NMDAR ratios solely in the medial territory (Fig. 1a and
77 Supplementary Fig. 1c) without affecting neurotransmitter release assessed
78 by trains of synaptic stimulation (Supplementary Fig. 1d). **Recordings**
79 **obtained 1 hour after the last MOR injection (without naloxone) yielded saline-**
80 **comparable AMPAR:NMDAR ratios (Fig. 1b). In contrast, spontaneous**
81 **MORwd persistently decreased AMPAR:NMDAR ratios in the ^{Med}LHb up to 30**
82 **days after the last MOR injection (Fig. 1b).**

83 To assess whether MORwd affects AMPAR conductance or number, we used
84 peak-scaled non-stationary fluctuation analysis of ^{Med}LHb-recorded sEPSCs⁶.
85 While estimated single-channel conductance remained unaffected in MORwd
86 slices, the number of channels opened at the peak positively correlated with
87 amplitude values (Supplementary Fig. 1e). MORwd failed to alter AMPAR-
88 EPSC rectification properties (Supplementary Fig. 1f), whereas it reduced
89 glutamate uncaging-evoked AMPAR:NMDAR ratios, yielding a decrease only
90 in absolute AMPAR currents (Supplementary Fig. 1g). Altogether, this
91 suggests that MORwd reduces, in a territory-specific fashion, the number of
92 AMPARs without affecting their biophysical properties, NMDARs or
93 presynaptic glutamate release.

94 MORwd-evoked glutamatergic plasticity occurs onto ^{Med}LHb neurons, which
95 innervate downstream structures including the raphe nucleus and the ventral
96 tegmental area (VTA)⁷. We therefore examined the strength of glutamatergic
97 synapses onto retrobeads-labeled Raphe– and VTA–projecting LHb neurons
98 (LHb_{Raphe} and LHb_{VTA} respectively). MORwd diminished AMPAR:NMDAR

ratios solely in LHb_{Raphe} neurons (Fig. 1c, d) suggesting that MORwd plasticity is specific for discrete habenular circuits.

Cytokine signaling in the LHb interfaces morphine withdrawal plasticity

Which induction mechanism gates MORwd-driven plasticity onto ^{Med}LHb neurons? Inflammatory responses and glial cell activation emerge during opioid withdrawal^{8,9}. Indeed, spontaneous morphine withdrawal drives adaptations in microglia and pro-inflammatory cytokine release (i.e. tumor necrosis factor- α (TNF α))¹⁰. Notably, cocaine also leads to reduced microglia arborization along with TNF α -dependent AMPAR internalization, partly underlying drug-mediated behavioral adaptations^{9,11}. We thereby examined microglia morphology and cytokine levels during MORwd. We found that, within the ^{Med}LHb, MORwd i. reduced microglial markers including Iba1 and CD68 and ii. diminished microglial cell volume (Fig. 2a–d). In parallel, naloxone- and spontaneous MORwd increased TNF α immunolabeling within the LHb (Fig. 2e and Supplementary Fig. 2a–d). Altogether, these findings support the engagement of inflammatory responses and cytokine signaling within the LHb during MORwd.

We then reasoned that if MORwd promotes TNF α release, artificially increasing its levels should prove sufficient to recapitulate MORwd-driven synaptic plasticity. Accordingly, incubating LHb-containing slices from saline-injected mice with exogenous TNF α reduced AMPAR:NMDAR ratios in the ^{Med}LHb. This effect was absent in ^{Lat}LHb, and occluded by naloxone-precipitated as well as spontaneous MORwd (Fig. 3a–b and Supplementary Fig. 3a). TNF α release may arise from microglial Toll-Like Receptor 4 (TLR-4) signaling¹². Systemically activating TLR-4 with the agonist MPLA in MOR-treated mice, instead of naloxone, mimicked MORwd plasticity (Fig. 3c). Moreover, MPLA application in acute slices from MOR-treated animals reduced AMPAR currents in ^{Med}LHb, but not in ^{Lat}LHb (Fig. 3d and Supplementary Fig. 3b). This MPLA-driven reduction in EPSCs did not occur in the presence of a dominant negative peptide, which blocks the soluble form of TNF α (XENP1595; Supplementary Fig. 3c)⁹. Furthermore, MORwd

132 occluded MPLA-driven synaptic depression (Fig. 3d) and systemic injection of
133 the dominant negative peptide XENP1595 prevented MORwd-induced
134 plasticity (Supplementary Fig. 3d). Altogether, this supports *i.* TLR-4
135 expression within the LHb (See Allen Brain Atlas), *ii.* its effect on AMPARs via
136 TNF α signaling, and *iii.* the necessity and sufficiency of TNF α for MORwd-
137 driven reduction of LHb glutamatergic transmission.

138

139 **Requirement of TNF-R1 for morphine withdrawal synaptic and** 140 **behavioral adaptations**

141 TNF α triggers its central effects partly through TNF receptor-type-1 (TNF-
142 R1)¹³. To test whether this applies to MORwd-driven plasticity in the LHb, we
143 employed TNF-R1^{fl/fl} mice to Cre-dependently knock-down TNF-R1
144 expression in LHb neurons (Fig. 4a–c). After viral injection, AAV_{Cre}-TNF-R1^{fl/fl}
145 mice failed to show MORwd-driven AMPAR:NMDAR ratio reduction compared
146 to AAV_{Control}-infused mice (Fig. 4d). This highlights the necessity of neuronal
147 TNF-R1 for MORwd-driven depression of synaptic AMPARs in LHb.

