SUPPLEMENTAL DATA

Title: **A central amygdala–ventrolateral periaqueductal gray matter pathway for pain in a mouse model of depression-like behavior**

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Supplemental Figure 1 to 7

Supplemental Table 1

**Supplemental Figure 1**

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**Supplemental Figure 1|** Identification of outputs of Amygdala neurons. (**A**) Schematic diagram of AAV-Cre-eGFP injected into the amygdala for anterograde tracing. (**B**) Typical examples of green fluorescent protein-expressing post-synaptic neurons in some of the main downstream targets of amygdala. bed nucleus of the stria terminalis (BNST); zona incerta (ZI); parafascicular thalamic nucleus (PF); mesencephalic reticular formation (mRt); parabrachial nucleus (PB); parvicellular reticular nucleus (PCRt) of C57BL/6J mice. (**C**) Quantification of green fluorescent protein-expressing post-synaptic neurons for fig. 1B and supplemental 1B in the whole imaging area per slice (0.54 mm2/slice, *n* = 4 slices from 2 mice). aca: anterior commissure, anterior; Amy: amygdala; DRN: dorsal raphe nucleus; fr: fasciculus retroflexus; GFP: green fluorescent protein; IRt: intermediate reticular nucleus; LPAG: lateral periaqueductal gray; scp: superior cerebellar peduncle, basal part; Sp5O: spinal trigeminal nucleus, oral part; vlPAG: ventrolateral periaqueductal gray. *Scale bar*, 100 μm.

**Supplemental Figure 2**

**Figure S3**

**Supplemental Figure 2|** Identification of outputs of central amygdala γ-aminobutyric acid–mediated (GABAergic) neurons. Representative images of ChR2-expressing central amygdala GABAergic fibers in bed nucleus of the stria terminalis (BNST); lateral hypothalamic area (LH); zona incerta (ZI); parafascicular thalamic nucleus (PF); parasubthalamic nucleus (PSTh); substantia nigra, reticular part (SNR); mesencephalic reticular formation (mRt); parabrachial nucleus (PB); parvicellular reticular nucleus (PCRt) of *GAD2*-*Cre* mice. acp: anterior commissure, posterior; CeA: central amygdala; ChR2, channelrhodopsin-2; cp: cerebral peduncle, basal part; fr: fasciculus retroflexus; IRt: intermediate reticular nucleus; ns: nigrostriatal bundle; opt: optic tract; scp, superior cerebellar peduncle; SNC: substantia nigra, compact part; STh: subthalamic nucleus; 7N: facial nerve. *Scale bar*, 100 μm.

**Supplemental Figure 3**

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**Supplemental Figure 3|** Chronic restraint stress induces depressive-like behaviors and pain sensitization. (**A**) Experimental design: mice underwent a 3-week restraint stress protocol, 6 hours per day; the sucrose preference, mechanical and thermal nociception were tested pre- and post-chronic restraint stress, and the forced swim test was carried out one day after the last Hargreave’ test. (**B**) Chronic restraint stress decreased the sucrose preference of female mice (*n* = 10 control and 9 chronic restraint stress mice; two-way repeated-measures ANOVA: time, *F*[1, 17] = 17.28, *P* = 0.001; chronic restraint stress, *F*[1, 17] = 4.158, *P* = 0.057; time × chronic restraint stress, *F*[1, 17] = 22.34, *P* < 0.001; Bonferroni *post hoc* test). (**C**) Chronic restraint stress decreased time struggling in the forced swim test (*n* = 8 per group; two-tailed unpaired Student’s *t*-test, *P* = 0.004). (**D**) The von Frey test showed that chronic restraint stress induced mechanical hypersensitivity in female mice (*n* = 10 control and 9 chronic restraint stress mice; two-way repeated-measures ANOVA: time, *F*[1, 93] = 64.72, *P* < 0.001; chronic restraint stress, *F*[1, 93] = 129.8, *P* < 0.001; time × chronic restraint stress, *F*[1, 93] = 63.2, *P* < 0.001; Bonferroni *post hoc* test). (**E**) The Hargreaves’ test showed that chronic restraint stress induced thermal hypersensitivity in female mice (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[1, 14] = 0.166, *P* = 0.69; chronic restraint stress, *F*[1, 14] = 0.449, *P* = 0.514; time × chronic restraint stress, *F*[1, 14] = 0.072, *P* = 0.793; Bonferroni *post hoc* test). (**F**) The Hargreaves’ test showed that chronic restraint stress induced thermal hypersensitivity in male mice (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[1, 14] = 8.108, *P* = 0.013; chronic restraint stress, *F*[1, 14] = 1.867, *P* = 0.193; time × chronic restraint stress, *F*[1, 14] = 7.099, *P* = 0.019; Bonferroni *post hoc* test). BL, paw withdrawal threshold baseline; CRS, chronic restraint stress; FST, forced swim test; SPT, sucrose preference test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. All data are expressed as mean ± SD.

