Molecular cartography studies of how chronic pain and opioid treatment transform RNA expression patterns in the mammalian amygdala

Daniel Berg[1-4,7], Xiqian Jiang[2-4,7], Yuxi Ke[2-4,7], David Lee[1], Michelle Chen[3,5], Stephen Quake[3,5-6], Mark Schnitzer[2-4,7], Grégory Scherrer[1,8]

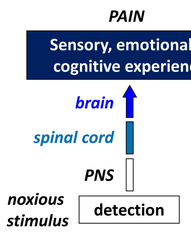
[1]Department of Cell Biology and Physiology, UNC Neuroscience Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA; [2]Department of Biology, Stanford University, Stanford, CA, USA; [3]Department of Applied Physics, Stanford University, Stanford, CA, USA; [4]Howard Hughes Medical Institute, Stanford University, Stanford, CA, USA; [5]Department of Bioengineering, Stanford University, Stanford, CA, USA; [6]Chan Zuckerberg Biohub, San Francisco, CA, USA; [7]CNC Program, Stanford University, Stanford, CA, USA; [8]New York Stem Cell Foundation – Robertson Investigator.

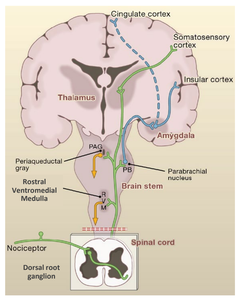
1. ABSTRACT

Neural activity in the amygdala has a crucial role in shaping the unpleasantness of painful experiences. For example, we recently found that a subset of neurons in the basolateral subdivision of the amygdala (BLA) respond selectively to painful stimuli; indicating the causal role of these cells in the experience of pain, chemogenetic inhibition of these BLA nociceptive neurons in mice in a chronic neuropathic pain state markedly reduced affective-motivational pain-related behaviors (Corder, Ahanonu et al., Science, 2019). Notably, several types of amygdalar neurons express opioid receptors, raising the possibility that modifications to neural signaling in the amygdala may be a primary means by which opioid treatment alleviates the experience of pain. Similarly, alterations in amygdala neural activity may contribute to the unpleasantness that accompanies withdrawal from opioid treatment. Given the joint epidemics of both chronic pain and opioid addiction, achieving a better understanding of how both pain and opioids affect the brain is an important step toward developing non-addictive methods for treating pain. Thus, to identify molecular events that are evoked in amygdalar neurons by the experience of pain, opioid treatment, and withdrawal from opioid treatment, we are applying molecular cartography, a highly multiplexed form of fluorescence in situ hybridization that provides the ability to spatially localize single RNA molecules at sub-cellular resolution, using up to 100 different RNA probes with individual tissue slices. The set of genes that we are targeting with molecular cartography includes a set of marker genes that will allow us to differentiate the distinctive types of excitatory and inhibitory neurons in the amygdala, a set of genes that are indicative of neural plasticity (such as immediate early genes), as well as genes encoding opioid peptide precursors, opioid receptors and other G protein-coupled receptors. By mapping the spatially resolved patterns in which these genes are organized in the amygdala of control mice and mice that have undergone either a persistent painful experience, morphine exposure, morphine treatment after a painful experience, or withdrawal from morphine exposure, we aim to uncover fundamental molecular aspects of how the brain responds to these extraordinarily salient experiences.

2. SCIENTIFIC PREMISE: A NOVEL APPROACH TO CONCEPTUALIZE AND TREAT CHRONIC PAIN

Pain is a multidimensional experience with sensory and affective components. The aversive quality of pain, i.e. its inherent unpleasantness, causes a majority of chronic pain patients' suffering and often leads to comorbid disorders such as anxiety and depression. Despite their addictive qualities, opioid analgesics remain clinically useful since they can profoundly dampen pain affect (Corder, Castro et al., Annu Rev Neurosci, 2018). Even at low opioid dosage, patients describe their pain as less bothersome, and previous studies have shown that this analgesic effect correlates with opioid receptor occupancy in affective neural circuits. Thus, discovering targets that could alter neural activity selectively in neural circuits that generate pain aversion—but not in the reward or breathing circuits that opioids also alter—is an attractive strategy to develop novel, safer analgesics. We postulate that chronic pain is a neurological disorder in which somatosensory information is miscoded in emotional circuits. Specifically, chronic pain causes abnormal assignment of negative emotional valence to innocuous stimuli (e.g., light touch and cool allodynia)