148

149 MORwd drives negative emotional symptoms among which social
150 detachment¹. Similarly, LHb dysfunction contributes to the negative
151 behavioural states emerging in mood disorders and addiction, although its
152 implications for sociability remains poorly addressed^{14,15,16}. Hence, we
153 examined the contribution of the LHb-to-Raphe pathway, the locus of MORwd
154 plasticity, for social behavior. We employed an intersectional chemogenetic
155 viral approach to reduce the efficiency of the LHb-to-Raphe projection. This
156 combined the retrograde expression of cre-recombinase (HSV-Cre in dorsal
157 raphe) with cre-dependent expression of hM4Di (rAAV-hM4Di-mCherry,
158 DREADDi, in LHb; Fig. Supplementary 4a). Reducing LHb-to-Raphe
159 efficiency with clozapine-N-oxide diminished social preference (Fig.
160 Supplementary 4b), supporting LHb contribution to social behaviors.
161 Next, we recapitulated MORwd-driven reduction in social preference in
162 C57Bl6 mice (Fig. 4e and Supplementary Fig. 4c–f)¹. We then prepared slices
163 from MORwd mice showing low or high sociability scores, and found that
164 AMPAR:NMDAR ratios, recorded in ^{Med}LHb, positively correlated with the

165 social score (Fig. 4f). This indicates that reduced synaptic strength in the LHb
166 predicts opiate-WD-driven sociability deficits.

167

168 Notably, microglia and TNF α signaling also contributes to social behaviors^{17–}
169 ¹⁹. We thereby predicted that LHb TNF-R1 ablation, not only prevents MORwd
170 synaptic plasticity (Fig. 4d), but also MORwd low sociability. Indeed, MORwd-
171 driven sociability deficits were absent after cre-dependent LHb TNF-R1
172 knock-down (Fig. 4g and Supplementary Fig. 4g–k). This genetic intervention
173 did not affect locomotion (Supplementary Fig. 3l).

174

175 Discussion

176 We found that MORwd-driven TNF α release requires neuronal TNF-R1 to
177 reduce AMPAR transmission onto raphe-projecting, medially-located, LHb
178 neurons. This ultimately gates MORwd-driven social impairment, a negative
179 symptom typical of opiate withdrawal.

180

181 The TNF α -TNF-R1 engagement within the LHb represents a previously
182 unidentified mechanism underlying precise cellular and behavioural aspects of
183 MORwd. Yet this is consistent with the following: *i.* drugs and drug-
184 withdrawal-mediated modulation of AMPAR transmission partly rely on
185 cytokine signaling⁹; *ii.* inhibition of TLR-4 attenuates MORwd symptoms²⁰; *iii.*
186 TNF-Rs contribute to social behaviors¹⁸. Notably, in pyramidal neurons of the
187 hippocampus and cortex, TNF α regulates AMPAR exocytosis and their
188 surface expression^{21,22}. This phenomenon is opposite at striatal synapses
189 where, similarly to the LHb, TNF α application results in decreased AMPAR
190 transmission^{9,23}. This divergence may arise from different TNF α release
191 dynamics, TNF receptors expression and signaling, or alternatively the
192 properties of AMPAR anchoring at postsynaptic compartments within the LHb.
193 MORwd modifies the morphology of microglia in the LHb. This is, at least
194 partly, consistent with previous findings⁹, yet it remains correlative with
195 respect to TNF α levels. This heightens the need to fill the gap in
196 understanding microglia function and its relationship with TNF α within the
197 habenular complex. Overall, while pharmacotherapies targeting pro-

198 inflammatory pathways in substance abuse are missing, our data further
199 support cytokine signaling as a cellular pillar for aspects of drug addiction and
200 more largely of psychiatric disorders^{9,24}.

201

202 MORwd-driven TNF α -dependent depression of AMPAR transmission occurs
203 at LHb_{Raphe} neurons. From a circuit standpoint, this may provide an ‘anti-
204 social’ signal²⁵ likely through reduced actions onto raphe neuronal
205 populations. This is consistent with the evidence reported here that
206 chemogenetic manipulation of the LHb-to-Raphe projection diminishes
207 sociability. Alongside, dopamine- and serotonin-containing neurons in the
208 raphe contribute to social behaviors, and medially-located LHb neurons
209 monosynaptically connect to the latter^{26–29}. Understanding the repercussions
210 of LHb activity onto raphe neuronal subtypes during MORwd remains an
211 important aspect for future investigation.

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213 In conclusion, our data support the participation of cytokine-mediated
214 plasticity for opiate-evoked negative symptoms, a mechanism by which LHb
215 ultimately contributes to the addiction spiral.

