# REPRESENTATION OF PAIN AFFECT IN THE CENTRAL NERVOUS SYSTEM UNDER NITROUS OXIDE-INDUCED ANESTHESIA

by
Cindy Danyang Liu

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Approved:

DocuSigned by:
Jon Levine
6835AD34857A49E...

Jon Levine
Chair

DocuSigned by:
Allan Basbaum
DocuSigned by:

Allan Basbaum

DocuSigned by:

DEVANAND MANOLI

DocuSigned by:
Tomasz Nowakowski
CAFC10E825A34A4...

Tomasz Nowakowski

Committee Members

*For Alex,*

*who believes in*

*the best of me.*

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# REPRESENTATION OF PAIN AFFECT IN THE CENTRAL NERVOUS SYSTEM UNDER NITROUS OXIDE-INDUCED ANESTHESIA

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## ABSTRACT

Approximately 0.2% of patients undergoing surgery with general anesthesia report experiencing intraoperative awareness, wherein they are aware of their surroundings and the procedure despite not showing signs of consciousness. These experiences are traumatic, can hinder recovery, and are underreported as general anesthetics cause amnesia. Improving both our understanding of mechanisms by which general anesthesia induces analgesia, separately from unresponsiveness or unconsciousness, and methods of accurately monitoring levels of consciousness in patients are necessary to ensure that patients receive adequate analgesia.

Nitrous oxide is a fast-acting general anesthetic that produces analgesia but does not induce loss of consciousness or impair sensory thresholds. As such, nitrous oxide anesthesia presents a unique opportunity to concurrently study the sensory-discriminative and the affective-motivational components of the pain experience. Interestingly, descriptions of nitrous oxide-induced analgesia match those of anterior cingulate cortex (ACC) lesions, which selectively inhibit pain aversion but not sensory-discrimination. Past studies agree that neuronal activity in the ACC correlates strongly with perceived pain intensity. We therefore predicted that nitrous oxide anesthesia, a known analgesic, would decrease neuronal activity in the ACC. Surprisingly, nitrous oxide exposure results in rapid, robust activation of ACC neurons. The ACC is highly interconnected to anesthesia- and pain-processing regions in the brain, including the midbrain periaqueductal gray (PAG), which has been hypothesized to mediate nitrous oxide analgesia by engaging descending inhibition of nociceptive input from the spinal cord. However,

whether nitrous oxide directly acts on PAG neurons, or indirectly via upstream structures like the ACC, is currently unknown.

Our objective is to investigate the circuit mechanisms by which nitrous oxide activates ACC neurons, which we hypothesize is critical for maintaining nitrous oxide-induced analgesia. To this end, we perform *in vivo* calcium imaging in the ACC of mice exposed to air or nitrous oxide before and after painful stimuli to characterize real-time changes in neuronal activity. We also use immunohistochemistry, *in situ* hybridization, and viral tracing techniques to characterize the pattern of nitrous oxide-induced neuronal activation throughout the central nervous system, using probes against the Fos protein (or *c-Fos* messenger ribonucleic acid) as a proxy for neuronal activation. We find that nitrous oxide-induced analgesia likely arises from activation of the ACC itself and not through PAG-mediated descending inhibition of the spinal cord. We propose that monitoring ACC activity can therefore be an effective way to determine that a patient is receiving sufficient analgesia even under intraoperative awareness where other metrics fail to do so.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ACC** | anterior cingulate cortex |
| **BLA** | basolateral amygdala |
| **dl, vlPAG** | dorsolateral, ventrolateral periaqueductal gray |
| **DOR** | delta opioid receptor |
| **GABA** | gamma-aminobutyric acid |
| **lLn** | intralaminar nuclei of the thalamus |
| **KOR** | kappa opioid receptor |
| **LHb** | lateral habenula |
| **lPBn** | lateral parabrachial nucleus |
| **MD** | mediodorsal thalamus of the nucleus |
| **MOR** | mu opioid receptor |
| **NMDA** | N-methyl-D-aspartate |
| **PFC** | prefrontal cortex |
| **PV** | parvalbumin (-expressing inhibitory interneurons) |
| **(m)RNA** | (messenger) ribonucleic acid |
| **SCDH** | spinal cord dorsal horn |
| **SST** | somatostatin (-expressing inhibitory interneurons) |
| **VIP** | vasoactive intestinal peptide (-expressing inhibitory interneurons) |

# CHAPTER 1

# INTRODUCTION TO NITROUS OXIDE ANESTHESIA

## 1.1 OVERVIEW OF GENERAL ANESTHESIA

General anesthesia is used to reversibly induce analgesia (loss of pain), amnesia (loss of memory), hypnosis (loss of consciousness), akinesia (loss of voluntary movement), and areflexia (loss of reflexive movement) prior to and during surgical procedures [1]. Analgesia before and during procedures is assessed by monitoring responses, often reflexive movements, to noxious stimuli; however, areflexia does not necessarily equate to loss of pain perception [2]. Brain and spinal cord activity can be detected at deeper levels of anesthesia than clinical indicators of responsiveness (heart rate, blood pressure, movement) would otherwise indicate [3]. Indeed, an estimated 0.2% of patients recall intraoperative awareness despite not showing signs of consciousness, and this number is likely underreported as amnesia is a common characteristic of general anesthetic agents [4]. Such experiences can be detrimental to patients' recovery, in many cases leading to the development of posttraumatic stress disorder [5]. Understanding the circuitry underlying anesthesia-induced analgesia, but not unresponsiveness or unconsciousness, is therefore critical to providing sufficient analgesia and improving patient outcomes.

Many general anesthetics, including intravenous anesthetics like propofol and etomidate and volatile anesthetics like isoflurane, desflurane, and sevoflurane, primarily activate gamma-aminobutyric acid A ($GABA_A$) receptors, while a few, most notably ketamine, which is injected intravenously or intramuscularly, and nitrous oxide, which is inhaled, are primarily N-methyl-D-aspartate (NMDA) receptor antagonists [6]. Both mechanisms of action inhibit neuronal signaling and thereby dampen overall neuronal activity [7,8], which could explain how general anesthetics induce unresponsiveness and unconsciousness [9]. Since sleep shares similar characteristics, it has been hypothesized that general anesthetics hijack the neuronal

pathways that regulate sleep and wakefulness [10]. Indeed, general anesthetics induce neuronal activation in some, but not all, brain areas that regulate sleep and arousal [11–13], and deep sleep and stages of deep anesthesia share similar characteristics in electrocorticogram and electroencephalogram readings [9]. However, these readings surprisingly cannot reveal if someone is experiencing intraoperative awareness, meaning that mimicking deep sleep is not always sufficient to prevent such an individual from experiencing pain under a state of supposed deep anesthesia.

General anesthetics are often administered as a cocktail to better leverage aspects of different general anesthetics and other drugs, which allows the use of lower doses for each component of the cocktail and diminished risk of adverse side effects [14]. However, such an approach would confound studies seeking to identify key regions that regulate anesthesia susceptibility. Since nociception, the detection of noxious stimuli, elicits activity in both the brain and spinal cord [3] even under deep anesthesia, it is important to consider what impact general anesthesia may have on pain perception, and in particular, the affective-motivational aspect of the pain experience [15–17]. Some studies in humans have identified general anesthesia-induced activity changes in brain regions associated with pain perception, including the thalamus and hypothalamus [6,18–21]. Research conducted in rodents is insufficient to answer such a question, as they cannot report intraoperative awareness after the fact, although one study has demonstrated that some general anesthetics have been shown to alter activity in the central amygdala, one brain region that contributes to pain perception [18–21].

### 1.2 CHARACTERISTICS OF NITROUS OXIDE ANESTHESIA

Nitrous oxide is a rapidly acting volatile general anesthetic that induces analgesia and amnesia without loss of consciousness, and, interestingly, without loss of somatosensation [22,23]. Indeed, in humans, inhaling nitrous oxide does not alter detection threshold of a noxious electrical stimulation but does increase the threshold at which pain is reported [24]. When

administered as general anesthesia, patients report - either afterwards, if they remember, or intraoperatively using the isolated forearm technique, which allows a patient to use a tourniquet-isolated hand to report feeling pain in real time [25] - that while they can feel the procedure, they feel significantly less, or even no anxiety accompanying pain or the procedure, and thus are not experiencing *pain* [26,27]. Importantly, nitrous oxide can be easily administered - even self-administered by children as young as three years old - and requires no postoperative monitoring, making nitrous oxide an effective anesthetic for minor procedures in pediatric patients [28,29]. In a cocktail of general anesthetics, nitrous oxide synergizes with other anesthetic and analgesic agents to potentiate psychomotor effects [30,31], sedation and depth of anesthesia or with opioids to potentiate analgesic effects [32], thereby reducing the required doses of each drug to achieve the same effect of a single drug. Nitrous oxide therefore provides a unique opportunity to study general anesthesia-induced analgesia without the confounds of sedation.

Early studies in animal models suggest that nitrous oxide impairs nociceptive signaling from the spinal cord; however, an important caveat is that nitrous oxide is almost never administered alone due to its inability to impart akinesia. As such, sedating general anesthetics like halothane are included during induction or maintenance of nitrous oxide general anesthesia. Neuronal responses in the brain to sensory input in the periphery are dampened and delayed by administration of nitrous oxide [33,34]. However, a far more significant driver of nitrous oxide-induced analgesia is the facilitation of descending inhibition of the spinal cord, evidence for which includes spinal cord transections blocking nitrous oxide-induced antinociception [35]. Supporting the involvement of descending inhibition, nitrous oxide has been shown to facilitate the release of endogenous opioids in the ventrolateral periaqueductal gray (vlPAG), a midbrain structure crucial for initiating the descending control of pain [36,37], to induce analgesia. It is hypothesized that activation of opioid receptors in the vlPAG then induces the activation of descending serotonergic [38] and noradrenergic [35,39] systems, both of which can be targeted

in treating chronic pain conditions [39–42]. Curiously, however, immunohistochemical studies of Fos, a protein marker for recently active neurons [43], reveal no difference in Fos expression in the spinal cord projection neuron layer [44–46]. These results may be confounded by the presence of sedating general anesthetics. If nitrous oxide analgesia indeed relies on descending inhibition of the spinal cord, a major unanswered question is how opioidergic neurons in the vlPAG are targeted by nitrous oxide [47]: specifically, whether nitrous oxide acts directly at the level of the vlPAG or indirectly, through brain regions that project to the vlPAG [48].

### 1.2.1 Lesion of the anterior cingulate cortex (ACC) resembles nitrous oxide analgesia

Thus far, there is limited evidence as to where in the brain nitrous oxide might act to induce analgesia [49]. Curiously, though, descriptions of nitrous oxide-induced analgesia parallel those of an anterior cingulotomy, a surgical treatment for intractable chronic pain [50,51]. The anterior cingulate cortex (ACC) has long been associated with the affective-emotional aspect of pain [52]; specifically, the literature has historically agreed that activity levels of the ACC correlates to perceived pain affect in humans [16,53] and can be manipulated to alter pain affect in rodents [54–56].

Indeed, infusion of morphine into the ACC [57], which locally inhibits neuronal signaling through activation of the mu opioid receptor (MOR) [58], induces analgesia in a neuropathic pain model but does not alter sensory thresholds [57]. The pharmacology of nitrous oxide - namely, the depression of synaptic signaling by antagonizing NMDA receptors - suggests that nitrous oxide should, like morphine, inhibit signaling in the ACC. The ACC also projects directly to the vlPAG [59], making the ACC a region of interest when considering how nitrous oxide influences vlPAG signaling. Specifically, in this case, nitrous oxide would decrease ACC activity, thereby disinhibiting opioidergic signaling in the vlPAG. Interestingly, a positron emission tomography scan of humans inhaling nitrous oxide suggests an increase in metabolic activity in

the ACC [60], although metabolism alone does not reveal the nature of neuronal activity in the area.

