An amygdalar neural ensemble that encodes the unpleasantness of pain

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Pain is an unpleasant experience. How the brain’s affective neural circuits attribute this aversive quality to nociceptive information remains unknown. By means of time-lapse in vivo calcium imaging and neural activity manipulation in freely behaving mice encountering noxious stimuli, we identified a distinct neural ensemble in the basolateral amygdala that encodes the negative affective valence of pain. Silencing this nociceptive ensemble alleviated pain affective-motivational behaviors without altering the detection of noxious stimuli, withdrawal reflexes, anxiety, or reward. Following peripheral nerve injury, innocuous stimuli activated this nociceptive ensemble to drive dysfunctional perceptual changes associated with neuropathic pain, including pain aversion to light touch (allodynia). These results identify the amygdalar representations of noxious stimuli that are functionally required for the negative affective qualities of acute and chronic pain perception.

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Noxious heat, cold, and pin prick stimuli elicited significant Ca²⁺ responses in 15 ± 2% (SEM), 13 ± 3%, and 13 ± 2% of active BLA neurons, respectively ([3979 neurons (117 ± 8 neurons per session)]) (Fig. 1, F to H, and table S1). Innocuous light touch induced Ca²⁺ activity in a smaller subset of neurons (7 ± 1%) (Fig. 1, F and I, and fig. S5E). Alignment of all stimulus-evoked ensemble responses to the noxious heat trials revealed an overlapping population of principal neurons that encoded nociceptive information across pain modalities (i.e., noxious heat, cold, pin), which we refer to here as the BLA nociceptive ensemble (24 ± 2% of active BLA neurons) (Fig. 1, F to I).

This ensemble was composed of multimodal responsive neurons, as well as a unique population that appeared to encode nociception selectively and no other sensory information (6 ± 1% of all imaged neurons) (Fig. 1K and fig. S5G). Pin behavioral responses evoked by noxious stimuli closely mirrored the activity of this nociceptive neural ensemble (Fig. 1, E and G, and fig. S4). The nociceptive ensemble contained a subset of neurons that maintained their nociceptive response properties for more than a week (11% of 3223 cross-day–aligned neurons) (fig. S6). Increasingly salient stimuli, from light touch (38 ± 3% of the nociceptive ensemble) to mild touch (31 ± 4%), activated larger subsets of the nociceptive ensemble (Fig. 1, G and I, and fig. S5, D and E). The nociceptive ensemble contained a subset of neurons that maintained their nociceptive response properties for more than a week (11% of 3223 cross-day–aligned neurons) (fig. S6). Increasingly salient stimuli, from light touch (38 ± 3% of the nociceptive ensemble) to mild touch (31 ± 4%), activated larger subsets of the nociceptive ensemble (Fig. 1, G and I, and fig. S5, D and E).

Previous studies attempting to define pain affect mechanisms recorded the acute nociceptive responses of single amygdalar neurons in anesthetized animals (11, 18). However, recent work has shown that the BLA encodes information via the coordinated dynamics of neurons within large ensembles (19); it is therefore important to resolve how the BLA processes pain affect at the neural ensemble level in awake, freely behaving animals. We first performed fluorescence in situ hybridization studies and used the immediate-early gene marker of neural activity, c-Fos, to determine that c-Fos⁺ neurons activated by nociceptive stimuli comprised a population of mid-anterior BLA Camk2a⁺ principal neurons (fig. S1). To identify how the BLA encodes nociceptive information, we used a head-mounted miniature microscope to track the somatic Ca²⁺ dynamics of individual BLA Camk2a⁺ principal neurons in freely behaving mice presented with diverse noxious and innocuous stimuli (Fig. 1, A to D, and figs. S2 and S3) (20). We monitored pain-related behaviors by measuring each animal’s locomotor acceleration, which allowed us to track both reflexive withdrawal and affective-motivational behaviors that include attendance to the stimulated tissue and escape (Fig. 1, A and E, and fig. S4).