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231 **Figure legends**

232 **Figure 1 MORwd-driven projection-specific synaptic depression in LHb.**

233 (a) Naloxone-precipitated MORwd (NP-MORwd) effect on AMPAR:NMDAR
234 ratios from ^{Lat}LHb ($n_{\text{mice/cells}}=7-8/11$; saline (gray) versus NP-MORwd (orange):
235 $t_{20}=0.0548$, $P=0.957$) and ^{Med}LHb: ($n_{\text{mice/cells}}=7-8/12-13$; saline (black) versus
236 NP-MORwd (red): $t_{23}=2.210$, $*P=0.037$) (b) Left: schematic of spontaneous
237 withdrawal timeline. Right: AMPAR:NMDAR ratios recorded from ^{Med}LHb 1
238 hour, 10, 20 or 30 days after saline or MOR treatment ($n_{\text{mice/cells}}=3-6/11-22$,
239 saline 1 hour and 10 days after last injection pooled (black) versus MOR
240 withdrawal 1 hour (open red) and MOR 10, 20 and 30 days withdrawal (red),
241 $F_{(4, 62)}=3.90$ one-way ANOVA, $**P=0.007$). (c) Retrobeads in Raphe (left) and
242 retrogradely-labeled LHb_{Raphe} neurons (right) images. AMPAR:NMDAR ratios
243 from LHb_{Raphe} neurons ($n_{\text{mice/cells}}=5/10-11$, saline (black) versus NP-MORwd
244 (red), $t_{19}=3.153$, $**P=0.005$). (d) Same as c but in VTA ($n_{\text{mice/cells}}=2-4/6-7$,
245 saline (black) versus NP-MORwd (red), $t_{11}=0.575$, $P=0.577$). Sal, saline; Mor,
246 morphine; Nlx, naloxone; PAG, periaqueductal gray; DG, dentate gyrus; MHb,
247 medial habenula; SNr, substantia nigra pars reticulata.

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
249 **Figure 2 Microglia and cytokine signaling in the LHb during MORwd.**

250 (a) Representative max-projection of confocal acquisition of the LHb,
251 depicting Iba1-positive microglia (left). Representative max-projection of
252 microglia obtained in the ^{Med}LHb of mice in saline and NP-MORwd (top right).
253 Representative 3D reconstruction of Iba1 positive microglia, containing CD68-
254 positive structures, acquired in the ^{Med}LHb of mice in saline and NP-MORwd
255 (bottom right). (b) Analysis of relative intensity of Iba1 microglia
256 immunoreactivity in ^{Med}LHb in the different experimental groups (Saline (black)
257 $n_{\text{mice/cells}}=4/366$, NP-MORwd (red) $n_{\text{mice/cells}}=4/328$, $t_{692}=3.305$, $***P=0.001$). (c,
258 d) Quantitative analysis of microglial cell volume (based on Iba1-
259 immunoreactivity) and volume of CD68-positive structures. Values are
260 normalized to Saline control (Saline (black) $n_{\text{mice/cells}}=4/89$, NP-MORwd (red)
261 $n_{\text{mice/cells}}=4/83$; Iba1 volume, $t_{170}=3.05$, $**P=0.003$; CD68/Iba1 volume,
262 $t_{170}=2.65$, $**P=0.008$). (e) TNF α (cyan) and DAPI (magenta) immunostaining

263 and normalized TNF α optical density in the LHb ($n_{\text{mice/samples}}=7-8/3-6$, saline
264 (black) versus NP-MORwd (red), $t_{13}=2.991$, $*P=0.0104$).

265

266 **Figure 3 TNF α signaling modulates habenular AMPAR-mediated**
267 **transmission.**

268 (a) AMPAR:NMDAR ratios  from naloxone-injected saline mice
269 in ^{Lat}LHb without (–) or with (+) exogenous TNF α ($n_{\text{mice/cells}}=2/7-9$,
270 saline+naloxone (–) TNF  vs saline+naloxone (+)TNF α  lighter
271 gray : $t_{14}=0.37$, $P=0.717$). (b) AMPAR:NMDAR ratios without (–) or with (+)
272 exogenous TNF α   from naloxone-injected saline (black and
273 gray) and morphine (red and pink) mice in ^{Med}LHb ($n_{\text{mice/cells}}=3-5/10-15$,
274 interaction factor $F_{(1, 42)}=4.90$ two-way ANOVA, $*P=0.039$). (c) ^{Med}LHb
275 AMPAR:NMDAR ratios from saline or MPLA-treated, instead of naloxone,
276 mice ($n_{\text{mice/cells}}=3-4/10-11$, morphine/saline (open red) versus morphine/MPLA
277 (pink), $t_{19}=3.070$, $**P=0.006$). (d) MPLA (1 g/ml) effect on AMPAR-EPSCs in
278 ^{Med}LHb (baseline (1) vs post-MPLA (2)) (MOR (open red), $n_{\text{mice/cells}}=4/11$,
279 $t_{10}=5.168$, $***P=0.0004$; NP-MORwd (filled red), $n_{\text{mice/cells}}=3/10$ cells, $t_9=0.779$,
280 $P=0.456$; MOR versus NP-MORwd, $t_{19}=2.419$, $*P=0.026$).

281

282 **Figure 4 TNFR1 is required for MORwd-driven synaptic and behavioural**
283 **adaptations.**

284 (a) Experimental protocol using TNF-R1^{fl/fl} mice. (b) Image and (c)
285 quantification of AAV-Cre-infected LHb neurons (magenta) and total LHb
286 neurons (cyan) from three mice (M1, M2 and M3). (d) AMPAR:NMDAR ratios
287 from ^{Med}LHb of: AAV_{Control}-TNF-R1^{fl/fl} (saline (gray) versus NP-MORwd (red))
288 or AAV_{Cre}-TNF-R1^{fl/fl} (saline (open gray) versus NP-MORwd (open pink))
289 ($n_{\text{mice/cells}}=7-8/9-14$, interaction factor $F_{(1,44)}=4.887$ two-way ANOVA,
290 $*P=0.032$). (e) Tracking plots of social preference test (SPT) in C57/Bl6 mice.
291 Box/scatter plots showing social preference score (saline (black) versus NP-
292 MORwd (red), $n_{\text{mice}}=22$ /group, $t_{42}=2.559$, $*P=0.014$). Animals indicated with
293 pink filled circles were used for AMPAR:NMDAR ratio recordings shown in (f).
294 (f) Correlation of AMPAR:NMDAR ratios and social preference score
295 ($n_{\text{mice/cells}}=4/12$; Pearson's $r^2=0.954$; $*P=0.023$). (g) Tracking and box/scatter