### 1.4 SUMMARY

In summary, nitrous oxide is a general anesthetic with analgesic properties that presents a unique opportunity to study general anesthesia-induced analgesia without loss of consciousness. There is significant evidence that nitrous oxide induces analgesia by engaging descending inhibition of the spinal cord, mediated by opioidergic signaling in the midbrain vlPAG. However, how nitrous oxide induces such activity is as yet unclear. A compelling hypothesis is that nitrous oxide inhibits neuronal signaling in the ACC, which then disinhibits the vlPAG to facilitate descending inhibition. Understanding how nitrous oxide alters cortical circuitry to produce analgesia will further our understanding of general anesthesia-induced analgesia to ensure that patients receive sufficient analgesia and, overall, improve patient outcomes.

In the following chapters, we characterize the pattern of neuronal activity induced by nitrous oxide anesthesia throughout the central nervous system. In Chapter 2, we use *in vivo* calcium imaging to monitor how spontaneous and noxious stimulus-induced activity in the ACC changes under nitrous oxide exposure, and Fos immunoreactivity to identify the cell types that drive such changes. In Chapter 3, we use viral tracing strategies and Fos immunoreactivity to investigate possible circuit mechanisms underlying nitrous oxide-induced analgesia and activation of the ACC. Finally, in Chapter 4, we discuss further potential studies of nitrous oxide-mediated analgesia and the future of nitrous oxide use in general anesthesia and beyond.

# CHAPTER 2

# PARADOXICAL INCREASES IN ANTERIOR CINGULATE CORTEX ACTIVITY DURING NITROUS OXIDE-INDUCED ANALGESIA REVEAL A SIGNATURE OF PAIN AFFECT

## 2.1 INTRODUCTION

The general consensus is that increases in neuronal activity in the anterior cingulate cortex (ACC) contribute to pain's negative affect. Here, using *in vivo* imaging of neuronal calcium dynamics in mice, we report that nitrous oxide, a general anesthetic that reduces pain affect, paradoxically, increases ACC spontaneous activity. As expected, a noxious stimulus also increased ACC activity. However, as nitrous oxide increases baseline activity, the relative change in activity from pre-stimulus baseline was significantly less than the change in the absence of the general anesthetic. We suggest that this relative change in activity represents a neural signature of the affective pain experience. Furthermore, this signature of pain persists under general anesthesia induced by isoflurane, at concentrations in which the mouse is unresponsive. We suggest that this signature underlies the phenomenon of connected consciousness, in which use of the isolated forelimb technique revealed that pain percepts can persist in anesthetized patients.

General anesthetics are potent regulators of pain processing and can produce effects ranging from diminished or absent pain perception (i.e., analgesia [61–64]) to the total abolition of reflexive responses to ongoing surgical stimuli [62,65]. Importantly, pain is a conscious, multidimensional percept that includes both sensory-discriminative (modality, location, intensity) and affective-motivational (unpleasantness) features [15]. Studies in awake, responsive patients inhaling nitrous oxide, a general anesthetic gas with analgesic properties [61,66,67], report a preferential reduction of the affective-motivational aspects of pain [24,68]. Currently, however, there is limited information as to the neural mechanisms that underlie the ability of nitrous oxide

to modulate affective-motivational aspects of pain.

The anterior cingulate cortex (ACC) is critical to processing the affective-motivational features of the pain percept [16]. In humans, primates, and rodents, ACC neurons respond to noxious, but not innocuous, thermal and mechanical stimuli [69–72]. Human neuroimaging studies have shown that increased ratings of the unpleasantness of pain correlate with increased ACC activity, and that analgesia correlates with decreased ACC activity [16,73]. Interestingly, after targeted ACC manipulations, including ablations [74–76] or deep brain stimulation [77,78], patients still sense noxious stimuli, but report that the stimuli are less painful or less “bothersome”. Consistent with clinical findings, ablative [79,80] or pharmacological [55,81,82] manipulations of the ACC in rodents produced selective reduction in affective-motivational responses to pain without influencing sensory thresholds [55–57,79–86]. Importantly, these studies led to the conclusion that *inhibition [79–81]* of the ACC is critical to generating pain control and that *excitation [55,87–89]* produces increased pain aversion.

Unexpectedly, and indeed paradoxically given the general consensus [15,90], cerebral blood flow and metabolism-based measurements of neural activity report that nitrous oxide increases activity in frontal cortical regions [91], and in particular in the ACC [60]. However, because nitrous oxide has a strong vasodilatory effect that confounds interpretations of data from neuroimaging studies [92,93], these findings are controversial. If true, these studies would suggest that increases in ACC activity do not necessarily lead to increased pain aversion and may even contribute to the analgesic effects of nitrous oxide. To date, however, there have been no direct measurements (electrophysiology or *in vivo* calcium imaging) of neural activity in the ACC during inhalation of nitrous oxide.

In this present work, using *in vivo* imaging of the calcium dynamics of ACC neurons in mice, we report that nitrous oxide, in fact, produces profound increases in ACC activity. Furthermore, in studies of molecularly distinct subsets of cortical neurons, we discovered that nitrous oxide preferentially activates excitatory ACC neurons, with no effect on inhibitory or

opioidergic (mu-, kappa-, or delta-opioid receptor expressing) interneurons. In behavioral studies, we confirmed that nitrous oxide produces a potent analgesia that preferentially diminishes affective-motivational pain endpoints. And in awake, freely moving mice, we demonstrated that by increasing spontaneous ACC activity, nitrous oxide reduces the relative magnitude of noxious stimulus-induced ACC activation. Importantly, this reduction in noxious stimulus-evoked ACC activation correlates with nitrous oxide-induced reductions in affective-motivational, but not reflexive behaviors. Lastly, using these changes in ACC as a neural biomarker for affective-motivational aspects of pain, we demonstrate the presence of neural signatures of pain even in an isoflurane-anesthetized, behaviorally unresponsive mouse.

## 2.2 RESULTS

### 2.2.1 Nitrous oxide induces paradoxical increases in ACC activity

We used head-mounted miniature microscopes [94,95] to monitor the calcium dynamics [96] of individually identified ACC neurons during exposure to nitrous oxide or control gas (oxygen) (**Figure 2.4.1A**, **B, Figure 2.4.6**). Across the two separate exposures, we identified 1,364 neurons (nitrous oxide: 795, oxygen: 569). Consistently, we found that inhalation of nitrous oxide drove sustained and significant increases in spontaneous ACC activity (**Figure 2.4.1C**).

Next, we used clustering to identify distinct activity patterns that occur during inhalation of nitrous oxide or control gas. Dimensionality reduction of neural activity patterns using t-distributed stochastic neighbor embedding (tSNE) reveals that neural activity patterns during inhalation of nitrous oxide are highly divergent and largely nonoverlapping with those observed during inhalation of control gas (**Figure 2.4.1D**, **E**). Density-based spatial clustering after tSNE analysis identified 4 unique clusters of activity (**Figure 2.4.1D**, **2.4.1F**). Neurons identified during nitrous oxide recordings predominately populated clusters defined by large increases in activity (**Figure 2.4.1F**: clusters 1, 2). Neurons from control gas recordings are largely confined to

clusters with minimal activity changes (**Figure 2.4.1F**: cluster 3) or slightly decreased activity (**Figure 2.4.1F**: cluster 4).

Using immunohistochemistry and consistent with our calcium imaging observations, we found that nitrous oxide increased the number of cells expressing the Fos protein [97], a surrogate marker of overall neuronal activity (**Figure 2.4.7**). Thus, in sharp contrast to the established literature, we conclude that increased ACC activity can occur during inhalation of a known analgesic.

### 2.2.2 Nitrous oxide preferentially activates excitatory ACC neurons in cortical layer 2/3

Cortical circuits are comprised of functionally distinct neurons with unique molecular identities that underlie cortical information processing [98–105]. Using a combinatorial viral/genetic strategy that enables the selective expression of genetically encoded calcium indicators within specific cell types, we monitored the activity of principal (excitatory) neurons (vGluT2 expressing; VG2), and molecularly distinct subpopulations of cortical inhibitory interneurons that express parvalbumin (PV), somatostatin (SST), or vasoactive intestinal peptide (VIP) (**Figure 2.4.2A**, **B**). During separate exposures to nitrous oxide or control gas (oxygen) (**Figure 2.4.6**), we identified 1,771 neurons (by subtype: VG2: 846, PV: 296, SST: 260, VIP: 369; by gas: nitrous oxide: 1049, oxygen: 722).

**Figures 2.4.2C** and **2.4.7** show that compared to oxygen, nitrous oxide preferentially activated excitatory neurons. Although we do observe PV-, SST-, and VIP-expressing interneurons with increased activity (**Figure 2.4.2D**), the proportion of neurons with increased activity did not differ between oxygen and nitrous oxide (**Figures 2.4.2C** and **2.4.8**). An assessment of the recruitment of subtypes of neurons to clusters with distinct activity profiles confirmed that excitatory, but not inhibitory (PV, SST, VIP), neurons are preferentially recruited to clusters with increased activity (**Figure 2.4.2E, F**: clusters 1 and 2).

Next, to further investigate the spatial distribution and molecular identity of ACC neurons

activated by nitrous oxide, we used multiplexed *in situ* hybridization of message to map changes in *c-Fos* message as well as that of other relevant genes [106] (**Figures 2.4.3A** and **2.4.9**). *In situ* hybridization shows that nitrous oxide increases *c-Fos*-expressing cells in cortical layers 2/3 but not other layers (**Figure 2.4.3B**). Also, consistent with the calcium imaging recordings (**Figure 2**), we found that nitrous oxide does not increase the percentage of inhibitory interneurons (*Gad1*-expressing cells) that express *c-Fos* (**Figure 2.4.3C**). This result indicates that the increased Fos expression observed in layer 2/3 is largely due to activation of excitatory neurons. We conclude that nitrous oxide preferentially activates excitatory neurons in the ACC, effects that we hypothesize profoundly alter the experience of pain.

The ACC is a known target of opioid analgesics [57,82,107], and interestingly, naloxone, an opioid receptor antagonist, partially reverses nitrous oxide-induced analgesia [108]. Therefore, we investigated whether nitrous oxide alters *c-Fos* expression within ACC neurons that express the mu-, kappa-, or delta-opioid receptors. We found no change in *c-Fos* expression in mu (**Figure 2.4.3D**), kappa (**Figure 2.4.3E**), or delta (**Figure 2.4.3F**) opioid receptor-expressing ACC neurons. We conclude, therefore, that the effects of nitrous oxide in the ACC are not due to local opioidergic circuit mechanisms (i.e., circuit disinhibition [109]).

**2.2.3 Nitrous oxide reduces affective-motivational pain-related behaviors**

Mice produce complex nocifensive behaviors to noxious stimuli. As noted above, alterations in ACC activity are postulated to influence affective-motivational, but not reflexive, responses to noxious stimuli. Here, we asked whether nitrous oxide-induced changes in ACC activity translate to preferential reduction of affective-motivational, rather than reflexive, indices of pain. In these studies, we compared the effects of nitrous oxide inhalation on the production of reflexive versus affective-motivational behaviors during presentation of a noxious stimulus (**Figure 2.4.4A**, **B**).