Responses during fear or pain (5). Damage to the basolateral amygdala (BLA) can induce a rare phenomenon in which noxious stimuli remain detected and discriminated but are devoid of perceived unpleasantness and do not motivate aversion (6, 7). Conversely, impairment of somatosensory cortex function reduces the ability to both localize noxious stimuli and describe their intensity, without altering aversion or avoidance (8, 9). Thus, BLA afferent neural circuits might link nociceptive inputs to aversive perceptions and behavior selection.

Patients with chronic pain often suffer allodynia, a pathological state in which an intense unpleasant percept arises in response to innocuous stimuli such as light touch (10). Notably, the BLA displays heightened activity during chronic pain (11), and longitudinal functional magnetic resonance imaging studies in humans and rodents show that neural hyperactivity and altered functional connectivity in the amygdala parallel the onset of chronic pain, suggesting that the BLA might play a critical role in shaping pathological pain perceptions (12–14). However, it remains unclear how the BLA influences the unpleasant aspects of innate acute and chronic pain perceptions (15), while the role of nociceptive circuits in the central amygdala are better understood (16, 17).

To determine whether the BLA nociceptive ensemble broadly encodes stimulus valence (22, 23), we presented mice with an appetitive stimulus (10% sucrose). Sucrose consumption was encoded by a distinct ensemble (38 ± 3% of all neurons) that only overlapped with a subset of neurons in the nociceptive ensemble (7% of total neurons) (Fig. 1J and fig. S5E) (19). Similar to conditioned responsive valence networks (23), neurons encoding unconditioned nociceptive and appetitive information were spatially intermingled (fig. S5, F, H, and I). Consistent with these results, nociceptive c-Fos⁺ neurons

Corder et al., Science 363, 276–281 (2019) 18 January 2019

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expressed the negative valence marker gene *Rspo2* but not the positive valence marker gene *Pppr1b* (24) (fig. SI, D and E).

We next determined if the nociceptive ensemble was engaged during aversive experiences other than pain by presenting a panel of sensory, but nonsomatosensory or nonnaturalistic, aversive stimuli, including repulsive odor, bitter taste, loud tone, facial air puff, and electric shock. We found that while there was overlap between the neural ensembles that encode nociceptive, aversive, and electric shock stimuli (~10% of all imaged neurons), there remained a subset of BLA neurons (~6% of imaged neurons) that responded only to naturalistic nociceptive stimuli (Fig. 1K and fig. S8).

By analyzing the neural ensemble dynamics with pattern classification methods, we were able to classify and distinguish with high accuracy noxious stimuli from other aversive stimuli (fig. S8E), supporting the finding that noxious stimuli induce a distinct mode of BLA activation (supplementary text S1). Moreover, sensory stimuli of different valences, intensities, and modalities are represented by unique activity codes. Noxious stimuli were encoded distinctively from one another and could be distinguished...
with even higher fidelity from innocuous, non-nociceptive averse, and appetitive stimuli (Fig. 1L and fig. S9, A and B), indicating that there is a core set of BLA neurons that encodes nociceptive stimuli via specific dynamic neural codes. One crucial finding was that greater activation of this BLA nociceptive ensemble was predictive of increased pain behaviors, suggesting that BLA nociceptive processing influences the magnitude of pain behaviors (Fig. 1M and fig. S7, H and I).