296 plots of SPT in TNF-R1^{fl/fl} mice (AAV_{Control}: saline (black), NP-MORwd (red),
297 n_{mice}=20/23 mice; AAV_{Cre}: saline (open gray), NP-MORwd (open pink),
298 n_{mice}=13 mice/group, interaction factor $F_{(1, 65)} = 7.20$ two-way ANOVA,
299 **P=0.009). S, social stimulus; O, object.

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344 **Methods**

345 **Animals and morphine treatments.** C57Bl/6J wild-type (male) and 129-
346 Tnfrsf1atm3Gkl (male and female, referred as TNF-R1^{fl/fl}) mice of 4–10 weeks
347 were group-housed (three to five per cage) on a 12:12 h light cycle (lights on
348 at 7 a.m.) with food and water ad libitum. All procedures aimed to fulfill the 3R
349 criterion and were approved by the Veterinary Offices of Vaud (Switzerland;
350 License VD3172). Part of the current study was carried out in the Institut du
351 Fer a Moulin, Paris and experiments were in accordance with the guidelines
352 of the French Agriculture and Forestry Ministry. Morphine withdrawal was
353 either precipitated with naloxone or was induced naturally. For naloxone-
354 precipitated morphine withdrawal, mice were subjected to six-day
355 intraperitoneal (i.p.) morphine (20mg/kg, Cantonal Hospital of Lausanne,
356 CHUV, Switzerland) or saline injections (saline and morphine-treated animals
357 were housed together). On day 6, the last morphine/saline injection was given
358 in a separate cage, thirty minutes after which animals received an i.p. injection
359 of naloxone hydrochloride (2mg/kg, Abcam). Morphine withdrawal
360 dependence symptoms were allowed to develop in the following thirty
361 minutes, after what mice were either sacrificed for *ex vivo* electrophysiological
362 recordings or were subjected to behavioral tests.

363

364 For spontaneous withdrawal, mice were treated with morphine or saline for 6
365 days and were sacrificed for recordings 10-13, 20 or 30 days after the last
366 injection. For recordings in morphine-treated animals not in withdrawal, mice
367 were sacrificed one hour after the last morphine injection on day 6. To assess
368 TNF α involvement in morphine withdrawal plasticity, part of the animals were
369 subjected to an i.p. injection of MPLA (Monophosphoryl Lipid A, 10 μ g, a Toll-
370 like receptor 4 activator dissolved in DMSO and saline) or saline (containing
371 the same amount of DMSO as control) instead of naloxone thirty minutes after
372 the last morphine injection on day 6. Another portion of the animals received
373 an i.p. injection of a dominant negative peptide blocking the soluble form of
374 TNF α (XENP1595, 30mg/kg, Xencor, US) one hour prior the last morphine
375 or saline injection on day 6. Thirty minutes after the morphine/saline injection
376 these animals received naloxone and were sacrificed for recordings as
377 described above.

378
379 **Surgery.** Animals of at least 4 weeks were anesthetized with ketamine (150
380 mg/kg)/xylazine (100 mg/kg) i.p. (Veterinary office University of Lausanne)
381 and were placed on a stereotactic frame (Kopf, Germany). Bilateral injections
382 of 200-400 nl volume were performed through a glass needle, at a rate of
383 approximately 100 nl min⁻¹. The injection pipette was withdrawn from the
384 brain 10 min after the infusion. Retrobeads (Lumafluor) were infused into the
385 dorsal raphe nucleus (A-P:-3.5; M-L:0;D-V:-3.8 mm) or ventral tegmental area
386 (A-P:-2.4; M-L:±0.65;D-V:-4.9 mm) of C57Bl6 mice. 129-Tnfrsf1atm3Gki mice
387 were injected with either rAAV2-hSyn-eGFP or rAAV2-hSyn or CMV-Cre-
388 eGFP into the LHb (A-P:-1.35; M-L: ±0.45;D-V:-3.00 mm). In another set of
389 experiments C57Bl6 mice were injected with a herpes simplex virus derived
390 hEF1α-cre vector (MGA Gene delivery technology core, Cambridge, MA,
391 USA) in the raphe nucleus and with rAAV-DJ-EF1α-Flex-hM4D(Gi)-mCherry
392 (Gene vector and virus core, Stanford medicine, CA, USA) in the LHb.
393 Animals were allowed to recover for about 5-7 days after retrobeads injections
394 or 5 weeks after viral infusion before being submitted to morphine/saline
395 treatment. The injection sites were carefully examined for all electrophysiology
396 experiments and only animals with correct injections were used for
397 recordings. Similarly, for behavioral studies only animals with correct injection
398 sites were included in the analysis. Brain slices from mice injected with
399 retrobeads or viruses were directly examined under an epifluorescence
400 microscope.