**Supplemental Figure 4**

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**Supplemental Figure 4|** Pattern and quantification of c-Fos expression in *c*-*fos*-*tTA* mice brains provoked by chronic restraint stress. (**A**) Representative images of chronic restraint stress-induced c-Fos in the infralimbic cortex (IL); paraventricular nucleus (PVN); arcuate hypothalamic nucleus (Arc); basolateral amygdala (BLA); central amygdala (CeA); hippocampus (Hipp); paraventricular thalamic nucleus (PV); ventrolateral periaqueductal gray (vlPAG) and locus coeruleus (LC) of *c-fos-tTA* mice. (**B**) Quantification of c-Fos expression for (A) in 0.04 mm2 imaging area per slice (*n* = 4 slices from 3 mice). Aq: aqueduct; D3V: dorsal 3rd ventricle; LA: lateral amygdala; me5: mesencephalic trigeminal tract; 3V: 3rd ventricle; 4V: 4th ventricle. *Scale bar*, 100 μm.

**Supplemental Figure 5**

未标题-1

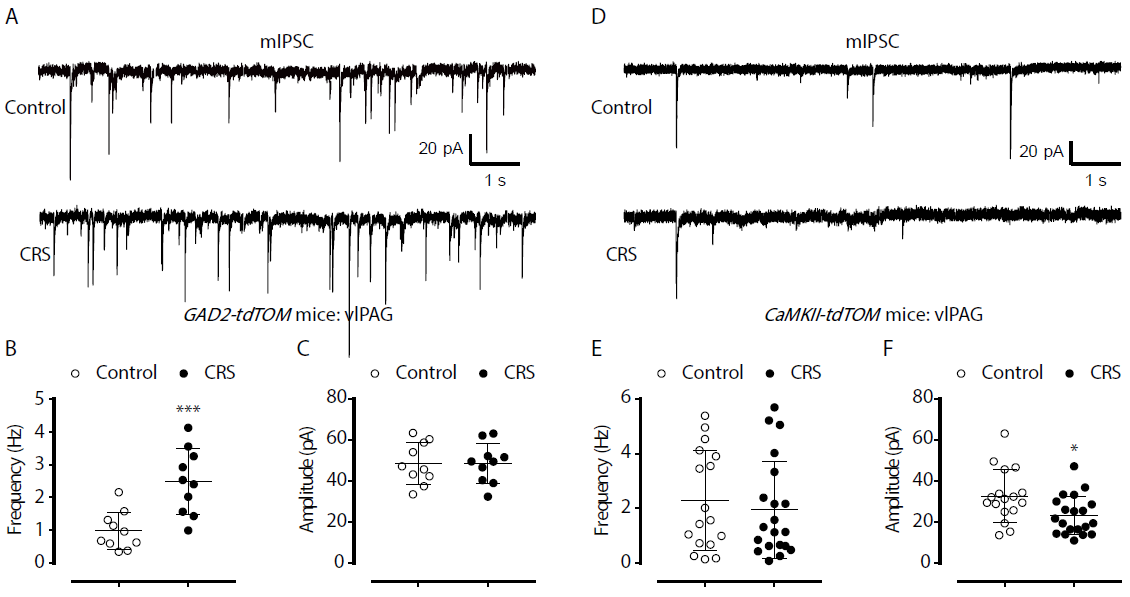
**Supplemental Figure 5|** Disinhibition of ventrolateral periaqueductal gray glutamatergic neuronsresults from activation of central amygdala–ventrolateral periaqueductal graypathway in spare nerve injury mice. (**A**) Schematic showing whole-cell recording of ventrolateral periaqueductal gray-projecting central amygdala neurons and experimental timeline for (B). (**B**) The effect of spare nerve injury on the firing rate of ventrolateral periaqueductal gray-projecting central amygdala γ-aminobutyric acid–mediated (GABAergic) neurons (*n* = 10 sham and 9 spare nerve injury cells from three mice per group; two-way repeated-measures ANOVA: current intensity, *F*[7, 119] = 303.2, *P* < 0.001; spare nerve injury, *F*[1, 17] = 9.213, *P* = 0.008; current intensity × spare nerve injury, *F*[7, 