To test the causal role of the BLA nociceptive ensemble for pain behaviors, we expressed a Cre-dependent inhibitory DREADD neuromodulator (hM4-mCherry) in mutant TRAP mice (FoxCreERTT) by applying noxious pin pricks that induced activity-dependent, spatially, and temporally controlled DNA recombination and hM4-mCherry expression (noci-TRAPhM4 mice) (Fig. 2, A to C, and fig. S10) (25, 26). Since the BLA encodes multiple modalities of nociceptive stimuli within a core ensemble (Fig. 1H), we hypothesized that silencing the neurons activated by noxious pin pricks would alter behavioral responses to all types of noxious stimuli. Indeed, the hM4 agonist clozapine-N-oxide (CNO; 10 mg/kg) significantly reduced both attending and escape behaviors, but not stimulus detection and withdrawal, for both mechanical and thermal noxious stimuli (Fig. 2, D to G, and fig. S11, A and B). CNO alone had no effect on pain behaviors in control mice (fig. S12C). To test operant pain behavior, we next allowed noci-TRAPhM4 mice to explore a thermal gradient track in which the polar ends were set at noxious cold (5 to 1°C) and hot (42 to 48°C) temperatures (Fig. 2I). The noci-TRAPhM4 mice injected with control saline rapidly acquired an adaptive avoidance strategy of the noxious zones. In contrast, noci-TRAPhM4 mice treated with CNO visited the noxious zones more frequently and for prolonged periods (Fig. 2, H to J, and fig. S12). Similarly, inhibition of the BLA nociceptive ensemble eliminated pain affective-motivational behaviors induced by the optogenetic activation of peripheral primary afferent nociceptors (fig. S13).

Whether pain and anxiety rely on common or distinct BLA ensembles is unknown; therefore, we placed noci-TRAPhM4 mice within an elevated plus maze, in which anxiety drives avoidance of the open arms (Fig. 2K). The noci-TRAPhM4 mice given either saline or CNO displayed equivalent visits to and occupancy of the open arms (fig. S14, A and B). Since nociceptive and...
sucrose reward-related information were encoded in divergent networks (Fig. 1J), we tested the contribution of the nociceptive ensemble to appetitive motivational drive during sucrose preference training. CNO enhanced sucrose reward in sucrose-naïve conditions (28) but had no retarding effects on preference development or on lick rates, relative to controls (Fig. 2L and fig. S4A). Thus, this BLA nociceptive ensemble transforms emotionally inert nociceptive information into an affective signal that is necessary for the selection and learning of motivational protective pain behaviors.

We next investigated the contribution of BLA neural ensemble activity to chronic pain.

A hallmark of chronic neuropathic pain is the appearance of allodynia and hyperalgesia, both pathological perceptual states in which aversion is ascribed to innocuous somatosensory stimuli and exacerbated in response to noxious stimuli, respectively (Fig. 3A) (29). We hypothesized that this pathological perceptual switch might result from maladaptive transformations in BLA coding. We tracked the longitudinal dynamics of BLA ensembles before and after the development of neuropathic pain induced by sciatic nerve injury (17,306 neurons, n = 17 mice) (Fig. 3). Throughout the development of chronic neuropathic pain, a subset of neurons stably encoded the nociceptive ensemble for both noxious mechanical and cold stimuli (fig. S6). Nerve injury did not significantly increase the spontaneous activity of the nociceptive ensemble and overall BLA population (fig. S15, A and B). However, BLA neural activity elicited in response to light touch displayed a significant expansion within the nociceptive ensemble in neuropathic (291 ± 88% increase) but not in uninjured mice (38 ± 14% decrease) (Fig. 3, D to G, and fig. S15, C to E). The emergence of this neuropathic coding schema was accompanied by the development of reflexive paw withdrawal hypersensitivity and by enhanced affective-motivational pain behaviors (Fig. 3, B and C, and fig. S4, C to F). The magnitudes of