While mice inhaled nitrous oxide (60%) or control gas (air), we generated brief, noxious heat stimuli using infrared laser pulses targeted to the hindpaw. The laser stimulus produced

robust nocifensive responses in mice, including withdrawals, shakes, and licks [110] (**Figure 2.4.4B**). Importantly, in rodents, licking of the hindpaw following a noxious stimulus is an affective-motivational response indicative of the experience of pain [83,111–113], not merely a reflexive response, as is the case for withdrawals and shakes [112,114,115]. **Figure 2.4.4C** shows that nitrous oxide produces a potent suppression of affective-motivational measures of pain. Indeed, nitrous oxide almost completely abolished laser-evoked licks. In contrast, reflexive behaviors are minimally affected by nitrous oxide (**Figure 2.4.4C**).

In mice, a single laser stimulus often evokes multiple concurrent behaviors, with the production of licks usually coupled to reflexive behaviors (i.e., withdrawals and shakes). Nitrous oxide dramatically reduced this coupling, with laser-evoked reflexes producing proportionally fewer concurrent licks (**Figure 2.4.4D**). Taken together, we conclude that the reduction of affective-motivational behaviors produced by nitrous oxide is independent of any effects on reflexive behaviors.

### 2.2.5 Nitrous oxide-induced analgesia correlates with noxious stimulus-evoked ACC activity

Next, we investigated how paradoxical, nitrous oxide-induced increases in spontaneous ACC activity translate to an analgesic effect in response to a noxious stimulus. We hypothesized that nitrous oxide-induced increases in ACC activity create a ceiling effect, thereby attenuating the relative magnitude of noxious stimulus-evoked changes, which results in a diminished experience of pain. To test this hypothesis, we imaged neural activity in ACC neurons during inhalation of nitrous oxide (60%) or control gas (air) with concurrent delivery of the laser stimulus (**Figure 2.4.4E**). Given our finding that nitrous oxide predominantly increases activity in excitatory neurons of the ACC, here we used a viral approach to monitor activity exclusively in excitatory neurons (**Figure 2.4.4E**). Across the nitrous oxide and air exposures, we identified 1402 neurons (nitrous oxide: 825, air: 577).

During presentation of the laser stimulus, we found that noxious stimulus responsive ACC neurons accounted for 31.8±2.5% of the total population in the air condition and 23.4±3.0% under nitrous oxide (**Figure 2.4.4F**, **J**). In agreement with the findings described above, nitrous oxide significantly increased the pre-stimulus baseline event rate compared to air (**Figure 2.4.4G**, **I**). Interestingly, and as would be expected for a ceiling effect, we observed no difference in the absolute magnitude of laser-evoked activity between nitrous oxide and air conditions (**Figure 2.4.4G**, **I**). However, when measures of noxious stimulus-evoked activity were normalized to pre-stimulus baseline activity there was a clear reduction in the nitrous oxide exposure as compared to air (**Figure 2.4.4H**, **4I**). In other words, nitrous oxide reduces the relative magnitude of noxious stimulus-evoked activity compared to air.

Importantly, the degree of laser-evoked licking behavior, but not reflexes, correlates not only with the relative magnitude of the laser-evoked maximum event rate, but also with the percentage of ACC neurons activated by the laser (**Figure 2.4.4I**, **4J**). These relationships suggest that noxious stimulus-evoked neural activity of the ACC can indeed be used as a proxy for the affective-motivational aspects of the pain experience in mice.

### 2.2.5 Neural signatures of pain are present during isoflurane-induced general anesthesia

To date, aside from directly asking patients to rate levels of ongoing pain, there is no neural biomarker that can be used as a proxy of the affective experience of pain. This lack of an adequate pain biomarker is particularly problematic during general anesthesia, where patients (and animals) are immobilized and thus behaviorally unresponsive and incapable of reporting their pain experience [2]. Rather disturbingly, clinical studies using the isolated forearm technique reveal that patients often report the experience of pain under general anesthesia [116,117]. Fortunately, perhaps, the amnestic effects of general anesthetics largely render them unable to recall such events postoperatively [118]. As nitrous oxide does not produce loss of behavioral responsiveness in mice under normal conditions (concentrations greater than 100%

would be required), we could not determine whether there is persistent brain activity in a nitrous oxide anesthetized mouse. Therefore, we initiated studies using isoflurane, a widely used volatile anesthetic that readily produces general anesthesia. Our specific question is whether there can be persistent ACC activity consistent with the experience of pain in an otherwise anesthetized, behaviorally unresponsive mouse.

We first assessed the influence of isoflurane on the spontaneous activity of ACC neurons. In contrast to changes recorded during nitrous oxide inhalation, we found that isoflurane decreased spontaneous ACC activity in a dose-dependent manner, and completely abolished ACC activity at the highest concentrations.

We then assessed noxious stimulus-evoked ACC activity in isoflurane-anesthetized mice. As expected, mice tested at 2% isoflurane, a concentration where nociceptive reflexes withdrawals are abolished, we observed a complete absence of spontaneous and evoked activity.

Next, we tested mice during inhalation of 1% isoflurane in air, a concentration where mice are immobilized and lack righting reflexes (a rodent measure of awareness), yet still retain neural activity (**Figure 2.4.5A**, **B**). Although laser stimulation increased the activity of ACC neurons in both air and 1% isoflurane conditions (**Figure 2.4.5C**), we recorded significantly fewer laser responsive neurons under isoflurane (**Figure 2.4.5G**). However, we found no significant differences between baseline or laser-evoked ACC activity (i.e., event rate-based) measurements (**Figure 2.4.5E-G**).

Lastly, using the relationship between noxious stimulus-evoked ACC activity and the generation of affective-motivational behaviors (licks) recorded in nitrous oxide and air exposed mice (**Figure 2.4.10**), we assessed the presence of neural activity patterns indicative of an affective-motivational pain experience in isoflurane-anesthetized mice. Surprisingly, noxious stimulus-evoked ACC activity during inhalation of 1% isoflurane, when mice could not perform licks due to isoflurane-induced immobilization, not only persists, but is consistent with the

experience of pain (**Figure 2.4.5H**). We conclude that a brain signature of affective-motivational aspects of the pain experience can be preserved under general anesthesia.

## 2.3 DISCUSSION

In this study, we explored the influence of nitrous oxide, an inhalational anesthetic with analgesic properties, on neural activity of the ACC, a cortical region that is a major contributor to the affective-motivational aspects of pain [15]. In contrast to the prevailing view, namely that inhibition of the ACC reduces pain affect [79–81], we discovered that nitrous oxide profoundly increases spontaneous ACC neuronal activity. Clearly, our results present a paradox: how is it that nitrous oxide increases spontaneous ACC activity and produces analgesia, but does not, as the literature would predict, increase affective-motivational indices of pain. Unexpectedly, we discovered that the absolute magnitude ACC activity provoked by a noxious stimulus did not differ between nitrous oxide and air, even though nitrous oxide reduced measures of the affective pain experience. Rather, we demonstrate that it is the relative magnitude of noxious stimulus-evoked ACC activity, compared to activity immediately prior to the stimulus, that best correlates with the production of affective-motivational pain behaviors.

In other words, what underlies the affective-motivational aspects of pain by the ACC is a circuit mechanism of gain control [119], namely one that adjusts the signal-to-noise ratio of stimulus-evoked ACC neuronal activity [120]. In this model, spontaneous activity (i.e., noise) can be tuned, for example, by nitrous oxide, to modulate the relative change of activity provoked by a noxious stimulus (i.e., signal). Based on our findings, we conclude that increased activity *per se* is not necessarily indicative of a pain experience. Rather, it is the change between resting (spontaneous) ACC activity and that evoked by a noxious stimulus (e.g., laser or surgical intervention) that determines whether pain affect is generated. Of course, this conclusion is consistent with the fact that despite ongoing activity in the naïve mouse (and human), there is no pain affect until the introduction of a noxious stimulus.

Unclear is the mechanism underlying the selective increase in the activity of excitatory ACC neurons by nitrous oxide, purportedly a non-competitive inhibitor of the NMDA receptor [47]. Although one would expect that blocking NMDA receptors would decrease neuronal activity, previous recordings in the prefrontal cortex reported that selective NMDA receptor antagonists, in fact, increase excitatory neuronal activity, not directly, but by decreasing the activity of inhibitory interneurons [121]. As we did not observe decreases in inhibitory interneuron activity, we suggest that nitrous oxide's effects on the ACC involve alternative mechanisms[47]. For example, nitrous oxide could influence ACC neuronal activity via direct actions on upstream brain regions [122], such as medial-dorsal thalamus or basolateral amygdala [123].

Also unclear are the direct downstream consequences of nitrous oxide-induced increases in ACC activity, and how this translates to behavioral analgesia in tests of pain affect. The ACC is a major hub that is highly connected to other elements of the so-called "pain matrix" [124]. Thus, nitrous oxide-induced activation of ACC projection neurons would produce wide-ranging effects on other components of the matrix [125], thereby altering the experience of pain. Other studies reported that nitrous oxide analgesia is naloxone reversible [47], however, as we did not uncover changes in neural activity in opioid receptor-expressing ACC neurons, we conclude this effect is unlikely due to direct opioid-mediated actions on the ACC. In ongoing studies we are examining whether the downstream circuits engaged by the ACC contribute to the naloxone-reversible aspects of nitrous oxide-induced analgesia, potentially by direct actions on endorphin-mediated inhibitory controls [59,126].

Particularly surprising was the persistence of noxious stimulus-evoked activity in the ACC of isoflurane-anesthetized mice, at concentrations that blocked behavioral indices of pain affect, namely, licking in response to a noxious stimulus. As this activity was comparable to that recorded in awake mice, we suggest that it represents a neural biomarker, in effect a surrogate pain index that is specific for the affective component of the pain experience. In other words, the

brain can “experience” pain even under general anesthesia, an interpretation consistent with provocative clinical reports of the high prevalence of an intraoperative experience of pain in patients [116–118,127] under general anesthesia. These patients can communicate their pain experience in real time through the isolated forearm technique [2,116,117], a phenomenon known as connected consciousness [2].

Importantly, it is possible to establish a level of general anesthesia in which connected consciousness does not occur [128]. In fact, when we increased the depth of anesthesia using 2% isoflurane and then tested the mice, not only was there no behavioral response to a noxious stimulus, but we observed that both spontaneous and evoked ACC activity were abolished. This absence of activity at the deepest levels of anesthesia [19] creates, in essence, a functional “ablation” of the ACC, which blocks the experience of pain much in the same way that a physical lesion of the ACC provides pain relief in patients [74]. However, although similarly deep levels of general anesthesia (measured by EEG) can ensure the absence of connected consciousness [128], the associated increased risk of adverse postoperative outcomes (death, stroke, postoperative delirium) [129] likely outweigh any potential benefits. For this reason, our findings are particularly relevant to ongoing efforts to develop neural activity-based biomarkers that can reliably document adequate analgesia during surgery under general anesthesia [130], which, in turn, will support the development of novel general anesthetics that can safely block the experience of pain.