119] = 4.332, *P* < 0.001; Bonferroni *post hoc* test). (**C**) The experimental timeline for (D). (**D**) Activation of central amygdala–ventrolateral periaqueductal graypathway alleviates spare nerve injury-induced neuropathic pain (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[2, 28] = 208.1, *P* < 0.001; light, *F*[1, 14] = 57.18, *P* < 0.001; time × light, *F*[2, 28] = 53.41, *P* < 0.001; Bonferroni *post hoc* test). (**E**) Schematic showing whole-cell recording of ventrolateral periaqueductal grayGABAergic and glutamatergic neurons in the control and spare nerve injury mice. (**F**) The effect of spare nerve injury on the firing rate of ventrolateral periaqueductal grayGABAergic neurons (*n* = 13 cells from three mice per group; two-way repeated-measures ANOVA: current intensity, *F*[3, 72] = 226.1, *P* < 0.001; spare nerve injury, *F*[1, 24] = 24.71, *P* < 0.001; current intensity × spare nerve injury, *F*[3, 72] = 13.39, *P* < 0.001; Bonferroni *post hoc* test). (**G**) The effect of spare nerve injury on the firing rate of ventrolateral periaqueductal gray glutamatergic neurons (*n* = 11 sham and 10 spare nerve injury cells from three mice per group; two-way repeated-measures ANOVA: current intensity, *F*[3, 57] = 232.1, *P* < 0.001; spare nerve injury, *F*[1, 19] = 6.382, *P* = 0.021; current intensity × spare nerve injury, *F*[3, 57] = 3.812, *P* = 0.015; Bonferroni post hoc test). BL, paw withdrawal threshold baseline; CeA, central amygdala; CTB, cholera toxin B subunit; dl/lPAG, dorsolateral and lateral periaqueductal gray; SNI, spare nerve injury；vlPAG: ventrolateral periaqueductal gray. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. All data are expressed as mean ± SD.