401
402 **Ex-vivo electrophysiology.** Animals of 5 weeks were anesthetized with
403 ketamine/ xylazine; 150 mg/kg/100 mg/kg i.p. for preparation of LHb-
404 containing brain slices. Slicing was done in bubbled ice- cold 95% O₂/5%
405 CO₂-equilibrated solution containing (in mM): choline chloride 110; glucose
406 25; NaHCO₃ 25; MgCl₂ 7; ascorbic acid 11.6; sodium pyruvate 3.1; KCl 2.5;
407 NaH₂PO₄ 1.25; CaCl₂ 0.5. Coronal slices (250 μm) were prepared and
408 transferred for 10 min to warmed solution (34 °C) of identical composition,
409 before they were stored at ~22 °C in 95% O₂/5% CO₂-equilibrated artificial
410 cerebrospinal fluid (ACSF) containing (in mM): NaCl 124; NaHCO₃ 26.2;
411 glucose 11; KCl 2.5; CaCl₂ 2.5; MgCl₂ 1.3; NaH₂PO₄ 1. Recordings (flow

rate of 2.5 ml/min) were made under an Olympus-BX51 microscope (Olympus) at 32 °C. Patch-clamp experiments were performed using borosilicate glass pipettes (2.7–4 MΩ; Phymep, France). Currents were amplified, filtered at 5 kHz and digitized at 20 kHz (Multiclamp 200B; Molecular Devices, USA). Data were acquired using Igor Pro with NIDAQ tools (Wavemetrics, USA). Access resistance was monitored by a step of –4 mV (0.1 Hz). Experiments were discarded if the access resistance increased more than 20%. All recordings were made in voltage-clamp configuration. Spontaneous EPSCs were recorded either in the lateral or in the medial territory of the LHb at –60 mV in presence of picrotoxin (100 μM, Abcam) and APV (50 μM, Abcam). The internal solution contained (in mM): CsCl 130; NaCl 4; MgCl₂ 2; EGTA 1.1; HEPES 5; Na₂ATP 2; sodium creatine-phosphate 5; Na₃GTP 0.6; spermine 0.1. The liquid junction potential was –3 mV and was not compensated. For AMPAR:NMDAR ratios EPSCs were evoked through glass electrodes placed ~200 μm from the recording site using AMPI ISO-Flex stimulator. A mixture of AMPA and NMDA currents were evoked at +40 mV (in presence of picrotoxin). The two components were pharmacologically isolated by adding APV in the recording solution and by subsequent identification of the individual currents via digital subtraction. For glutamate uncaging experiments MNI-glutamate (4-methoxy-7-nitroindolinyll-caged L-glutamate 500μM, Tocris) was added to the recording solution. Uncaging was obtained via a single-path photolysis head (Prairie Technologies) connected to a solid-state laser (Rapp Optoelectronics, Germany; 405 nm, duration 1 ms, diameter 3–5μm, 250-300 μm from soma). AMPAR:NMDAR ratios in uncaging experiments were calculated as follows: AMPA-EPSC at –60 mV/NMDA-EPSCs at +40 mV and the individual components were identified as previously described, using the late component of the EPSC at 30 ms after the onset (Maroteaux and Mameli, 2012). Rectification index was computed by recording AMPA-EPSC at –70 and +40 mV and was calculated as follows: (AMPA-EPSC at –70/AMPA-EPSC at +40)/1.75. To assess presynaptic release properties, trains of AMPAR-EPSCs were evoked using extracellular stimulating electrode (5 pulses at 5Hz, 10Hz and 20Hz). The amplitudes of EPSCs trains were normalized to the amplitude of the first pulse. When indicated recordings were

performed from retrogradely labeled and fluorescently identified LHb neurons. Some experiments were performed in LHb-containing slices incubated for minimum one hour with exogenous TNF α (100ng/ml). To test the effect of MPLA on AMPAR transmission, neurons were patched either in the lateral or the medial territory of the LHb and EPSCs were evoked with extracellular stimulation. Following a ten-minute baseline, MPLA (1 μ g/ml) was added to the recording solution and EPSCs were recorded minimum 40 minutes after. Some experiments were performed in presence of the TNF α -dominant negative peptide α 6mg/1ml; XENP1595, Xencor, US) in the recording solution.

456

457 **Non stationary fluctuation analysis.**

458 A peak-scaled nonstationary fluctuation analysis (NSFA) was performed on
459 sEPSCs (# of events, 70–250) (Synaptosoft, USA). sEPSCs were selected
460 by: fast rise time alignment, stable baseline holding current, and the absence
461 of spurious fluctuations during the sEPSCs decay. The variance–amplitude
462 relationship of sEPSC decay was plotted and fitted with the equation
463 $\sigma^2 = iI - I^2/N + \sigma_b^2$ (where i is the mean single-channel AMPA current, I is the
464 mean current, N is the number of channels activated at the peak, $N = \text{mean}$
465 $\text{amplitude}/i$; and σ^2 is the baseline variance). i was estimated as the slope of
466 the linear fit of the first portion of the parabola of the fitted sEPSC decay. The
467 goodness-of-fit was assessed with a least-squares algorithm. The unitary
468 current was converted in conductance based on the reversal potential of
469 evoked EPSCs (0 mV) and the holding potential (–60 mV). Conductance and
470 average EPSC amplitude, mean rise time, mean decay time, access
471 resistance, or background noise variance had no correlation ($p > 0.4$)
472 (Maroteaux and Mameli, 2012).