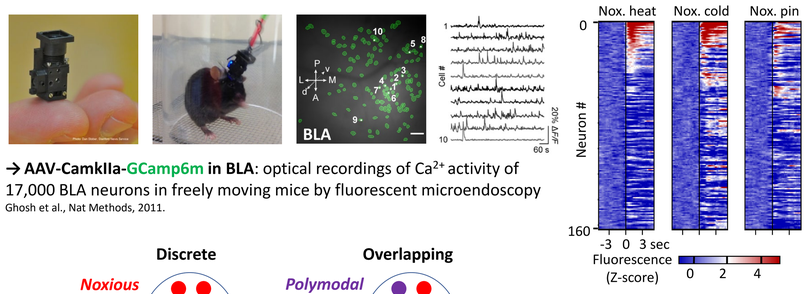
Basbaum, Bautista, Scherrer, and Julius. Cell. 2009.

How does the brain generate the unpleasantness that characterizes the pain percept?

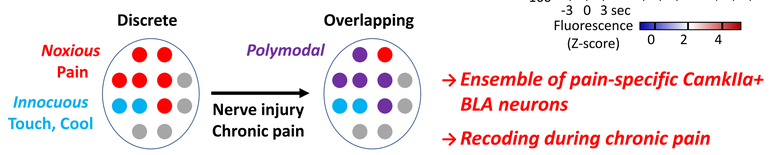
Because multiple cortical and subcortical brain areas shape pain emotions and affect, to date it has been challenging to identify the neuron-types that are most central to pain aversion. Recently, by combining *in vivo* imaging and chemogenetic manipulations of neural dynamics in the BLA of freely behaving mice encountering noxious stimuli, our collaboration discovered a distinct neural ensemble in the BLA that encodes the negative affective valence of pain (Corder, Ahanonu et al., Science, 2019). Chemogenetic inhibition of this 'nociceptive coding ensemble' using Gi/o-protein-coupled DREADDs (hM4Di) alleviated pain affective behaviors without altering withdrawal reflexes, anxiety or reward. Moreover, our functional studies of this nociceptive ensemble revealed a causal neural basis for the phenomenon of allodynia, in which a subject in chronic pain perceives previously innocuous stimuli as painful. Specifically, we found that after a peripheral nerve injury, innocuous stimuli activate this nociceptive ensemble to drive dysfunctional perceptual changes associated with neuropathic pain, including pain aversion to light mechanical and cool stimuli, as reported in patients.

3. RESULTS

3.1. Recording of amygdalar neural dynamics during pain perception



→ **AAV-CamkIIa-GCamp6m in BLA**: optical recordings of Ca^{2+} activity of 17,000 BLA neurons in freely moving mice by fluorescent microendoscopy
Ghosh et al., Nat Methods, 2011.



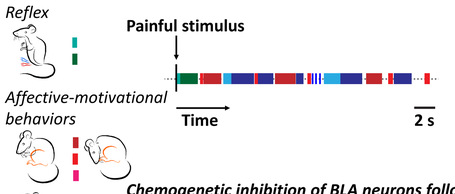
→ ***Ensemble of pain-specific CamkIIa+ BLA neurons***

→ ***Recoding during chronic pain***

3.2. Testing causality: chemogenetic inhibition of BLA nociceptive neurons

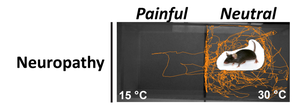
→ **hM4Di: inhibitory Designer Receptors Exclusively Activated by Designer Drug in BLA nociceptive neurons DREADD, Gi/o-protein-coupled receptor** activated by clozapine-N-oxide (CNO) Roth, Neuron, 2016.