## 2.4 FIGURES

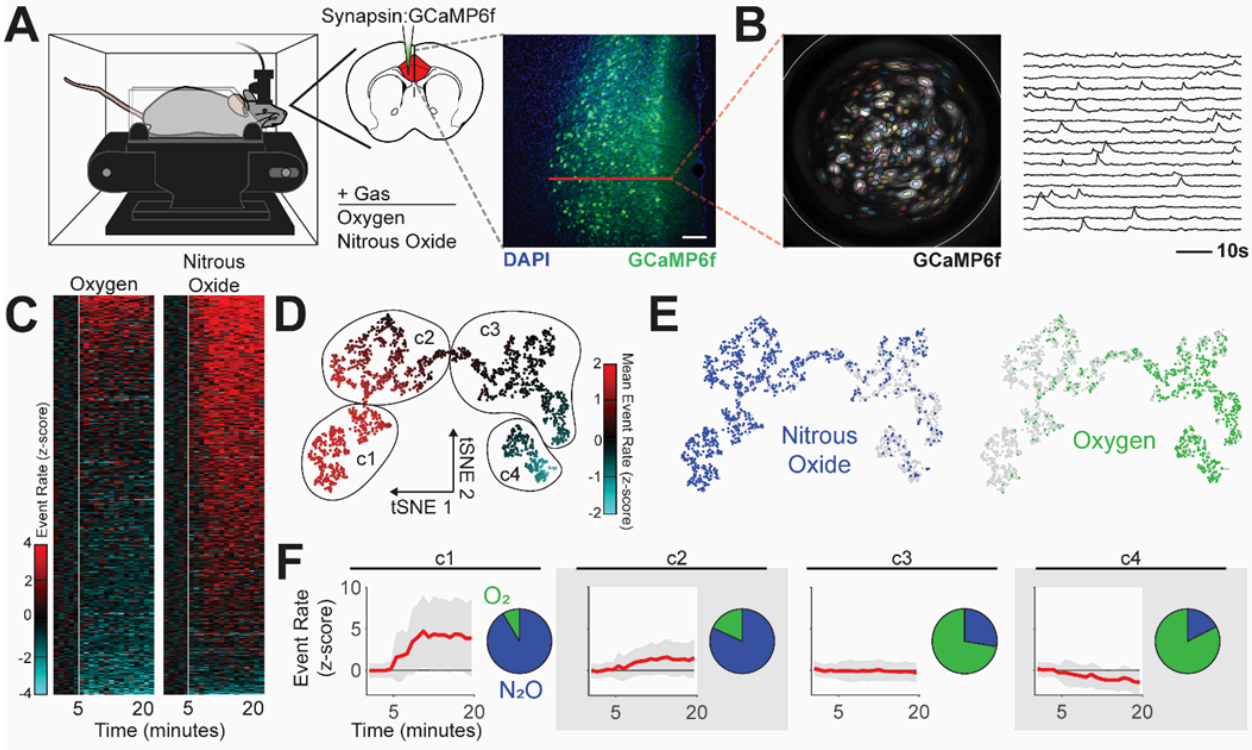


**Figure 2.4.1 Nitrous oxide increases ACC activity.**
(**A**) Left: Spontaneous ACC activity monitored during inhalation of nitrous oxide using a genetically encoded calcium indicator (GCaMP6f) and head-mounted miniature microscopes. Right: ACC targeted GCaMP6f neuronal expression. Red line indicates target for calcium imaging. Scale bar (white) equals 50 μm. (**B**) Left: Maximum projection of recording over time displays neuronal distribution and is overlaid with PCA/ICA cell segmentation (colored areas) and GRIN lens boundaries (white circle). Right: Single neuron traces of extracted calcium fluorescence over time. (**C**) Heatmap of changes in event rate induced by nitrous oxide or control gas (oxygen); z-score normalized to baseline activity, prior to gas exposure (white line). (**D** and **E**) Representation of neural activity patterns using t-distributed stochastic neighbor embedding (tSNE), colored by neural activity (**D**) and gas exposure (**E**). (**F**) Identified clusters, including the mean (red) and standard deviation (gray) of neural activity (baseline normalized z-scored event rate) and cluster makeup by gas exposure.

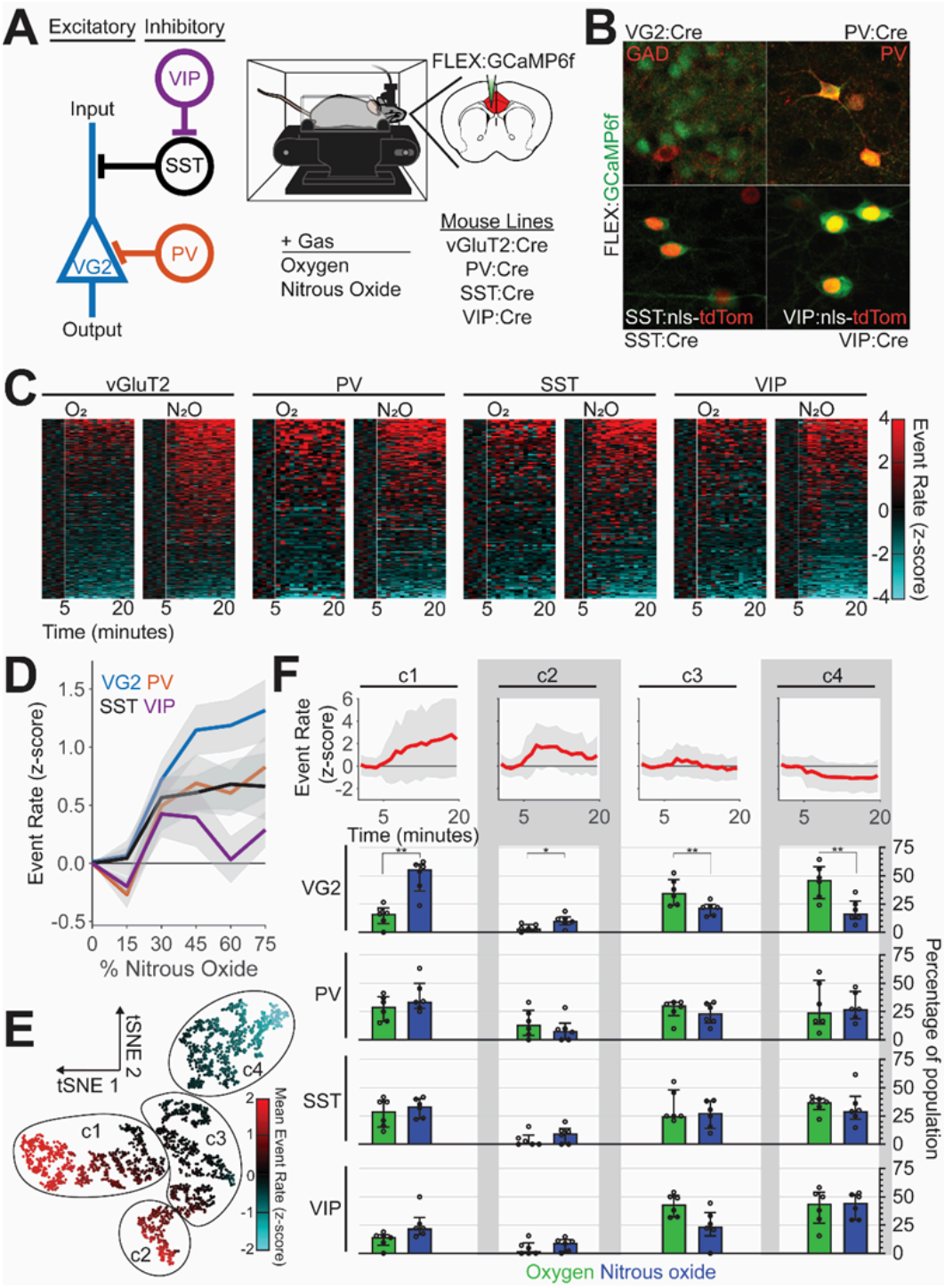


**Figure 2.4.2 Nitrous oxide preferentially activates excitatory ACC neurons.**
(**A**) Left: Simplified circuit illustrating the local connectivity of molecularly distinct cortical neurons. Right: Spontaneous activity of molecularly distinct ACC neurons monitored during inhalation of nitrous oxide, after their restricted labeling with GCaMP6f using a combinatorial viral/genetic approach. (**B**) Selective labeling of molecularly distinct populations. (**C**) Heatmap of
(*Figure caption continued on the next page.*)

(*Figure caption continued from the previous page.*)
changes in event rate induced by nitrous oxide or control gas (oxygen); z-score normalized to baseline activity prior to gas exposure (white line). (**D**) Changes in neural activity (z-scored event rate) across different neural subtypes as a function of nitrous oxide concentration (colored line: mean, gray area: SEM). (**E**) Representation of neural activity patterns using t-distributed stochastic neighbor embedding (tSNE), colored by neural activity. (**F**) Mean normalized event rate of identified clusters (top; line: mean, gray area: standard deviation) and the preferential recruitment of distinct molecular subtypes to individual clusters by gas exposure, displayed as median and interquartile range (two-way repeated measures ANOVA, FDR corrected).