**Supplemental Figure 6**

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**Supplemental Figure 6|** Activation of central amygdala–ventrolateral periaqueductal grayγ-aminobutyric acid–mediated (GABAergic) pathway induces analgesia in non-chronic restraint stress mice. (**A**) Schematic of viral injections and optic fiber implantations in ventrolateral periaqueductal gray for optogenetic manipulations and experimental timeline for (B-E). (**B**) The effect of photo-stimulating central amygdala–ventrolateral periaqueductal grayGABAergic pathway on mechanical pain threshold (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[1, 14] = 19.94, *P* = 0.001; light, *F*[2, 28] = 33.21, *P* < 0.001; time × light, *F*[2, 28] = 25.24, *P* < 0.001; Bonferroni *post hoc* test). (**C**) The effect of photo-stimulating central amygdala–ventrolateral periaqueductal grayGABAergic pathway on thermal pain threshold (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[1, 14] = 27.57, *P* < 0.001; light, *F*[2, 28] = 48, *P* < 0.001; time × light, *F*[2, 28] = 59.15, *P* < 0.001; Bonferroni *post hoc* test). (**D**) The effect of photo-stimulating central amygdala–ventrolateral periaqueductal grayGABAergic pathway on sucrose preference (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[1, 14] = 0.123, *P* = 0.731; light, *F*[2, 28] = 0.936, *P* = 0.404; time × light, *F*[2, 28] = 0.428, *P* = 0.656; Bonferroni *post hoc* test). (**E**) The effect of photo-stimulating central amygdala–ventrolateral periaqueductal grayGABAergic pathway on tail suspension immobility time (*n* = 8 mice per group; two-way repeated-measures ANOVA: time, *F*[2, 42] = 1.324, *P* = 0.277; light, *F*[2, 21] = 0.668, *P* = 0.523; time × light, *F*[4, 42] = 1.438, *P* = 0.238; Bonferroni *post hoc* test). CeA, central amygdala; NpHR, Natronomonas pharaonis halorhodopsin; TST, tail suspension test; SPT, sucrose preference test; vlPAG, ventrolateral periaqueductal gray. \*\*\**P* < 0.001. All data are expressed as mean ± SD.

**Supplemental Figure 7**



**Supplemental Figure 7|** Altered spontaneous miniature inhibitory postsynaptic current of ventrolateral periaqueductal gray γ-aminobutyric acid–mediated (GABAergic) and glutamatergicneurons after chronic restraint stress. (**A**) Spontaneous miniature inhibitory postsynaptic current traces of ventrolateral periaqueductal gray GABAergicneurons from the control and chronic restraint stress *GAD2*-*tdTOM* mice. (**B**) Summarized data of the effect of 3-week chronic restraint stress on miniature inhibitory postsynaptic current frequency in ventrolateral periaqueductal gray GABAergic neurons (*n* = 10 cells from three mice per group; two-tailed unpaired Student’s *t-test*, t[18] = 4.103, *P* = 0.001). (**C**) Summarized data of the effect of 3-week chronic restraint stress on miniature inhibitory postsynaptic current amplitude in ventrolateral periaqueductal gray GABAergicneurons (*n* = 10 cells from three mice per group; two-tailed unpaired Student’s *t-test*, t[18] = 0.015, *P* = 0.988). (**D**) Spontaneous miniature inhibitory postsynaptic current traces of ventrolateral periaqueductal gray glutamatergicneurons from the control and chronic restraint stress *CaMKII-tdTOM* mice. (**E**) Summarized data of the effect of 3-week chronic restraint stress on miniature inhibitory postsynaptic current frequency in ventrolateral periaqueductal gray glutamatergic neurons (*n* = 17 control and 20 chronic restraint stress cells from three mice per group; two-tailed unpaired Student’s *t-test*, t[35] = 0.557, *P* = 0.581). (**F**) Summarized data of the effect of 3-week chronic restraint stress on miniature inhibitory postsynaptic current amplitude in ventrolateral periaqueductal gray glutamatergicneurons (*n* = 17 control and 20 chronic restraint stress cells from three mice per group; two-tailed unpaired Student’s *t-test*, t[35] = 2.599, *P* = 0.014). CRS, chronic restraint stress; mIPSC, miniature inhibitory postsynaptic current; vlPAG, ventrolateral periaqueductal gray. \**P <* 0.05, \*\*\**P* < 0.001. All data are expressed as mean ± SD.