473

474 **Histology and immunofluorescence.** Mice were injected daily with
475 saline/morphine (20mg/kg, i.p.) for 6 days. Some mice were left to develop
476 spontaneous withdrawal, while others received naloxone (2mg/kg, i.p.)
477 injection 30 min after the last saline/morphine injection on day 6. After 10-13
478 days of spontaneous withdrawal or 30 min after naloxone injection mice were

479 anesthetized and perfused with cold 4% paraformaldehyde (PFA) in PBS
480 (phosphate-buffered saline). The brains were extracted, post-fixed in 4% PFA
481 in PBS, and incubated in 30% sucrose in PBS until they sank. 30 μ m slices
482 were cut at the cryostat, and stored in PBS containing 0.02% NaN₃ for future
483 analysis. For the immunofluorescence, the slices were incubated 2h in
484 blocking buffer (5% NGS, 0.3% Triton-X in PBS) and then 24h at 4°C with the
485 primary antibody solution (mouse anti-TNF α antibody, ab1793, Abcam, 1:100
486 in blocking buffer). After extensive rinses, the secondary antibody was applied
487 (goat anti-mouse IgG-conjugated Alexa 488, Invitrogen, 1:400 in blocking
488 buffer, 24h at 4°C). The slices were then incubated in a 1:400 DAPI solution in
489 PBS, extensively rinsed, mounted on glass slides with Pro-Long Gold Antifade
490 Reagent (Invitrogen) and coverslipped. Images were acquired with an
491 epifluorescent microscope with a 20x objective (AxioVision, Zeiss) using the
492 same parameters for all the samples. The images were analyzed and
493 processed with ImageJ software. Optical density was measured on the whole
494 LHb area, and normalized on the neighboring thalamus [LHb-
495 Thal/(LHb+Thal)]. 3-6 slices distributed in the rostrocaudal axis were analyzed
496 per each animal (8 morphine, 7 saline).

497

498 **Microglia analysis**

499 Mice were anesthetized and perfused with cold 4% paraformaldehyde (PFA)
500 in PBS (phosphate-buffered saline). The brains were extracted, post-fixed in
501 4% PFA in PBS, and incubated in 30% sucrose in PBS until they sank. 30 μ m
502 slices were cut at the cryostat, and stored in PBS containing 0.02% NaN₃ for
503 future analysis. Brain sections were permeabilized at room temperature (RT)
504 in 0.5% Triton X-100 (Sigma) for 1 hr RT, followed by 1 hr RT blocking in 2%
505 BSA 0.5% Triton X-100 and overnight incubation with primary antibody (Iba1
506 1:1000, Wako Chemicals, Cat. No. 019-19741 and CD68 1:400, Bio-Rad Cat.
507 No. MCA1957) at 4°C. Upon washing, sections were incubated 2 hr RT with
508 Alexa-fluorophore-conjugated secondary antibodies (Invitrogen), and
509 counterstained with DAPI (Invitrogen).
510 Confocal microscopy was performed with a TCS-SP5 (Leica) Laser Scanning
511 System, by using a 20X dry objective and images were processed and
512 analyzed by Fiji Software or Imaris Software (Bitplane, Switzerland), as

appropriate. Imaris was used for 3D rendering of confocal images for quantification of volumes. For density analysis, for each acquisition, the DAPI channel was max-projected and the medial and lateral portions of the lateral habenula were manually drawn as region of interest. Then, stacks ranging from 15 to 20 μm in thickness, with z-step size of 1 μm , were processed as follows: Iba1 and DAPI channels were thresholded in Fiji and multiplied to each other for each stack, with the image calculator function. The resulting thresholded stack was max-projected and the microglia nuclei were counted with Analyze Particle function. For cell soma size and Iba1 intensity, each acquisition was max-projected and the contour of cell somata in the medial portion of the lateral habenula were manually drawn based on the Iba1 immunoreactivity, and analyzed per size in μm^2 and intensity. 3D imaging analysis was performed by Imaris applying recorded algorithms (fixed thresholds for signal intensity) to all the images of the same experiment, in order to produce unbiased signal quantification. In each experiment, one brain slice per animal (n=4) per each group was acquired. The microglial cell volume and the volume of phagocytic structures were reconstructed based on the absolute intensity of Iba1 and CD68 signals, respectively. The volume of CD68 was then normalized for the Iba1 volume, to take in account the cell size.

535

536 **Behavior.**

537 **Social preference test.** A three-chambered social preference test was used, consisting in a rectangular Plexiglas arena (60 × 40 × 22 cm) (Ugo Basile, Varese, Italy) divided into three chambers. The walls of the center chamber had doors to allow free access to all compartments. The luminosity was around 10 lux. Thirty minutes after naloxone injection each mouse was placed in the arena for a habituation period of 10 min and was allowed to freely explore the whole empty arena. The social preference test was performed immediately after the end of the habituation: two enclosures with vertical bars were placed in the middle of the two lateral compartments, while the central chamber remained empty. One enclosure was empty (serving as an

547 inanimate object) whereas the other contained a social stimulus (unfamiliar
548 juvenile mouse 25 ± 1 days old). The enclosures allowed visual, auditory,
549 olfactory and tactile contact between the experimental mice and the social
550 stimuli mice. The juvenile mice in the enclosures were habituated to the
551 apparatus and the enclosures for 3 days before the experiment and each one
552 of them served as a social stimulus for no more than 2 experimental mice (at
553 least 6 weeks old). The test lasted 10 minutes where experimental mice were
554 allowed to freely explore the apparatus and the enclosures. The position of
555 the empty and juvenile-containing enclosures alternated and was
556 counterbalanced for each trial to avoid any bias effects. Every session was
557 video-tracked and recorded using Ethovision XT (Noldus, Wageningen, the
558 Netherlands) or AnyMaze (Stoelting, Ireland), which provided an automated
559 recording of the entries and time spent in the compartments, the distance
560 moved and the velocity. The time spent in each chamber was assessed and
561 then used to determine the preference score for the social compartment as
562 compared to the object compartment (social/(social + object)). The arena was
563 cleaned with 1% acetic acid solution and dried between trials.