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**Fig. 3. Convergence of BLA neural ensemble representations of innocuous and noxious information during chronic pain.** (A) Long-term tracking of BLA neural activity with microendoscopes throughout the development of chronic neuropathic pain. Peripheral nerve injury results in an increased sensitivity and perceived aversion to innocuous (allodynia) and noxious (hyperalgesia) stimuli. (B) Affective-motivational escape acceleration for neuropathic (top row; n = 5 mice) and uninjured (bottom row; n = 4 mice) animals in response to noxious pin or light touch stimuli before and after nerve injury. Dark lines, means; shaded regions, ±SEM. (C) Hyperalgesic and allodynic behavioral responses for neuropathic (n = 13 mice for paw withdrawal, n = 5 mice for escape acceleration) or uninjured (n = 4 mice for both measures) animals after application of light touch (0.07-g filament), noxious pin, or noxious cold (acetone or 5°C H2O drop) stimuli, respectively. Data were quantified by reflexive hypersensitivity (left axis) and affective-motivational escape acceleration (right axis). (D) Mean Ca2+ activity (Z-scored ΔF/ΔF per trial) of all neurons from the same animal for that imaging session, before and after nerve injury, in response to noxious pin prick, noxious cold, and light touch stimuli. Neuron identifications were consistent between stimuli within a day, but not across days (n = 157 and 156 neurons, for days −7 and 42, respectively). (E) Mean Ca2+ response within the nociceptive ensemble for neuropathic (top row; n = 13 mice, 12,026 total neurons imaged) and uninjured (bottom row; n = 4 mice, 5370 total neurons imaged) animals in response to noxious pin or light touch stimuli. (F) Venn diagrams of percentages of significantly responding neurons to noxious pin, noxious cold, and light touch before and after nerve injury. (G) Overlapping neural populations responsive to light touch within the nociceptive ensemble (pin prick and 5°C water or acetone responsive neurons) after nerve injury (n = 13 mice) or in uninjured animals (n = 4 mice). Numbers indicate means ± SEM. (H) Percentages of nociceptive ensemble activated and escape acceleration per imaging session (light-colored points) and across animal groups and conditions (dark, larger points) show significant correlations [Spearman’s p = 0.54 (normal), 0.33 (Neuropathic), and 0.58 (Uninjured) groups]. All tests results in the figure were analyzed via Wilcoxon rank-sum with Benjamini-Hochberg correction unless otherwise noted. Stars, P < 0.01.
Inhibition of neuropathic BLA ensemble activity reduces the aversive quality of chronic pain. (A) Utilization of light touch to gain genetic access to, and manipulate, the neuropathic nociceptive ensemble. (B) Quantification of light touch TRAP neurons in the BLA of neuropathic mice compared to uninjured mice; n = 7 per group. (C) Behavioral raster plots from neuropathic mice showing the effects of inhibiting the BLA nociceptive ensemble on reflexive and affective-motivational pain behaviors associated with cold allodynia. (D and E) Summary of the effects of ensemble inhibition against reflexive (D) and affective-motivational (E) pain behaviors in response to noxious pin prick, noxious cold (acetone drop), or formerly innocuous touch stimuli (0.07-g filament). Behavior was assessed before and 42 days after nerve injury and again at 60 min after CNO or saline administration on day 42; n = 14 per group. (F and G) Effects of neuropathic ensemble inhibition on adaptive avoidance during a cold plate aversion assay. (F) Group mean exploration paths, color coded for the relative occupancy time, following CNO or saline treatments; (G) summary of the effects in response to decreasing floor plate temperatures; n = 6 per group. Stars, P < 0.05 for all panels. In (G), the black star indicates P < 0.05 versus the uninjured + saline group; open star, P < 0.05 versus the neuropathic + saline group. Overlaid dots and lines represent individual subjects. Error bars, ±SEM. For (B), Student’s t test; (D and E), two-way ANOVA with Bonferroni correction; (G) three-way ANOVA with Bonferroni correction.

the behavioral responses and the BLA nociceptive ensemble Ca2+ activity were significantly correlated before and after injury (Fig. 3H and fig. S13F). These results suggest a role for the BLA in the emergence of alldynia in chronic pain states.