**Table 1**.Statistical analyses related to figures 1-9 and supplemental figures 1-7.

|  |  |  |  |
| --- | --- | --- | --- |
| **Figure** | **Sample size** | **Statistical Test** | **Values** |
| 2D | Control: 8 | Two-tailed Wilcoxon matched-pairs signed rank test | *P* = 0.008 |
| Bicuculline: 8 |
| 2H | Control: 8 | Two-tailed Wilcoxon matched-pairs signed rank test | *P* = 0.008 |
| Bicuculline: 8 |
| 2L | Control: 5 | Two-tailed paired Student’s t-test | t[4] = 3.98, *P* = 0.016 |
| Bicuculline: 5 |
| 3B | Control: 7 | Two-way repeated measures ANOVA |  |
| CRS: 7 | Factor 1: time | *F*[3, 36] = 0.713, *P* = 0.551 |
|  | Factor 2: CRS | *F*[1, 12] = 11.17, *P* = 0.006 |
|  | Interaction: time × CRS | *F*[3, 36] = 1.209, *P* = 0.320 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | day 14:Control vs CRS | *P* = 0.040 |
|  | day 21:Control vs CRS | *P* = 0.028 |
| 3C | Control: 7 | Two-tailed unpaired Student’s t-test | *P* = 0.029 |
| Bicuculline: 10 |
| 3D | Control: 7 | Two-way repeated measures ANOVA |  |
| CRS: 9 | Factor 1: time | *F*[2, 28] = 9.201, *P* = 0.001 |
|  | Factor 2: CRS | *F*[1, 14] = 8.83, *P* = 0.010 |
|  | Interaction: time × CRS | *F*[2, 28] = 4.482, *P* = 0.021 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | day 14: Control vs CRS | *P* = 0.004 |
|  | day 21: Control vs CRS | *P* = 0.023 |
| 4E | Control: 20 | Two-way repeated measures ANOVA |  |
| CRS: 18 | Factor 1: current intensity | *F*[7, 252] = 212.6, *P* < 0.001 |
|  | Factor 2: CRS | *F*[1, 36] = 10.09, *P* = 0.003 |
|  | Interaction: CRS × current intensity | *F*[7, 252] = 5.896, *P* < 0.001 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | 40 pA: Control vs CRS | *P* = 0.006 |
|  | 50 pA: Control vs CRS | *P* = 0.002 |
|  | 60 pA: Control vs CRS | *P* < 0.001 |
|  | 70 pA: Control vs CRS | *P* < 0.001 |
| 5E | GABA+:4  GABA-:4 | Two-tailed paired Student’s t-test | *P* = 0.004 |
| 5F  5F | non-CRS mCherry: 7 | Two-way repeated measures ANOVA |  |
| mCherry: 7 | Factor 1: time | *F*[8, 144] = 13.48, *P* < 0.001 |
| hM4Di: 7 | Factor 2: CNO | *F*[2, 18] = 56.87, *P* < 0.001 |
|  | Interaction: CNO × time | *F*[16, 144] = 3.962, *P* < 0.001 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | 30 min | *P* = 0.042 |
| 5G | non-CRS mCherry: 8 | Two-way repeated measures ANOVA |  |
| mCherry: 8 | Factor 1: time | *F*[1, 21] = 40.33, *P* < 0.001 |
| hM4Di: 8 | Factor 2: CNO | *F*[2, 21] = 5.219, *P* = 0.015 |
|  | Interaction: time × CNO | *F*[2, 21] = 10.54, *P* = 0.001 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | non-CRS mCherry vs mCherry | *P* = 0.033 |
|  | mCherry vs hM4Di | *P* = 0.039 |
| 5H | mCherry: 7  hM3Dq: 7 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: CNO × time  Post-hoc comparisons, Bonferroni  30 min  40 min  50 min  60 min | *F*[8, 96] = 23.14, *P* < 0.001  *F*[1, 12] = 19.97, *P* = 0.001  *F*[8, 96] = 8.522, *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* = 0.002  *P* = 0.020 |