564

565 **Analysis and statistics.** Animals were randomly assigned to experimental
566 groups. Compiled data are always reported and represented as whisker box
567 plots (whisker top/bottom represent 90/10th percentile, box top/bottom
568 represent 75/25th percentile and median) or mean \pm SEM, with single data
569 points plotted (single cell for electrophysiology and single animal for
570 behavioral experiments). Animals or data points were not excluded and
571 normality test was applied. When applicable, statistical tests were paired or
572 unpaired t-test and one-way or two-way ANOVA. Significance for correlations
573 was obtained applying Pearson's estimates. Testing was always performed
574 two-tailed with $\alpha = 0.05$.

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Author contributions

K.V., A.T. and M.M. performed and analyzed *ex vivo* electrophysiological recordings. A.L.L and J.C. contributed to *ex-vivo* recordings. K.V and A.T.

676 performed the behavioral experiments. M.T. I.M. and L.M. performed
677 molecular biology experiments. M.T., C.B. and S.T. provided support for
678 behavioral experiments. A.M. and R.C.P. performed and analyzed the
679 experiments related to microglia morphology. A.V. provided conceptual and
680 experimental input related to the TNF α signaling, including use of TNFR1^{fl/fl}
681 mice and their breeding. K.V. and M.M. conceptualized and designed the
682 study and wrote the manuscript with the help of all authors.

683

684 **Competing financial interests**

685 The authors declare no competing financial interests.

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687 **Statement on data availability**

688 The data sets generated during and/or analyzed during the current study are
689 available from the corresponding author on reasonable request.

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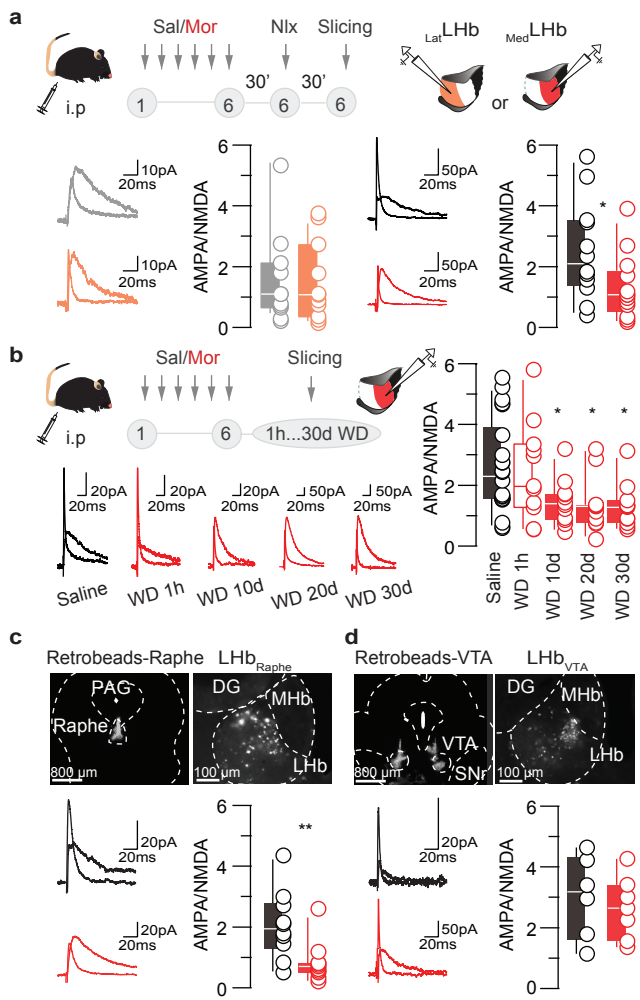


Fig 1., Valentinova, Tchenio et al.

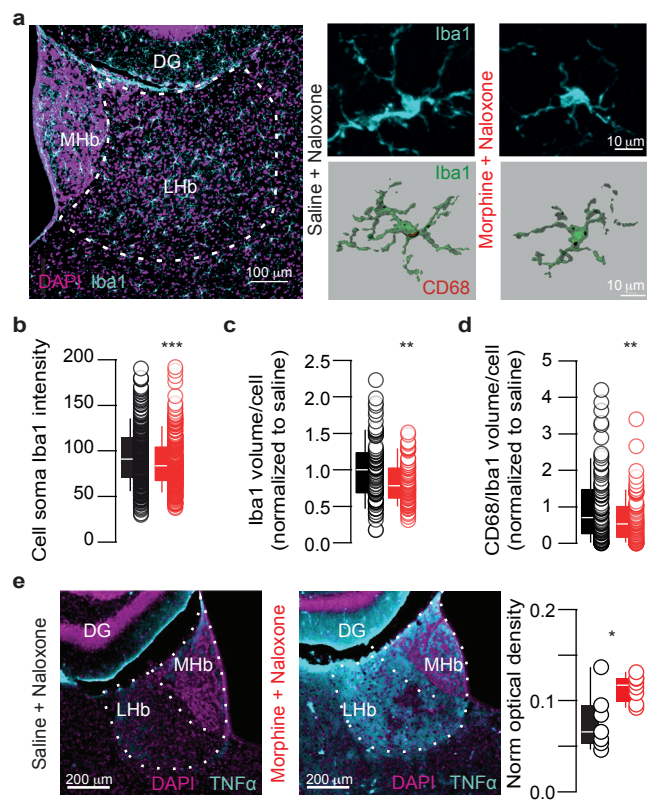


Fig 2., Valentinova, Tchenio et al.