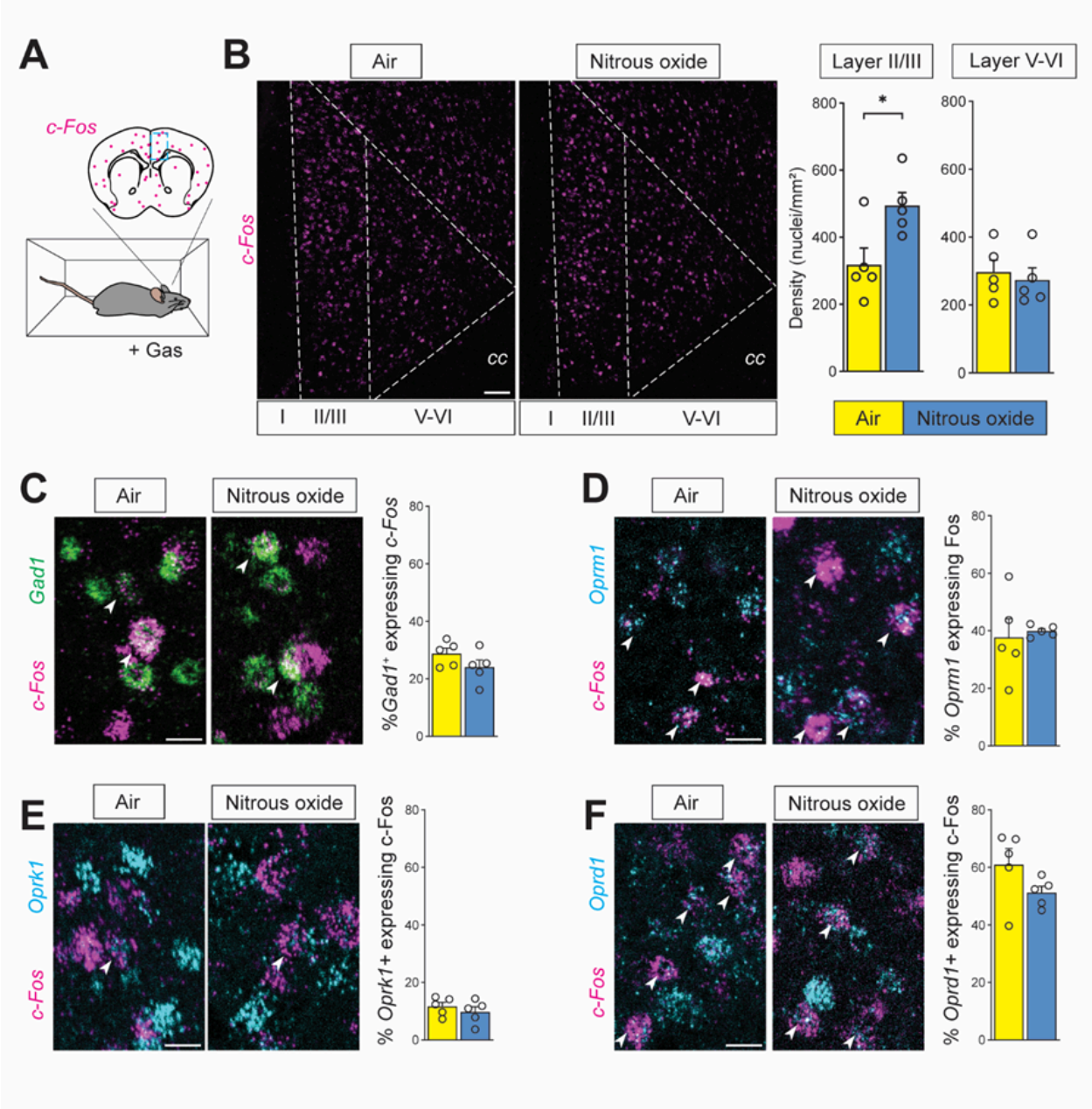


**Figure 2.4.3 Nitrous oxide predominantly activates excitatory ACC neurons in cortical layer 2/3.**

(**A**) *c-Fos* expression, a correlate of neuronal activity, after exposure to air or nitrous oxide (60%). (**B**) *In situ* labeling of *c-Fos*-expressing neurons (magenta; left) are quantified (right) in layer II/II (Student's t-test, $p \leq 0.0244$) and layers V-VI (Student's t-test, $p \leq 0.6621$). (**C**-**F**) Left: *in situ* labeling of *c-Fos* (magenta) within inhibitory (**C**, *Gad1*-expressing in green) or opioid receptor-expressing (mu: *Oprm1* (**D**), kappa: *Oprk1* (**E**), and delta: *Oprd1* (**F**) in cyan). Right: Quantification of percent of c-Fos co-expression in (**C**) *Gad1*+ cells (Student's t-test, $p \leq 0.1778$), (D**)** *Oprm1*+ cells (Welch's t-test, $p \leq 0.7512$), (**E**) *Oprk1*+ cells (Student's t-test, $p \leq 0.4271$), and (**F**) *Oprd1*+ cells (Student's t-test, $p \leq 0.1470$). White scale bars = 100 um (B) or 15 um (**C**-**F**). White arrows indicate double-labeled cells (**C**-**F**). Corpus callosum marked as cc. Data are displayed as mean ± SEM.

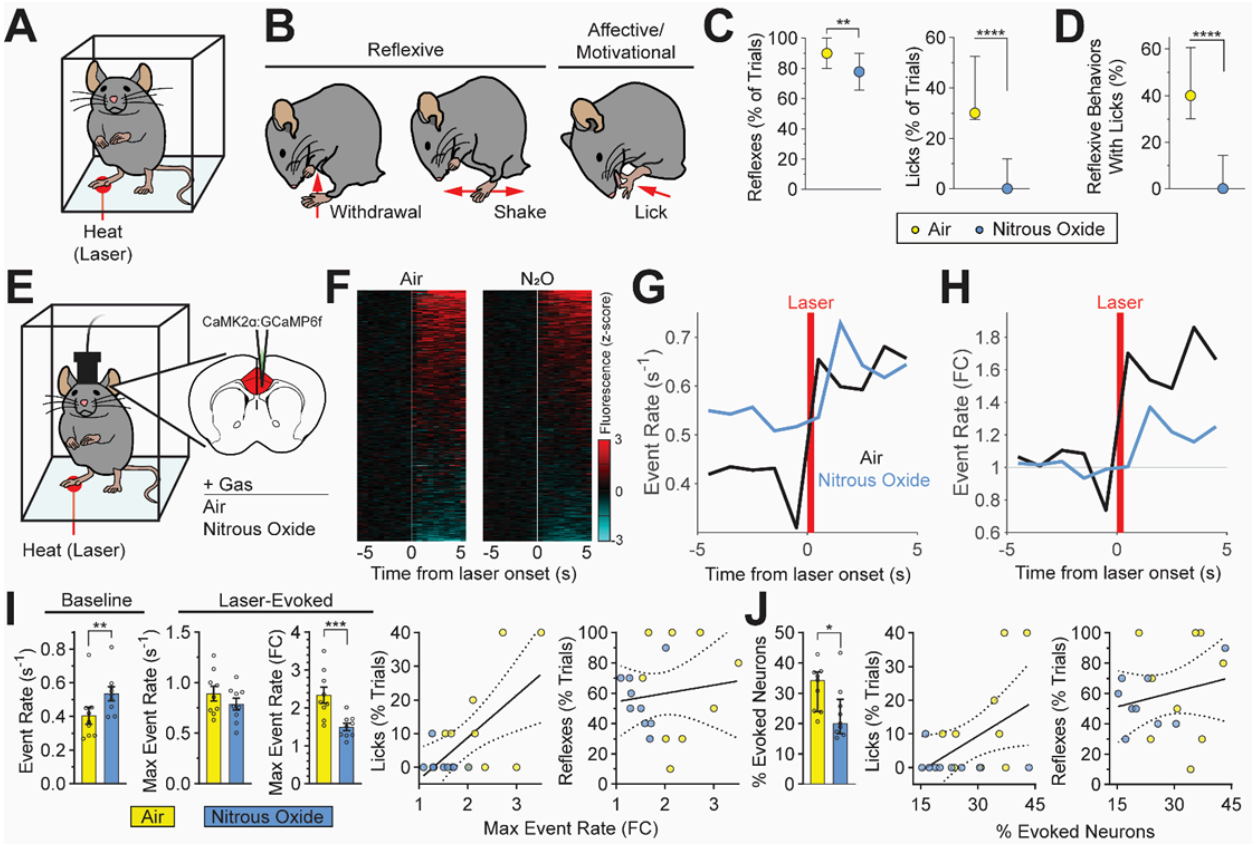


**Figure 2.4.4 Nitrous oxide-induced reduction of affective-motivational pain-related behaviors correlates with changes in noxious stimulus-evoked ACC activity.**
(**A**) Behavioral responses to noxious heat (high-power infrared laser) monitored during inhalation of control gas (air) or nitrous oxide (60%). (**B**) Heat-evoked reflexive and affective-motivational behaviors. (**C**) Reflexive and affective-motivational behavioral responses to noxious stimuli during inhalation of nitrous oxide, quantified as percent of trials (paired t-test, reflexes: $p < 0.0082$; licks: $p < 0.0001$). (**D**) Percentage of licks that occur with reflexive behaviors (paired t-test, $p < 0.0001$). (**E**) Noxious stimulus-evoked ACC activity monitored in awake, freely behaving mice during inhalation of nitrous oxide. GCaMP6f virally-expressed in excitatory neurons (CaMK2a). (**F**) Heatmap of changes per neuron (rows) in calcium dynamics (z-scored and averaged across trials) provoked by laser stimulus (white line) during nitrous oxide or air. (**G** and **H**) Noxious stimulus-evoked neural activity during inhalation of nitrous oxide or air displayed as absolute event rate (**G**, events/second) and baseline normalized event rate (**H**) (n = 9 mice). (**I**) Left: Quantification of baseline and laser-evoked neural activity illustrated in **G** and **H** (paired t-test). Right: Simple linear regression of normalized maximum event rate versus licks ($R^2 = 0.382$, $p < 0.006$) or reflexes ($R^2 = 0.017$, $p < 0.610$). (**J**) Left: Neurons with significantly altered calcium dynamics following laser stimulation as a percentage of the total number of neurons identified per mouse, quantified from (**F**) (paired t-test). Right: Simple linear regression of the percentage of neurons with altered activity versus licks ($R^2 = 0.235$, $p < 0.042$) or reflexes ($R^2 = 0.046$, $p < 0.390$). Data for plots in **C**, **D** and **J** displayed as median and interquartile range; bar graphs in **I** displayed as mean ± SEM; regressions in **I** and **J** displayed as best fit line and 95% confidence interval. N = 30 mice for panels **C** and **D**. N = 9 mice for panels **F** through **J**.

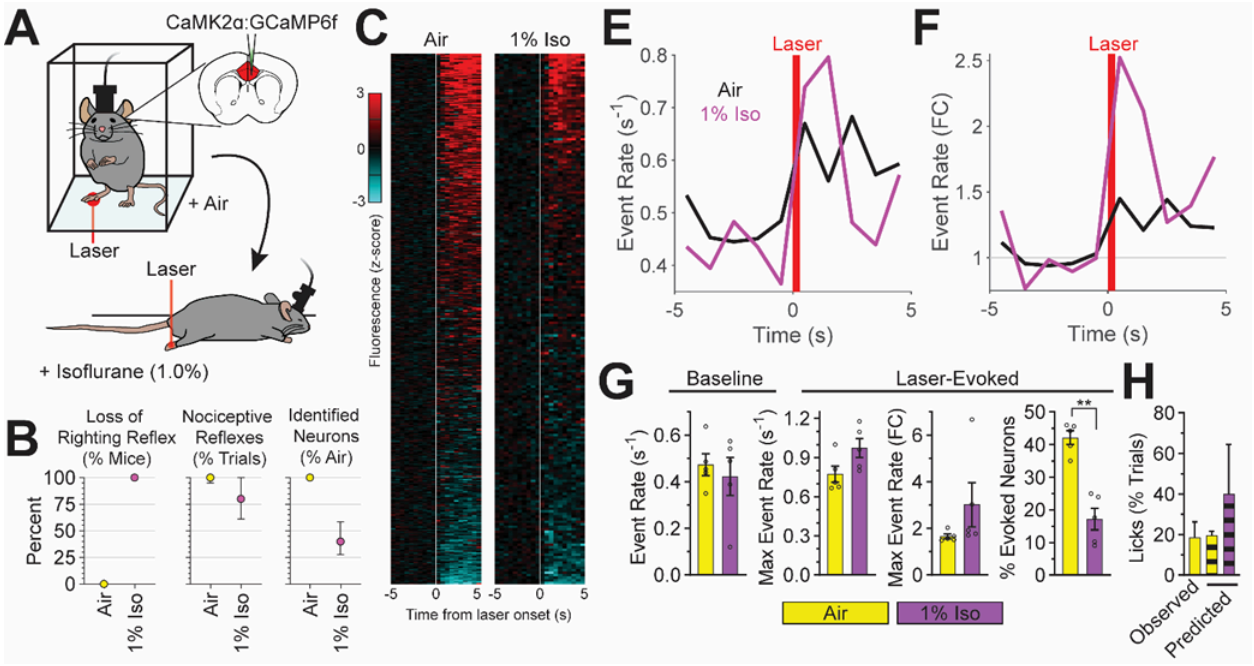


**Figure 2.4.5 Neural signatures of pain during general anesthesia.**
(**A**) Noxious (laser) stimulus-evoked ACC activity monitored in awake, freely behaving mice inhaling air and in anesthetized mice inhaling isoflurane (1%). GCaMP6f virally-expressed in excitatory neurons (CaMK2a). (**B**) Behavioral and imaging endpoints: left: loss of righting reflex/absence of volitional movements; middle: presence of nociceptive reflexes; right: percent of spontaneously active neurons (isoflurane compared to air). Data displayed as median and interquartile range. (**C**) Heatmap of calcium dynamics (z-scored and averaged across trials) per neuron in response to laser stimulus (white line) during isoflurane or air. (**E** and **F**) Laser-evoked neural activity during inhalation of isoflurane or air displayed as absolute event rate (**E**, events/second) and baseline normalized event rate (**F**). (**G**) Baseline and laser-evoked neural activity quantified from E (baseline and max event rate), F (fold change in max event rate), and **C** (percent of neurons with evoked activity) (paired t-test). (**H**) Noxious stimulus-evoked affective-motivational behaviors (licks) observed in awake mice (solid bar) and those predicted by noxious stimulus-evoked ACC activity (striped bars). N = 5 mice.