We next asked if we could prevent the neural transformation of light touch sensory information into an aversive signal and eliminate chronic pain unpleasantness by gaining genetic access to the nociceptive ensemble with innocuous stimuli in neuropathic TRAP mice. At 21 days post–nerve injury, when alldynia had fully developed (fig. S16, B to E), we delivered a light touch TRAP protocol to express hM4-mCherry in the BLA nociceptive ensemble (neuropathic TRAPm4 mice) (Fig. 4, A and B, and fig. S16). At day 42 post injury, neuropathic TRAPm4 mice displayed significant alldynia and hyperalgesia, for both reflexive and affective-motivational pain responses, relative to uninjured mice (Fig. 4, C to E). While the injection of CNO in neuropathic TRAPm4 mice did not alter reflexive hypersensitivity (Fig. 4D), we observed a profound decrease in neuropathic affective-motivational behaviors, regardless of stimulus intensity or modality (Fig. 4E and fig. S17, A and B). Uninjured TRAPm4 mice given the light touch TRAP protocol expressed levels of hM4-mCherry in the BLA that were similar to those of nonstimulated control mice (Fig. 4B and fig. 2C), presumably because the nociceptive ensemble does not strongly encode innocuous information under normal conditions (Fig. 1I). We observed neither CNO-mediated changes in affective-motivational pain behaviors in these uninjured mice nor CNO effects on neuropathic reflexive or affective-motivational behaviors in the absence of hM4 expression (Fig. 4, C to E, and fig. S17, A and B). In addition, to alleviate allodynia, patients with neuropathic pain often report intense pain in response to cold temperatures (cold allodynia). We therefore ran neuropathic TRAPm4 mice through a two-chamber thermal escape-avoidance assay in which the floor of one chamber was cooled (from 30°C to 10°C) (Fig. 4F). Uninjured TRAPm4 mice avoided the cold chamber, while mice with nerve injury showed enhanced avoidance, consistent with allodynia (Fig. 4, F and G). Notably, CNO administration to neuropathic TRAPm4 mice generated a near-total indifference between cold and neutral temperature chambers (Fig. 4, F and G). Together, these results indicate that the BLA nociceptive ensemble is also necessary for the pain aversion associated with allodynia and hyperalgesia during chronic pain states.

Thus, disrupting neural activity in a nociceptive ensemble in the BLA is sufficient to reduce the affective dimension of pain experiences, without altering their sensory component. The unconditioned nociceptive ensemble described here is a stable network of amygdalar principal neurons that is responsive to a diverse array of noxious stimuli. Within this ensemble, combinatorial neural ensemble codes distinguish the

Corder et al., Science 363, 276–281 (2019) 18 January 2019
we have identified in the BLA a critical neural ensemble target that mediates chronic pain unpleasantness. This finding may enable the development of chronic pain therapies that could selectively diminish pain unpleasantness, regardless of etiology, without influencing reward, and importantly, preserving reflexes and sensory-discriminative processes necessary for the detection and localization of noxious stimuli (44, 45). Collectively, our findings begin to refine the neural basis and coding principles underlying the multiple dimensions and complexity of the pain experience for developing more effective analgesic therapies.

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Competing interests: M.J.S. is a consultant and scientific cofounder of Inscopix Inc., which makes the miniature microscope used for in vivo imaging in this study. Data and materials availability: Additional data relating to this paper are available upon request, because of the size (43 TB) of the data. Code used in this analysis is available at (46).

SUPPLEMENTARY MATERIALS

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Supplementary Text

Materials and Methods

Figs. S1 to S17

Table S1

References (47–94)

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Corder et al., Science 363, 276–281 (2019)
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The emotional dimension of pain
The unpleasantness of pain is an emotional phenomenon distinct from pain's sensory qualities. To study how the brain processes pain-related emotions, Corder et al. used in vivo neural calcium imaging in freely behaving mice. They identified brain circuits that respond to pain and directly tested their causal role in motivational behaviors associated with acute and chronic pain.

Science, this issue p. 276