→ **Categorization of pain behaviors**



→ **PPA: Painful Place Avoidance**

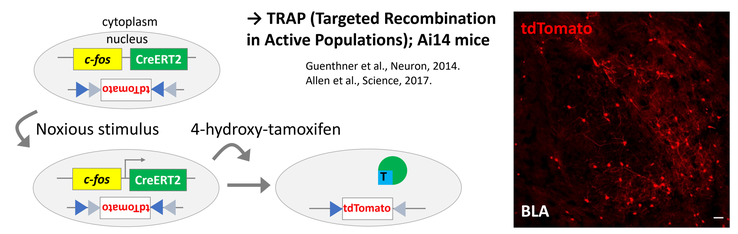
Video tracking of mouse trajectory



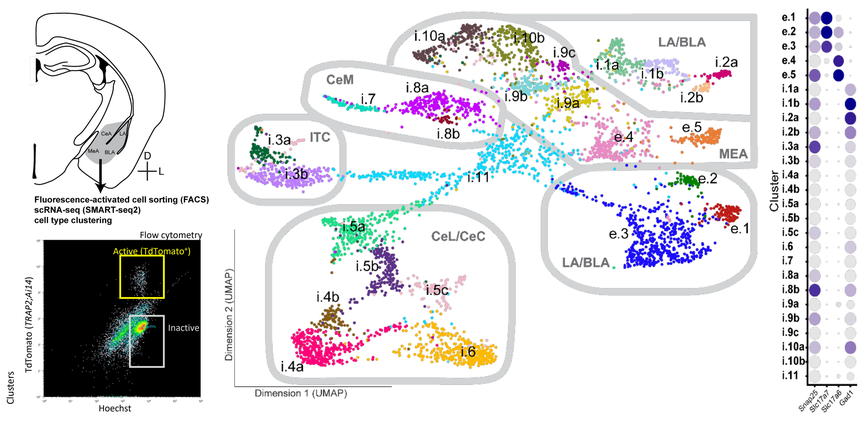
Chemogenetic inhibition of BLA neurons following CNO administration:

→ ***BLA nociceptive neurons are necessary for acute and chronic pain unpleasantness, across acute and chronic pain types***

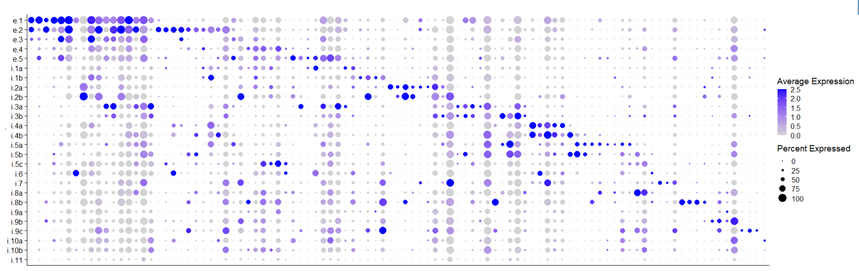
3.3. Genetic labeling of amygdalar nociceptive neurons with TRAP mice



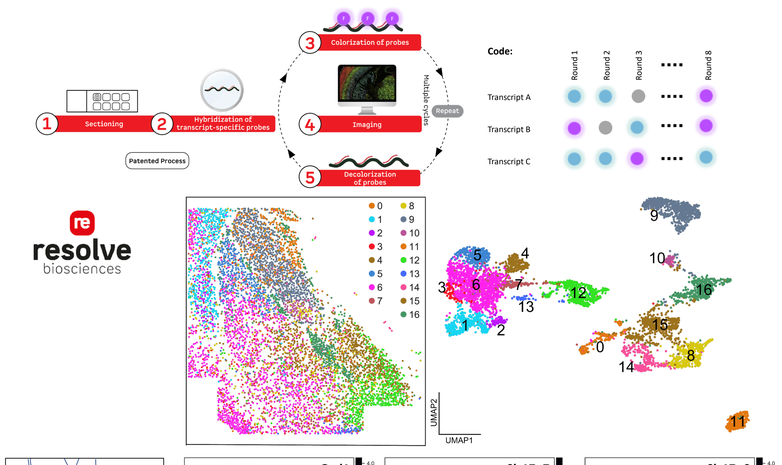
3.4. Single-amygdalar neuron preparation, sorting, and sequencing