| 5I | non-CRS mCherry: 8  mCherry: 8  hM3Dq: 7 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: time × CNO  Post-hoc comparisons, Bonferroni  non-CRS mCherry vs mCherry  mCherry vs hM3Dq | *F*[1, 21] = 10.47, *P* = 0.004  *F*[2, 21] = 32.36, *P* < 0.001  *F*[2, 21] = 55.62, *P* < 0.001  *P* = 0.008  *P* < 0.001 |
| 5J | non-CRS mCherry: 7  mCherry: 9  hM4Di: 8  hM3Dq: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: time × CNO  Post-hoc comparisons, Bonferroni  non-CRS mCherry vs mCherry  mCherry vs hM4Di  mCherry vs hM3Dq  hM4Di vs hM3Dq | *F*[1, 28] = 46.19, *P* < 0.001  *F*[3, 28] = 3.914, *P* = 0.019  *F*[3, 28] = 3.833, *P* = 0.020  *P* = 0.005  *P* > 0.999  *P* > 0.999  *P* > 0.999 |
| 5K | non-CRS mCherry: 7  mCherry: 9  hM4Di: 8  hM3Dq: 8 | Ordinary one-way ANOVA  Factor : CNO  Post-hoc comparisons, Bonferroni  non-CRS mCherry vs mCherry  mCherry vs hM4Di  mCherry vs hM3Dq  hM4Di vs hM3Dq | *F*[3, 30] = 4.965, *P* = 0.007  *P* = 0.031  *P* = 0.838  *P* > 0.999  *P* = 0.846 |
| 6E | eYFP: 8 | Two-way repeated measures ANOVA |  |
| NpHR: 7 | Factor 1: time | *F*[8, 104] = 14.53, *P* < 0.001 |
|  | Factor 2: light | *F*[1, 13] = 0.147, *P* = 0.708 |
|  | Interaction: light × time | *F*[8, 104] = 0.791, *P* = 0.611 |
| 6I | mCherry: 8 | Two-way repeated measures ANOVA |  |
| ChR2: 7 | Factor 1: time | *F*[8, 104] = 17.6, *P* < 0.001 |
|  | Factor 2: light | *F*[1, 13] = 85.64, *P* < 0.001 |
|  | Interaction: time × light | *F*[8, 104] = 16.47, *P* < 0.001 |
|  | Post-hoc comparisons, Bonferroni |  |
|  | 30 min | *P* < 0.001 |
|  | 40 min | *P* < 0.001 |
|  | 50 min | *P* < 0.001 |
|  | 60 min | *P* < 0.001 |
|  | 70 min | *P* < 0.001 |
| 6K | ChR2: 6 | Two-way repeated measures ANOVA |  |
|  | Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni | *F*[5, 25] = 23.63, *P* < 0.001  *F*[1, 5] = 423.5, *P* < 0.001  *F*[5, 25] = 7.835, *P* < 0.001 |
|  | 0 min | *P* = 0.001 |
|  | 10 min | *P* = 0.003 |
|  | 20 min | *P* < 0.001 |
|  | 30 min | *P* < 0.001 |
|  | 40 min | *P* < 0.001 |
| 6M | ChR2: 6 | Two-way repeated measures ANOVA |  |
|  | Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni | *F*[5, 30] = 2.505, *P* = 0.052  *F*[1, 6] = 38.8, *P* = 0.001  *F*[5, 30] = 2.988, *P* = 0.026 |
|  | 0 min | *P* =0.001 |
|  | 10 min | *P* = 0.004 |
|  | 20 min | *P* = 0.001 |
|  | 30 min | *P* = 0.018 |
|  | 40 min | *P* < 0.001 |
| 7C | Control: 19  CRS: 15 | Two-way repeated measures ANOVA  Factor 1: current intensity  Factor 2: CRS  Interaction: current intensity × CRS  Post-hoc comparisons, Bonferroni  40 pA: Control vs CRS  60 pA: Control vs CRS  80 pA: Control vs CRS | *F*[3, 96] = 200.9, *P* < 0.001  *F*[1, 32] = 7.87, *P* = 0.009  *F*[3, 96] = 3.806, *P* = 0.013  *P* = 0.032  *P* = 0.010  *P* = 0.006 |
| 7E | hM4Di:5 | Two-tailed paired Student’s t-test | t[4] = 2.99, *P* = 0.04 |