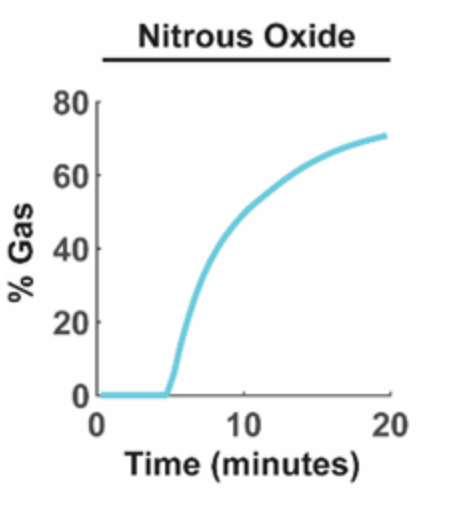


**Figure 2.4.6 Nitrous oxide concentrations during recordings of spontaneous ACC activity.**
Related to Figures 2.4.1 and 2.4.2. Nitrous oxide concentrations averaged across all mice in figures 1 and 2 (mean ± SEM; N = 30 mice).

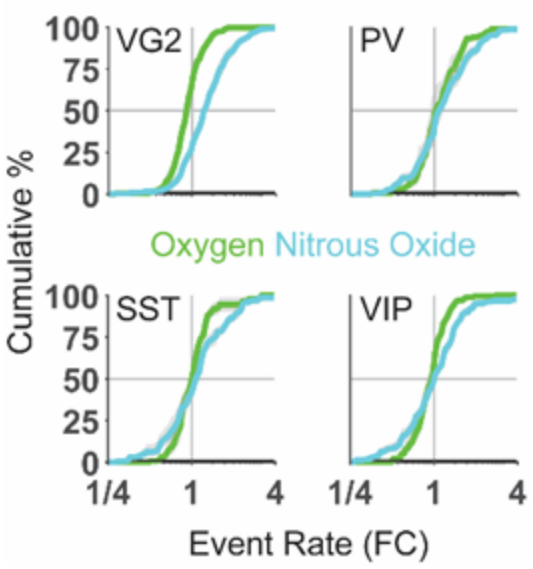


**Figure 2.4.7 Nitrous oxide differentially influences molecularly distinct subpopulations of ACC neurons.**
Related to Figure 2.4.2. Fold change in event rate quantified as cumulative percent (mean ± SEM) for distinct populations of ACC neurons (neurons expressing vGluT2 (VG2), parvalbumin (PV), somatostatin (SST), or vasoactive intestinal peptide (VIP). N = 6 mice per genotype.

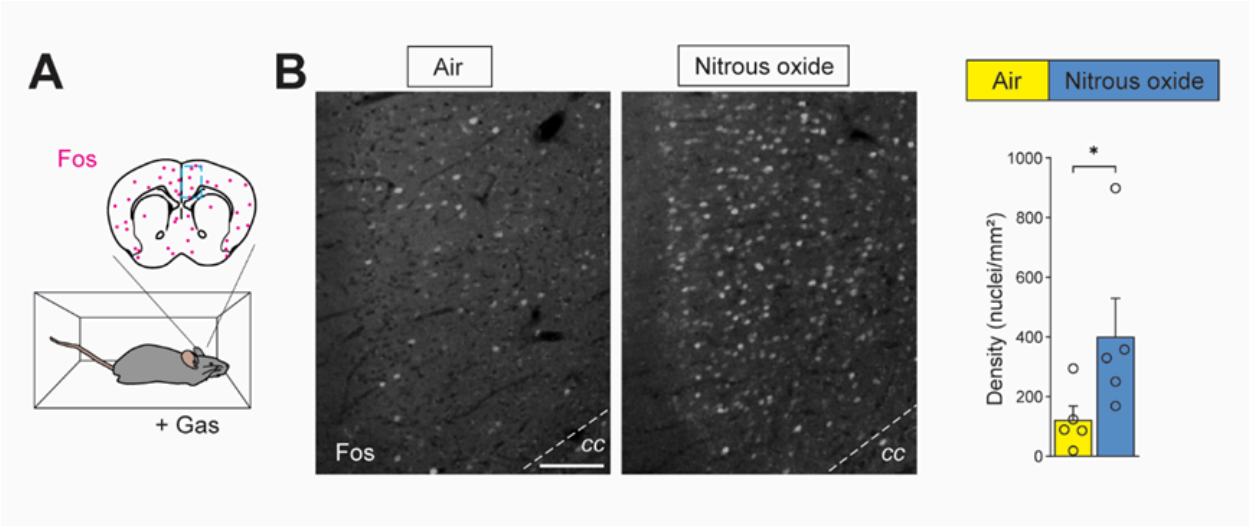


**Figure 2.4.8 Nitrous oxide increases expression of Fos.**
Related to Figure 2.4.3 (**A**) Fos expression, a correlate of neuronal activity, after exposure to air or nitrous oxide (60%). (**B**) Left: Immunofluorescence labeling of Fos-expressing neurons (gray). Right: Quantification of ACC Fos-expressing cells (Mann-Whitney U test, $p \leq 0.0317$). White scale bars = 100 um (B). Corpus callosum marked as cc.

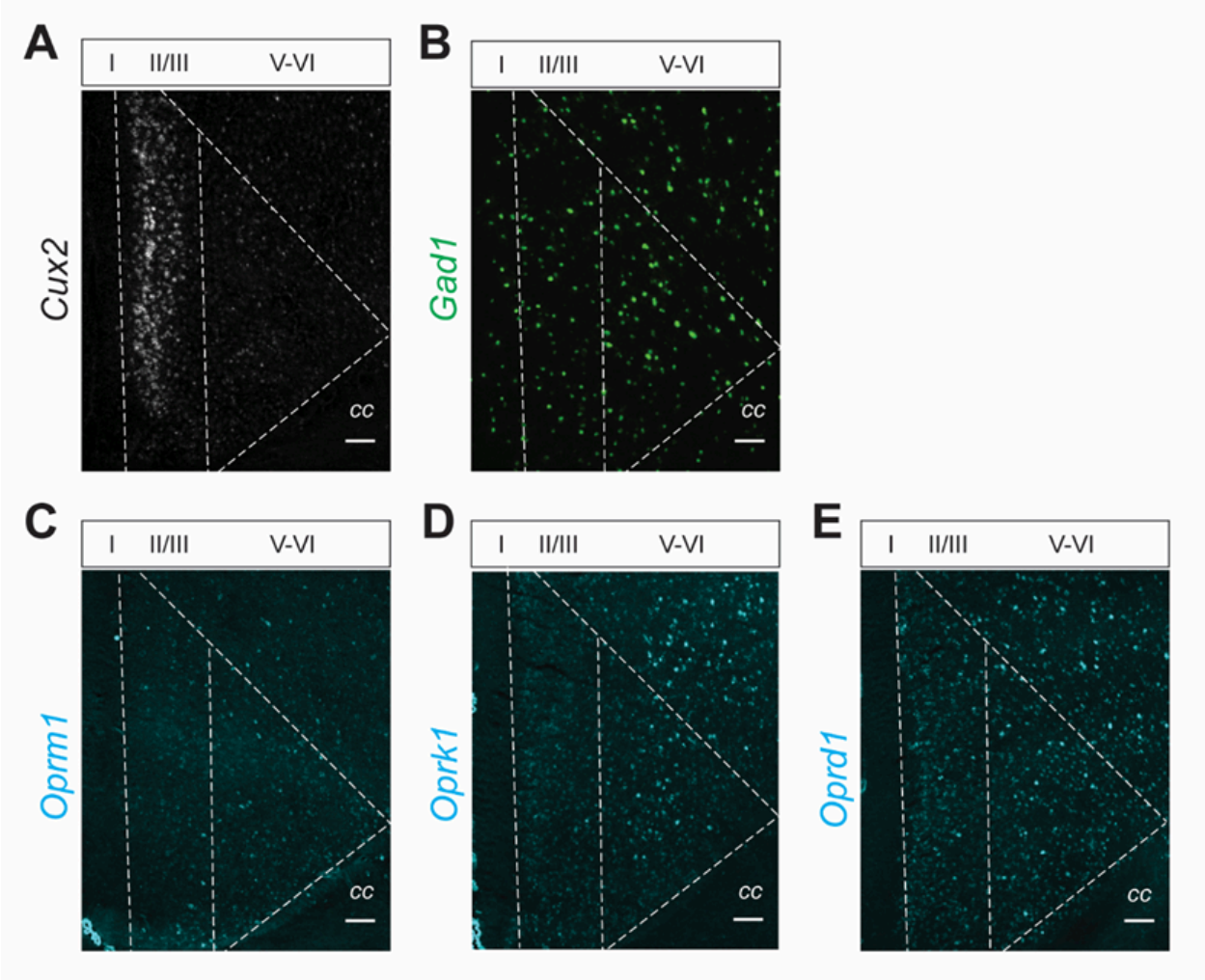


**Figure 2.4.9 Representative images of ACC distribution of molecularly distinct neurons quantified in Fig. 2.4.3.**
(**A**) *Cux2* mRNA distribution in layer II/III but not layers I or V-VI. (B) Distribution of *Gad1* mRNA, a molecular marker of inhibitory interneurons. (**C**-**E**) Distribution of mRNA for opioid receptor subtypes: **C**, *Oprm1*; **D**, *Oprk1*; **E**, *Oprd1*. White scale bars = 100 um (**B**). Corpus callosum marked as cc.

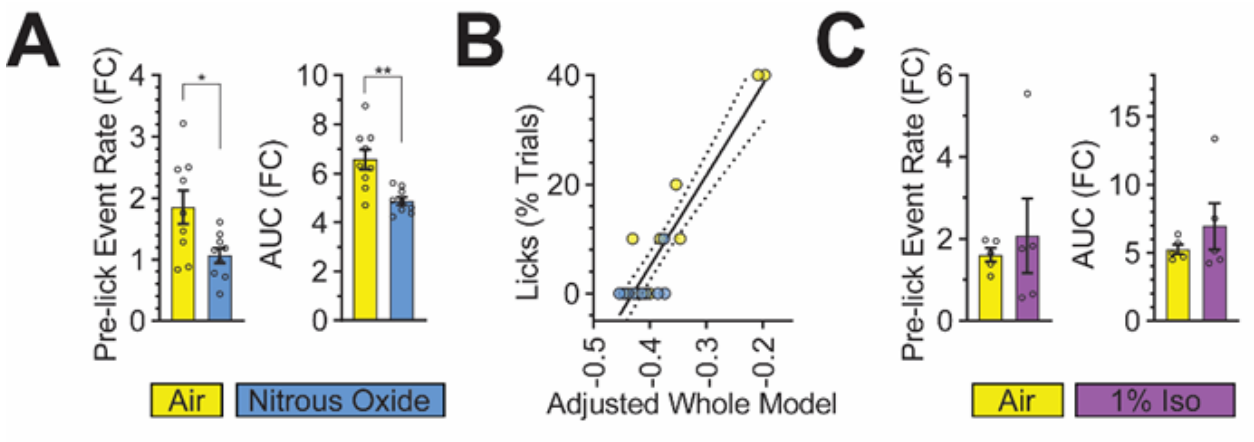


**Figure 2.4.10 ACC neural activity correlates with production of noxious stimulus-evoked affective-motivational behaviors.**
Related to Figure 2.4.5. (**A**) Nitrous oxide-induced changes to event rate post laser and prior to licking; area under the curve (AUC) quantified from **Fig. 4H** (paired t-test, n = 9). (**B**) Stepwise linear regression with interaction effects of the Adjusted Whole Model (pre-lick event rate (FC), maximum event rate (FC), AUC (FC), and percentage of laser responsive neurons vs percentage of licks; adjusted $R^2$ = 0.736, $p < 0.004$). (**C**) Isoflurane-induced changes to event rate prior to licking and AUC quantified from Fig. 5F (paired t-test, n = 5).