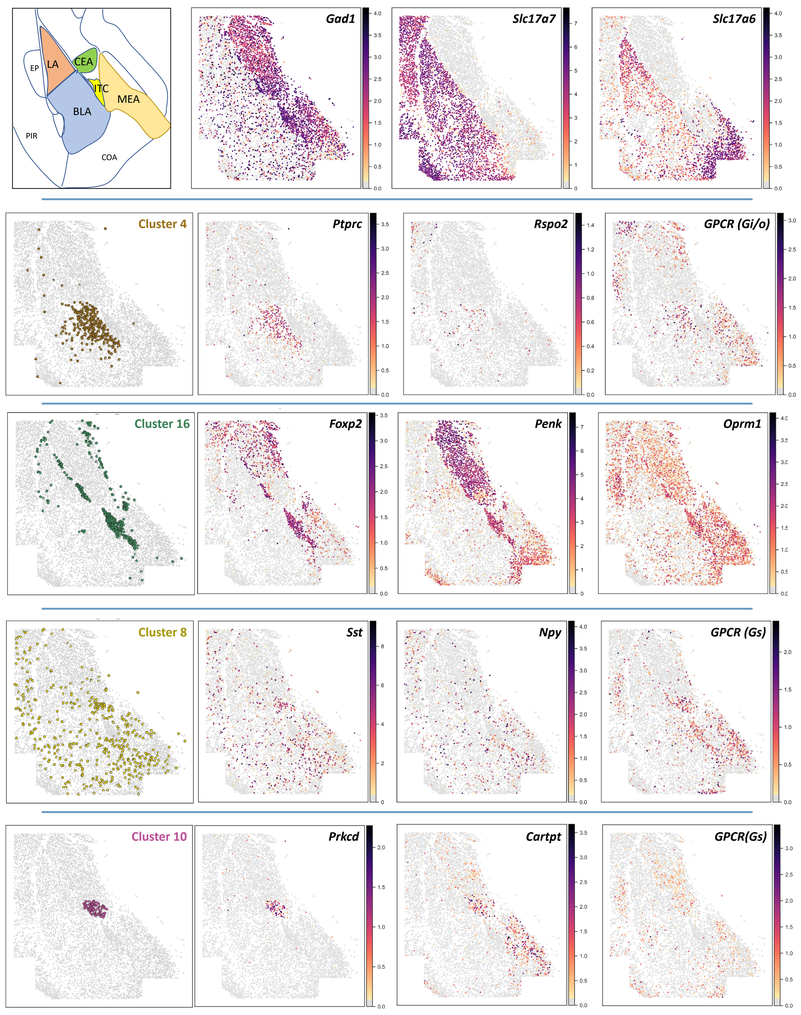


3.5. Bioinformatics to identify GPCRs in nociceptive amygdalar cell clusters



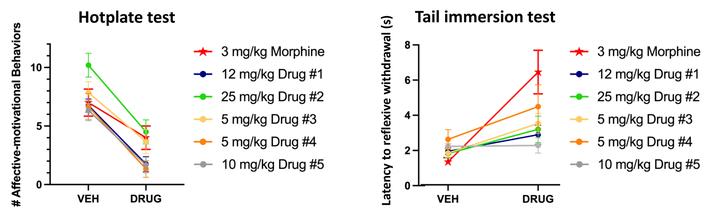
3.5. Molecular cartography to determine amygdalar cell type and GPCR spatial organization





3.6. In vivo pharmacology to test analgesic efficacy and safety

Drug effect on affective-motivational pain behaviors versus reflexive withdrawal



4. REFERENCES

1. Endogenous and Exogenous Opioids in Pain. Corder G, Castro DC, Bruchas MR, Scherrer G. *Annu Rev Neurosci*. 2018 Jul 8;41:453-473. doi: 10.1146/annurev-neuro-080317-061522. Epub 2018 May 31. PMID: 29852083
2. An amygdalar neural ensemble that encodes the unpleasantness of pain. Corder G, Ahanonu B, Grewe BF, Wang D, Schnitzer MJ, Scherrer G. *Science*. 2019 Jan 18;363(6424):276-281. doi: 10.1126/science.aap8586. PMID: 30655440
3. Miniaturized integration of a fluorescence microscope. Ghosh KK, Burns LD, Cocker ED, Nimmerjahn A, Ziv Y, Gamal AE, Schnitzer MJ. *Nat Methods*. 2011 Sep 11;8(10):871-8. doi: 10.1038/nmeth.1694. PMID: 23396101
4. DREADDs for Neuroscientists. Roth BL. *Neuron*. 2016 Feb 17;89(4):683-94. doi: 10.1016/j.neuron.2016.01.040. PMID: 26889809
5. Permanent genetic access to transiently active neurons via TRAP: targeted recombination in active populations. Guenthner CJ, Miyamichi K, Yang HH, Heller HC, Luo L. *Neuron*. 2013 Jun 5;78(5):773-84. doi: 10.1016/j.neuron.2013.03.025. PMID: 23764283
6. Temporal evolution of cortical ensembles promoting remote memory retrieval. DeNardo LA, Liu CD, Allen WE, Adams EL, Friedmann D, Fu L, Guenthner CJ, Tessier-Lavigne M, Luo L. *Nat Neurosci*. 2019 Mar;22(3):460-469. doi: 10.1038/s41593-018-0318-7. Epub 2019 Jan 28. PMID: 30692687

5. CONCLUSIONS

1. Optimization of an amygdalar neuron isolation procedure to obtain highly viable cells.
2. Selective labeling of nociceptive amygdalar neurons with TRAP2 mice and resolution of their molecular identity using single-cell RNA-sequencing.
3. Computational identification of cell types and GPCRs in pain-relevant amygdalar cell types.
4. Using Resolve Biosciences Molecular Cartography platform, generation of high-definition spatial maps that establish amygdalar cell type marker and analgesic GPCR target architecture.
5. Validation of multiple amygdalar analgesic GPCRs for the development of new therapeutics to treat pain unpleasantness.