nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Images of neural tissue were collected using both Keyence BZ-X Analyzer and Viewer software (v. 1.4.0.1). Behavior data was collected using LUPE, an automated analysis system using DeepLabCut technology and machine learning approaches. Miniscope recordings were collected using the Inscopix nVista3.0 and nVoke2.0 Miniscope and Data Acquisition (DAQ) box.
Data analysis	Statistical analyses were performed using Graphpad Prism 9 & 10 software. Initial processing of Miniscope neural recordings were done using Inscopix Data Processing Software (IDPS), and further analyses utilized MATLAB (v. 9.14). Quantification of RNAscope images was performed by HALO (Indica Labs). Image data analyses were performed using both Fiji/ImageJ (v. 2) and Adobe Photoshop (v. 23.5) software suites.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All new mu-opioid receptor promoter viruses (AAV-mMORp) will be available for academic use from the Stanford Gene Vector and Virus Core (https://neuroscience.stanford.edu/research/neuroscience-community-labs/gene-vector-and-virus-core) and/or by contacting the lead authors. Upon manuscript peer-review publication, all data (e.g. 10X snRNAseq, LUPE video files, raw TIFF images from histology, etc.) will be unembargoed at Zenodo and GEO.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was not statistically determined prior to the studies. Rather, the group sizes were based off published literature for the type of manipulation (i.e. chemogenetics, site-specific genetic knockout, pharmacology) and measured outcome (e.g. pain behaviors) published in the field and/or by the authors involved (examples include PMID 37704594, 30655440, 34646022, 25948274). Sample sizes for all studies are included in the figure legends.
Data exclusions	For all imaging and behavioral studies, virus injected animals with either little or no evidence of viral transduction and/or incorrect viral targeting were excluded from any final analyses. No other mice or data points were excluded across analyses.
Replication	For many of the behavior studies, multiple cohorts were used due to the large number of animals in the final group sizes. All behavior results were consistent and replicated across cohorts. Individual data points or lines are included and indicate consistent trends across many mice in each behavior study.
Randomization	Mice were randomly assigned into control or experimental groups to the best of experimenter's abilities, with counterbalancing for age and sex as needed. In most of the included studies, the experimental and control groups only differ in the type of virus intracranially infused. The surgical protocol for all mice was identical in the amount, wait time, and location of the intracranial injection. Each surgery day was randomly assigned as a control/experimental surgery date and the according mice from the pre-determined groups underwent surgery that day. Viral spread maps are included for the mMORp-hm4di and mMORp-eYFP control groups in the supplement to demonstrate the similarity of the injection protocol and outcome. Once experimental/control groups were formed to comprise the studies cohort of mice, the cohort underwent all behavioral testing concurrently and experimenters were blinded as described in the "blinding" section.
Blinding	Throughout all studies, experimenters were blinded as to the group identity of the mice as needed, and multiple automated analysis systems were used to further reduce any chance of experimenter bias. LUPE is a machine-based behavior analysis system introduced in this work and is used in numerous of the included experiments. This automated system does not rely on the experimenter and thus heavily reduces experimenter bias for the quantification of pain behaviors and the need for blinding of experimenters. For non-LUPE behavioral testing, experimenters were blinded to the control/experimental status of the mice in the following manner via a separate experimenter that set up

the behavioral testing room and removed any identifying information prior to the experimenter performing the behavioral testing. For studies involving quantification of cell expression studies (e.g. RNAscope), HALO - a deep learning cell quantification software - was used.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Primary antibodies:
Chicken anti-GFP [1:1000, Abcam, ab13970],
Guinea pig anti-FOS [1:1000, Synaptic Systems, 226308]
Rabbit anti-FOS [1:1000, Synaptic System, 226008]
Rabbit anti-DsRed [1:1000, Takara Bio, 632496]
Rabbit anti-MOR [1:1000, abcam, ab134054]

Secondary Antibodies:
Alexa-Fluor 647 donkey anti-rabbit [1:500, Thermo Scientific, A31573]
Alexa-Fluor 488 donkey anti-chicken [1:500, Jackson Immuno, 703-545-155]
Alexa-Fluor 555 donkey anti-rabbit [1:500, Thermo Scientific, A31572]
Alexa-Fluor 647 donkey anti-guinea pig [1:500, Jackson Immuno, 706-605-148]

Validation

All Abcam antibodies have been tested and approved for use in immunohistochemistry for neural tissue across multiple species, with both validation specs and supporting references for their use in mammalian tissue available at: https://www.abcam.com/gfp-antibody-ab13970.html (for Chicken anti-GFP), and https://www.abcam.com/mu-opioid-receptor-antibody-umb3-c-terminal-ab134054.html (for Rabbit anti-MOR).

Synaptic System antibodies undergo testing for use in immunohistochemistry protocols in neural tissue of various species and at various concentrations to determine the efficacy and specificity. Data is available on the web pages for the antibodies. Links for antibodies used in this study included for convenience: Guinea pig anti-FOS (https://www.sysy.com/product/226308#list) and Rabbit anti-FOS https://www.sysy.com/product/226008#list).

Takara Bio antibodies, and the specifically the Rabbit anti-DsRed Primary antibody included in this study, undergo validation and are recommended for use in immunohistochemistry. Certificate of Analysis and other information is available at the product page (https://www.takarabio.com/products/antibodies-and-elisa/fluorescent-protein-antibodies/red-fluorescent-protein-antibodies?catalog=632496).

All Thermo Scientific Invitrogen antibodies are tested and validated for use in immunohistochemistry protocols, and data from these validation processes are listed on the product pages. Links for antibodies used in this study included for convenience: Alexa-Fluor 647 donkey anti-rabbit (https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31573) and Alexa-Fluor 555 donkey anti-rabbit (https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31572).

Jackson Immuno antibodies are validating for efficacy and specificity through multiple testing methodologies and data sheets are available on the product pages. Those included in this study are Alexa-Fluor 488 donkey anti-chicken (https://www.jacksonimmuno.com/catalog/products/703-545-155) and Alexa-Fluor 647 donkey anti-guinea pig (https://www.jacksonimmuno.com/catalog/products/706-605-148).

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals

Male and female wildtype C57BL/6J mice (Jackson Laboratory, 000664); ages 8-20 weeks
Male and female Fos-FOS-2A-iCreERT2 mice (Fostm2.1(icre/ERT2)Luo)Luo/Jackson Laboratory, 030323); ages 8-20 weeks

	Male and female Oprm1Cre/Cre B6.Cg-Oprm1tm1.1(cre/GFP)Rpa/J mice (Jackson Laboratory, 035574); ages 8-20 weeks, used at homozygosity to produce an Oprm1 "knockout" phenotype for re-expression studies Male and female Oprm1 fl/fl (Jackson Laboratory, 030074); ages 8-20 weeks Male and female, heterozygous and homozygous Oprm1-2A-Cre:Sun1-sfGFP mice (provided by the lab of Dr. Julie Blendy, UPenn); ages 8 -20 weeks
Wild animals	No wild animals were used in this study.
Reporting on sex	Both sexes were used in all studies with the exception of the miniscope, whole brain clearing, and RNAsequencing datasets. For all studies, the breakdown of sexes are listed in the figure legends along with the total number of animals and test statistics. Numerous studies in the main figures include distinguishable data point shapes for male and female animals, and further sex-specific reporting is available in the supplemental figures.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	All procedures and manipulations conducted in mice were approved by the Institutional Animal Use and Care Committees of the University of Pennsylvania.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A