## 2.5 METHODS

### Animal husbandry

All mouse husbandry and surgical procedures adhered to the regulatory standards of the Institutional Animal Care and Use Committee of the University of California San Francisco (UCSF; protocol AN199730). The following mouse strains were used: vGluT2-IRES-Cre [131] (Jax # 028863), PV-IRES-Cre [132] (Jax #017320), SST-IRES-Cre [133] (Jax # 028864), VIP-IRES-Cre [133] (Jax # 031628), Ai75D (ROSA26-nls-tdTomato; Jax # 025106), and GAD67-GFP [134]. The health and wellbeing of the mice were monitored daily.

### Calcium imaging of spontaneous ACC activity

*GECI expression strategy*

We used two strategies to express genetically encoded calcium indicators (GECI; GCaMP6f[96]) in neurons: (1) viral pan-neuronal expression, and (2) viral/genetic expression within molecular distinct subsets of neurons. For experiments with pan-neuronal expression, we delivered GCaMP6f under control of the synapsin promoter (AAV1/9-SYN-GCaMP6f; Addgene, #100837)[51]. The restricted delivery of GCaMP6f to molecularly distinct populations of neurons was achieved using the vGluT2-Cre [131], PV-Cre [132], SST-Cre [133], or VIP-Cre [133] mouse lines in combination with the Cre-inducible viral expression of GCaMP6f in the ACC (AAV1/9-SYN-FLEX-GCaMP6f; Addgene, #100833).

*Surgical preparation for ACC calcium imaging*

Briefly, mice were anesthetized with isoflurane (2% in oxygen) and placed on a stereotaxic frame (Kopf). After craniotomy above the left ACC (Bregma, x: -0.33mm, y:1.27mm), we injected virus (depth: -1.75mm), and chronically implanted a gradient index (GRIN) lens (0.5x4mm ProView, Inscopix; depth: -1.7mm). The GRIN lens and titanium headbar (custom made, eMachineShop.com) were affixed to the skull with dental cement (Metabond). Mice were provided with postoperative analgesia (carprofen and slow-release buprenorphine). One week

after implantation surgery, under isoflurane anesthesia, a baseplate was affixed above the GRIN lens with dental cement. To provide time for sufficient GCaMP6f expression, mice recovered for 3 to 4 weeks before experiments began.

*Behavioral apparatus and anesthesia delivery*

Mice were headfixed to a passive treadmill [135], which was modified to provide heating that kept the mice isothermic during exposure to anesthesia, and then placed in a modified anesthetic induction chamber (VetEquip, 7L). Before the start of the experiment, the atmosphere of the chamber was replaced with oxygen. During experimental sessions, the mice were exposed to continually increasing concentrations of isoflurane or nitrous oxide, or for control conditions, continued exposure to oxygen. For all experiments, the concentration of oxygen never fell below 21%. Isoflurane was delivered via an Isoflurane Vaporizer (DRE Veterinary). Gas concentrations were monitored by a Datex Ohmeda S/5 anesthesia patient monitor and recorded by VSCapture software.

*Calcium imaging and behavior monitoring*

Changes in GCaMP6f fluorescence were captured with Inscopix miniscopes (nVista 3.0 or nVoke 2.0) at 20 frames per second (fps). Imaging parameters (excitation LED power, digital gain, and focus depth) were individually set for each mouse. Calcium imaging data were recorded via Inscopix Data Acquisition Software (IDAS), and recordings were triggered via TTL input. The behavior of the mouse was monitored with a Logitech webcam and recorded via ffmpeg software (https://ffmpeg.org/). Recording sessions were coordinated by Arduino/MATLAB, which triggered the start and end of data acquisition via miniscopes, ffmpeg, and VSCapture.

*Tissue processing*

After completion of the *in vivo* imaging experiments, the mice were anesthetized with Avertin (2.5% in saline) and transcardially perfused with phosphate-buffered saline (PBS) and then 4% formaldehyde (37% formaldehyde; Acros Organics, 11969-0100) diluted in PBS. Whole heads

were postfixed in 4% formaldehyde at 4°C overnight, then brains were extracted from the skull and postfixed overnight at 4°C. Following postfixation, the brains were cryoprotected at 4°C overnight in 30% sucrose in PBS, and embedded in specimen matrix (Optimal Cutting Temperature (OCT) compound, Tissue-Tek) and stored at -80°C. Confocal microscopy confirmed GCaMP6f expression and proper GRIN lens targeting.

**Assessing induction of immediate early genes by nitrous oxide**

*Fos induction*

Adult GAD67-GFP mice (6-10 weeks old) were habituated to an anesthetic induction chamber (7L, VetEquip) for 30-minute sessions on 3 separate days. The following day, after an additional 30 minute habituation, the mice were exposed to 2L/minute of 60% nitrous oxide or medical air. After 2 hours, the mice were anesthetized with Avertin and transcardially perfused as described above. The brain was then removed, post-fixed in 4% formaldehyde for 4 hours at 4°C and then cryoprotected in 30% sucrose, embedded in OCT, frozen on dry ice, and stored at -80°C.

*In situ hybridization*

*In situ* hybridization was performed as in Wercberger et al., 2021 [106]. Briefly, 10 micron-thick coronal sections of ACC were collected directly onto Superfrost microscopy slides. Slides were then processed with RNAscope Fluorescent Multiplex Detection (Advanced Cell Diagnostics). The RNAscope probes used were: *c-Fos* (#316921), *Gad1* (#400951), *Cux2* (#469551), *Oprm1* (#315841), *Oprd1* (#427371), and *Oprk1* (#316111). RNAscope processed brain sections were imaged with an Olympus FV3000 confocal laser scanning microscope. For studies on ACC layer specificity, the location of layers II/III was identified by the presence of *Cux2* expression, and layers V/VI by the absence of *Cux2* expression. The position of cells expressing *c-Fos*, *Gad1*, *Oprm1*, *Oprk1*, or *Oprd1* was identified within ImageJ by a scorer blinded to group identity (i.e., air vs nitrous oxide). Custom written MATLAB code was used to quantify the intensity of *in situ* labeling per identified cell, which was then thresholded to determine whether a cell positively

expressed a gene.

*Fos immunohistochemistry*

Frozen brains were coronally sectioned (30 microns) with a Hacker cryostat (Bright OTF series). ACC sections were slide mounted, washed with PBS (3 times for 5 minutes), and blocked with 10% normal goat serum (NGS) in PBS for 1 hour. Slides were incubated overnight at room temperature in rabbit anti-Fos primary antibody (1:1000, Cell Signaling Technology) diluted in PBS with 0.3% Triton-X and 1% NGS (PBST). Slides were then washed with PBS (3 times for 5 minutes), incubated in AlexaFluor 594-conjugated goat anti-rabbit secondary antibody (1:1000, Invitrogen) diluted in PBST at room temperature for one hour, and washed again with PBS (3 times for 5 minutes). Sections were coated with mounting media (DAPI Fluoromount-G, Southern Biotech, #0100-20), and then coverslipped with #1.5 glass (Epredia, #152460). Fos immunofluorescence was captured using an Olympus FV3000 confocal laser scanning microscope. Fos immunolabeling was quantified within ImageJ by a blinded scorer.

**Assessing noxious stimulus-evoked responses to infrared laser pulses**

*Behavioral apparatus and volatile anesthetic delivery*

Mice were placed inside a modified anesthetic induction chamber (VetEquip, 2L) with a high-transmittance glass floor that allowed for the presentation of noxious heat stimuli during the concurrent inhalation of nitrous oxide. During experimental sessions, mice were exposed to nitrous oxide (60%) or control gas (medical air). We used subhypnotic concentrations of nitrous oxide that allowed awake, weightbearing mice to freely respond to noxious stimuli [22], but below concentrations that induce unconsciousness (i.e., $MAC_{awake}$) [136,137]. The concentration of oxygen was held equivalent to atmospheric concentrations (21%) during nitrous oxide inhalation. Concentrations of nitrous oxide, oxygen, and carbon dioxide were monitored by a Datex Ohmeda S/5 anesthesia patient monitor and recorded using VSCapture software [138]. Individual mice were tested with different gasses during separate experimental sessions using a

crossover study design with a minimum washout period of 7 days.

*Generation of acute noxious thermal stimuli*

Acute noxious thermal stimuli were generated using a fiber-attached infrared diode laser (LASMED (Lass-7M) 7W 975nm laser) that produced brief pulses that rapidly heat skin without causing injury [110,139]. Mice received 10 trials of laser stimuli, with one laser pulse per trial. The laser power and pulse duration were set to 1750mA and 300 milliseconds, respectively. During presentation of the laser stimulus, a focused beam (2.0mm, $1/e^2$ diameter) was shone on the central portion of the plantar surface of the hindpaw [110]. The laser stimulus was manually triggered via a footswitch and laser firing time was recorded via Arduino/MATLAB. Behavioral responses were recorded with a digital camera (Imaging Source, DMK 37BUX252) at 200 frames per second (fps) using StreamPix (Norpix) software.

**Calcium imaging of noxious stimulus-evoked ACC activity**

*ACC calcium imaging preparation*

Mice were prepared for calcium imaging as described above, with the following differences: (1) GCaMP6f was virally expressed in excitatory neurons using the CaMKIIa promotor (AAV1/9-CaMKIIa-GCaMP6f, Inscopix), (2) viral injection and implantation of an integrated GRIN lens/baseplate (0.5x4mm, Inscopix) occurred within a single surgery.

*Calcium imaging and behavior monitoring*

Changes in GCaMP6f fluorescence were monitored and noxious heat stimuli were generated as described above. For nitrous oxide recordings, anesthesia and behavioral monitoring were performed as for the laser experiments. For recordings under isoflurane anesthesia, mice were first tested during inhalation of air as described above. Then, mice were briefly anesthetized with 1.0% isoflurane within the recording chamber, and then moved from the chamber to a heating pad where isoflurane was administered via nosecone. Mice were equilibrated to isoflurane anesthesia for 30 minutes to ensure steady-state concentrations within the brain [140]

before testing resumed. This equilibration process was repeated for recordings conducted under 2% isoflurane. Miniscope recording, laser pulses and behavioral camera recording signals were coordinated via TTL pulse via Arduino/MATLAB and synchronized by monitoring TTL signals via Inscopix DAQ (data acquisition) box.

**Data processing**

*Calcium imaging data processing*

Calcium imaging data were processed with Inscopix Data Processing Software (IDPS), MATLAB-IDPS API, and custom MATLAB code. Briefly, raw videos are spatially cropped, downsampled (2X), and bandpass filtered [141]. Processed videos are then motion corrected, normalized (dF/F), and individual cells segmented using principal component analysis and independent component analysis (PCA/ICA) [142]. Cell segmentation is manually confirmed in the IDPS GIU for each identified cell. Changes in calcium fluorescence per cell are extracted, and individual calcium transients within a trace are extracted as events, using either event detection algorithm in IDPS or custom MATLAB code.