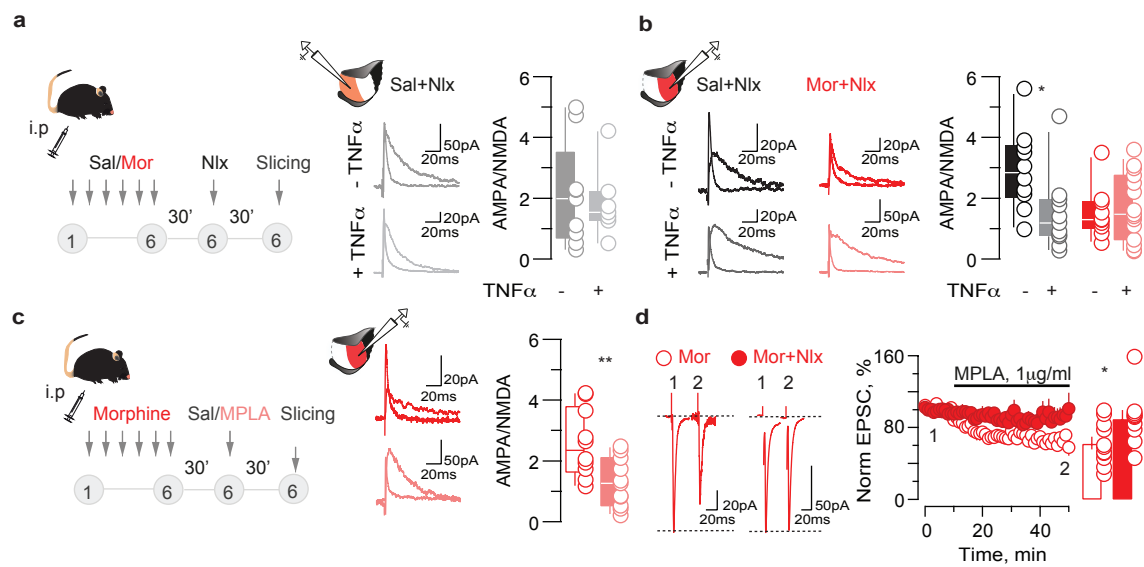


Fig 3., Valentinova, Tchenio et al.

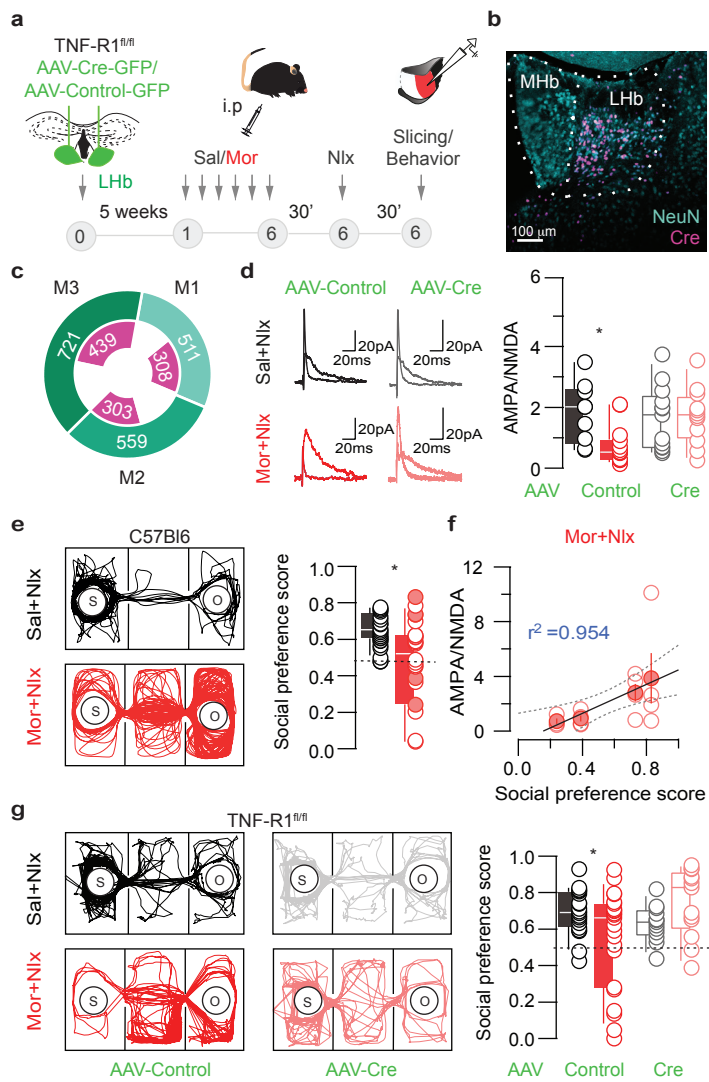


Fig 4., Valentinova, Tchenio et al.