| 7F | mCherry: 7  hM4Di: 8  hM3Dq: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: time × CNO  Post-hoc comparisons, Bonferroni  30 min: mCherry vs hM4Di  40 min: mCherry vs hM4Di  50min: mCherry vs hM4Di  60min: mCherry vs hM4Di  70 min: mCherry vs hM4Di | *F*[8, 160] = 16.78, *P* < 0.001  *F*[2, 20] = 99.18, *P* < 0.001  *F*[16, 160] = 18.71, *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001 |
| 7H | Control: 14  CRS: 31 | Two-way repeated measures ANOVA  Factor 1: current intensity  Factor 2: CRS  Interaction: current intensity × CRS  Post-hoc comparisons, Bonferroni  70 pA: Control vs CRS | *F*[3, 129] = 164.3, *P* < 0.001  *F*[1, 43] = 5.543, *P* = 0.023  *F*[3, 129] = 1.302, *P* = 0.277  *P* = 0.033 |
| 7I | mCherry: 7  hM4Di: 8  hM3Dq: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: time × CNO  Post-hoc comparisons, Bonferroni  30 min: mCherry vs hM3Dq  40 min: mCherry vs hM3Dq  50min: mCherry vs hM3Dq  40min: mCherry vs hM4Di  50 min: mCherry vs hM4Di | *F*[8, 160] = 17.54, *P* < 0.001  *F*[2, 20] = 28.43, *P* < 0.001  *F*[16, 160] = 11.62, *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* = 0.029  *P* = 0.031 |
| 8C | mCherry:7  hM4Di: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CNO  Interaction: time × CNO  Post-hoc comparisons, Bonferroni  30 min  40 min  50min  60min  70 min | *F*[9, 117] = 10.85, *P* < 0.001  *F*[1, 13] = 24.51, *P* < 0.001  *F*[9, 117] = 8.595, *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* = 0.003  *P* = 0.008 |
| 8D  8D | mCherry:7 | Two-way repeated measures ANOVA |  |
| hM3Dq: 8 | Factor 1: time | *F*[9, 117] = 1.192, *P* = 0.306 |
|  | Factor 2: CNO | *F*[1, 13] = 0.278, *P* = 0.607 |
|  | Interaction: time × CNO | *F*[9, 117] = 0.473, *P* = 0.890 |
|  | Post-hoc comparisons, Bonferroni |  |
| S3B | Control: 10  CRS: 9 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: CRS  Interaction: time × CRS  Post-hoc comparisons, Bonferroni  d21: Control vs CRS | *F*[1, 17] = 17.28, *P* = 0.001  *F*[1, 17] = 4.158, *P* = 0.057  *F*[1, 17] = 22.34, *P* < 0.001  *P* = 0.002 |
| S3C | Control: 8 | two-tailed unpaired Student’s t-test | *P* = 0.004 |
|  | CRS: 8 |  |  |
|  | Control: 10 | Two-way repeated measures ANOVA |  |
| S3D | CRS: 9 | Factor 1: time  Factor 2: CRS  Interaction: time × CRS  Post-hoc comparisons, Bonferroni  d23: Control vs CRS | *F*[1, 93] = 64.72, *P* < 0.001  *F*[1, 93] = 129.8, *P* < 0.001  *F*[1, 93] = 63.2, *P* < 0.001  *P* < 0.0001 |
|  | Control: 8 | Two-way repeated measures ANOVA |  |
| S3E | CRS: 8 | Factor 1: time  Factor 2: CRS  Interaction: time × CRS  Post-hoc comparisons, Bonferroni | *F*[1, 14] = 0.166, *P* = 0.690  *F*[1, 14] = 0.449, *P* = 0.514  *F*[1, 14] = 0.072, *P* = 0.793 |
|  | Control: 8 | Two-way repeated measures ANOVA |  |
| S3F | CRS: 8 | Factor 1: time  Factor 2: CRS  Interaction: time × CRS  Post-hoc comparisons, Bonferroni  d23: Control vs CRS | *F*[1, 14] = 8.108, *P* = 0.013  *F*[1, 14] = 1.867, *P* = 0.193  *F*[1, 14] = 7.099, *P* = 0.019  *P* = 0.032 |
| S5B | Sham: 10  SNI: 9 | Two-way repeated measures ANOVA  Factor 1: current intensity  Factor 2: SNI  Interaction: current intensity × SNI  Post-hoc comparisons, Bonferroni  30 pA: Sham vs SNI  40 pA: Sham vs SNI  60 pA: Sham vs SNI  70 pA: Sham vs SNI | *F*[7, 119] = 303.2, *P* < 0.001  *F*[1, 17] = 9.213, *P* = 0.008  *F*[7, 119] = 4.332, *P* < 0.001  *P* = 0.013  *P* = 0.004  *P* = 0.049  *P* = 0.001 |
| S5D | mCherry: 8  ChR2-mCherry: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni  Light: mCherry vs ChR2-mCherry | *F*[2, 28] = 208.1, *P* < 0.001  *F*[1, 14] = 57.18, *P* < 0.001  *F*[2, 28] = 53.41, *P* < 0.001  *P* < 0.001 |
| S5F | Sham: 13  SNI: 13 | Two-way repeated measures ANOVA  Factor 1: current intensity  Factor 2: SNI  Interaction: current intensity × SNI  Post-hoc comparisons, Bonferroni  40 pA: Sham vs SNI  60 pA: Sham vs SNI  80 pA: Sham vs SNI | *F*[3, 72] = 226.1, *P* < 0.001  *F*[1, 24] = 24.71, *P* < 0.001  *F*[3, 72] = 13.39, *P* < 0.001  *P* < 0.001  *P* < 0.001  *P* < 0.001 |
| S5G | Sham: 11  SNI: 10 | Two-way repeated measures ANOVA  Factor 1: current intensity  Factor 2: SNI  Interaction: current intensity × SNI  Post-hoc comparisons, Bonferroni  60 pA: Sham vs SNI  80 pA: Sham vs SNI | *F*[3, 57] = 232.1, *P* < 0.001  *F*[1, 19] = 6.382, *P* = 0.021  *F*[3, 57] = 3.812, *P* = 0.015  *P* = 0.026  *P* = 0.011 |
| S6B | mCherry: 8  ChR2: 8  NpHR: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni  Light: mCherry vs ChR2-mCherry | *F*[1, 14] = 19.94, *P* = 0.001  *F*[2, 28] = 33.21, *P* < 0.001  *F*[2, 28] = 25.24, *P* < 0.001  *P* < 0.001 |
| S6C | mCherry: 8  ChR2: 8  NpHR: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni  Light: mCherry vs ChR2-mCherry | *F*[1, 14] = 27.57, *P* < 0.001  *F*[2, 28] = 48, *P* < 0.001  *F*[2, 28] = 59.15, *P* < 0.001  *P* < 0.001 |
| S6D | mCherry: 8  ChR2: 8  NpHR: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni | *F*[1, 14] = 0.123, *P* = 0.731  *F*[2, 28] = 0.939, *P* = 0.404  *F*[2, 28] = 0.428, *P* = 0.656 |
| S6E | mCherry: 8  ChR2: 8  NpHR: 8 | Two-way repeated measures ANOVA  Factor 1: time  Factor 2: light  Interaction: time × light  Post-hoc comparisons, Bonferroni | *F*[2, 42] = 1.324, *P* = 0.277  *F*[2, 21] = 0.668, *P* = 0.523  *F*[4, 42] = 1.438, *P* = 0.238 |
| S7B | Control: 10  CRS: 10 | two-tailed unpaired Student’s t-test | t[18] = 4.103, *P* = 0.001 |
| S7C | Control: 10  CRS: 10 | two-tailed unpaired Student’s t-test | t[18] = 0.015, *P* = 0.988 |
| S7E | Control: 17  CRS: 20 | two-tailed unpaired Student’s t-test | t[35] = 0.557, *P* = 0.581 |
| S7F | Control: 17  CRS: 20 | two-tailed unpaired Student’s t-test | t[35] = 2.599, *P* = 0.014 |

ChR2, channelrhodopsin-2; CNO, clozapine-N-oxide; CRS, chronic restraint stress; hM3Dq, Gq-coupled human M3 muscarinic receptor; hM4Di, Gi-coupled human M4 muscarinic receptor; SNI, spared nerve injury.