*Calcium imaging data analysis*

To demonstrate nitrous oxide-induced increases in spontaneous activity, we Z-score normalized the activity of each cell to the baseline event rate. For clustering analysis, z-scored neuronal activity was transformed by dimensional reduction using tSNE algorithm. Clusters of neurons with unique activity patterns were identified from the tSNE mapping using DBSCAN. For stimulus-evoked changes, activity was extracted from 10 separate presentations of the noxious laser stimulus. The timing of stimulus presentation was determined using laser-generated TTL pulses recorded via the Inscopix nVoke DAQ box. Pre-stimulus activity is the time from -5 to 0 seconds before the laser stimulus; post-stimulus activity is measured from 0 seconds to 5 seconds after the laser stimulus. Baseline event rate is the mean pre-stimulus event rate per neuron per mouse. The maximum event rate is the maximum post-stimulus event rate. Where

noted, fold changes in measures of neural activity are calculated as post-stimulus activity divided by pre-stimulus activity. Fluorescence traces were used to calculate the percentage of neurons with stimulus-evoked activity. Traces were z-scored to pre-stimulus activity and considered to have evoked activity if the trace z-scored value was greater than 1.96 for the post-stimulus period. Stepwise linear regression with interaction effects was performed on data mean-centered to control gas condition (medical air only). A neural activity (biomarker)-based estimate of laser evoked licks that otherwise would occur in awake mice was predicted from ACC recordings in isoflurane anesthetized mice. The isoflurane data were mean-centered to the control gas (medical air), and a prediction was made using the linear regression model generated from nitrous oxide and air recordings.

*Scoring of stimulus-evoked behaviors*

We used custom MATLAB software to score behavioral videos (viewed at 1/5X speed). Behavioral responses to individual laser stimuli were categorized as: (a) no response, (b) reflexes, such as flinches (the stimulated hindpaw shifted position but did not leave the glass floor), withdrawals (the stimulated hindpaw is rapidly pulled off of the glass floor), or shakes (the stimulated hindpaw is moved in a repetitive oscillatory fashion), or (c) licks (the stimulated hindpaw is brought to the face and licked or bitten). Each video was scored independently by two individuals. All scorers were blind to experimental conditions.

**Statistical analyses**

Data were processed and analyzed in Mathwork's MATLAB (R2020a) software. Statistical tests were performed with Prism (GraphPad) software. The threshold for significance for all statistical tests was set at $p < 0.05$, and indicators of significance levels were as follows: ns (not significant; $p > 0.05$); *= $p < 0.05$; **=$p < 0.01$; *** =$p < 0.001$; and ****= $p < 0.0001$. Corrections for multiple comparisons were performed using the false discovery rate metho

d of Benjamini, Krieger, and Yekutieli, and noted within figure legends as "FDR corrected".

## 2.6 ACKNOWLEDGEMENTS

This work was funded by NIH NSR35NS097306 (AIB), Open Philanthropy (AIB), NIGMS fellowship F32GM131479 (JAPW), and Canadian Institutes of Health Research (CIHR) Doctoral Foreign Study Award DFD-170771 (CDL). We are grateful to: Dr. Eric Lam (UCSF) for aiding in the design and fabrication of experimental setups; Dr. Philip Bickler (UCSF) for advice on experimental setup; Dr. Racheli Wercberger (UCSF) for helpful advice on calcium imaging and analyses; Dr. Chris Tsang (Inscopix) for initial training for surgical implant of GRIN lenses; Dr. Julian Motzkin (UCSF) for helpful discussions on data analysis and statistics.

# CHAPTER 3

# MAPPING A NEURONAL CORRELATE OF NITROUS OXIDE-INDUCED ANALGESIA

## 3.1 INTRODUCTION

Nitrous oxide is a fast-acting general anesthetic that produces analgesia without loss of consciousness. Importantly, pain aversion, but not sensory thresholds, are impaired under nitrous oxide anesthesia [24], making nitrous oxide anesthesia a unique tool to study the affective components of the pain experience, separately from pain's sensory-discriminative features. One hypothesis is that nitrous oxide induces analgesia by engaging opioidergic circuits [143,144] in the midbrain periaqueductal gray (PAG) [37] that drive descending inhibition of the spinal cord, thus decreasing noxious input to the brain. However, whether nitrous oxide enacts its effects directly on the PAG or indirectly via upstream brain regions is unclear [47]. Moreover, where in the brain nitrous oxide might act to specifically affect the emotional-affective component of the pain experience is unknown.

We recently demonstrated that nitrous oxide robustly induces neuronal activation of the anterior cingulate cortex (ACC), a key region that processes pain aversion [145]. Previously, the consensus in the literature has been that neuronal activity in the ACC correlates with perceived pain unpleasantness [16,51], and additionally that pain unpleasantness can be modulated by manipulating ACC neuronal activity [54,146–148]. Our finding that nitrous oxide, a known analgesic, increases ACC activity, is therefore paradoxical given the literature. In the presence of noxious stimuli, however, we found that the maximum level of neuronal activity induced by noxious stimulation does not change under nitrous oxide exposure. Therefore, by increasing baseline line ACC activity, nitrous oxide lowers the net increase in neuronal activity induced by a noxious stimulus, an effect that we hypothesize is sufficient to induce analgesia.

What is currently unclear is whether the analgesia arising from increased ACC activity during nitrous oxide exposure is due to a direct action of nitrous oxide at the level of the ACC (i.e. by activating excitatory neurons or by disinhibiting inhibitory interneurons), or via activation of upstream structures [49]. This is significant as the ACC receives direct projections from numerous cortical and subcortical regions that are involved in processing pain affect [122]. To begin to address the overall impact of nitrous oxide on brain activity, here, we use Fos immunohistochemistry and viral tracing to characterize neuronal activity during nitrous oxide-induced analgesia throughout the mouse central nervous system [43]. Since previous work focused on the ACC, we now look at nitrous oxide-induced Fos activity both up- and downstream of the ACC, so as to better understand how changes in ACC activity translate to nitrous oxide-mediated analgesia.

## 3.2 RESULTS

### 3.2.1 Nitrous oxide activates some, but not all, regions in the brain that process pain aversion.

To assess the degree to which nitrous oxide broadly activates the brain, we quantified the expression of Fos protein, a marker for recently-active neurons, in select regions connected to the ACC [59,122]. These regions were previously demonstrated by our laboratory and others to mediate analgesia: the mediodorsal nucleus of the thalamus (MD) [123,149], intralaminar nuclei of the thalamus (ILn) [150,151], the lateral habenula (LHb) [152–154], the basolateral amygdala (BLA) [95,123], and the periaqueductal gray (PAG) [37].

*Mediodorsal nucleus (MD) and intralaminar nuclei of the thalamus (ILn)*

The MD and ILn receive sensory input from the dorsal horn of the spinal cord, directly via the spinothalamic tract [155] and indirectly via the spinoreticular tract [156]. Increased neuronal activity in response to noxious stimuli in normal and injury settings in the MD and ILn have long

been reported [149,157]. Both the MD and ILn additionally mediate arousal and awareness via input from brainstem nuclei [158,159] and projections to cortical and subcortical regions [160]. Furthermore, involvement of both the MD and ILn in the affective-motivational component of pain have garnered attention in more recent years. For example, previous work from our lab demonstrated that MD neurons projecting to the ACC mediate pain aversion [123], and other work has demonstrated the necessity of MD neurons receiving nociceptive input from the lPBn in sustaining so-called “coping” behaviors, like licking [113].

Since nitrous oxide preferentially reduces pain affect, we hypothesized that nitrous oxide decreases neuronal activity in the MD and ILn. Instead, as in the ACC, we observed a significant increase in MD and ILn Fos expression induced by nitrous oxide exposure (**Figure 3.4.1A**). As the ACC both projects to and receives projections from the MD and ILn, it is unclear to what extent nitrous oxide-induced activation of these regions might be influencing the ACC.

*Lateral habenula (LHb)*

The lateral habenula, a dorsal thalamic nucleus that receives projections from the ACC [59,161], mediates negative motivation and valence [162]. With respect to pain processing, the LHb exhibits increased activity [152,163]. Furthermore, infusion of mu opioid receptor (MOR) agonists directly into the LHb decreases neuronal activity and induces analgesia [164]. Although our previous work did not reveal differences in the activation of opioid receptor-expressing neurons in the ACC, we wondered if the partial opioid receptor-mediated reversal of nitrous oxide [144] could be explained by nitrous oxide acting on other brain regions. However, we did not observe a change in Fos expression induced by either air or nitrous oxide exposure (**Figure 3.4.1B**).

*Basolateral amygdala (BLA)*

The BLA has long been studied in the context of fear, which often accompanies pain as a protective measure [165,166]. In fact, activation of a subset of BLA neurons correlates with and enhances pain aversion [95]. Importantly, the BLA reciprocally connects to the ACC [59,122].

Studies have demonstrated that activation of ACC neurons projecting to the BLA reduces generalized fear responses [167,168], while BLA neurons projecting to the ACC mediate chronic pain-associated aversion, interestingly, in a layer-dependent manner [123,169,170]. Specifically, activating BLA-ACC layer 5 neurons reduces pain aversion, while activating BLA-ACC layer 2/3 neurons enhances pain aversion. Since we found that nitrous oxide selectively increases Fos expression in ACC layer 2/3 neurons, we hypothesized that nitrous oxide could decrease activity in the BLA, but surprisingly found no change in Fos expression (**Figure 3.4.1C**).

*Periaqueductal gray (PAG)*

The ventrolateral PAG (vlPAG) was one of the first structures identified in the pathway mediating the descending inhibition of pain [171]. Specifically, activation of the vlPAG induces analgesia by inhibiting spinal cord dorsal horn neurons [172]. Importantly, studies demonstrate that nitrous oxide analgesia can be reversed by lesioning the vlPAG [37] or partially reversed by local microinjection of opioid receptor antagonists [36]. Coupled with the fact that direct projections from the ACC mediate antinociceptive responses [173], we hypothesized that nitrous oxide exposure would induce Fos expression.

Surprisingly again, however, we did not observe a change in Fos expression in the ventrolateral PAG (vlPAG). We also observed no changes in Fos expression in the dorsolateral PAG (dlPAG), which potentiates fear responses [174] and could have been inhibited by nitrous oxide (**Figure 3.4.1D**).

### 3.2.2 Nitrous oxide does not preferentially activate upstream regions of the ACC.

Of the regions that we probed for nitrous oxide-induced Fos expression, we only observed changes in the MD and ILn. Both of these regions reciprocally connect to the ACC. Thus, whether Fos expression results from nitrous oxide directly activating these thalamic nuclei or from downstream ACC activation is unclear. Since we did not observe changes in the vlPAG, a known downstream target of the ACC, and the proposed target of nitrous oxide analgesia, we

hypothesized that the Fos activation in the thalamus was indeed upstream of the ACC. To test this hypothesis, we unilaterally injected a retrograde tracer (pAAVrg-CAG-tdTomato; Addgene plasmid # 59462, gifted from Edward Boyden) into the ACC and quantified the percent of tdTomato-expressing cells that expressed Fos after nitrous oxide exposure (**Figure 3.4.2A**). We focused on the contralateral ACC, the MD, and ILn, which are the regions that previously demonstrated nitrous oxide activation.

We observed no difference in Fos induction in tdTomato-expressing cells in any of our selected regions (**Figure 3.4.2B, C**), suggesting that nitrous oxide-induced activation of the ACC results from direct action on ACC neurons. We additionally did not observe a layer-specific difference in the ACC (**Figure 3.4.2D**), although the systemic action of nitrous oxide would make it impossible to determine whether layer-specific Fos expression in tdTomato-expressing cells is induced directly by nitrous oxide or by inputs from the contralateral ACC.

### 3.2.3 Nitrous oxide does not reduce ascending noxious input from the spinal cord.

Both the MD and ILn reside in the spinothalamic and spinoreticular tracts of ascending sensory information from the spinal cord dorsal horn. Although the increased Fos expression in both regions does not appear to be upstream of, and therefore influencing, nitrous oxide-induced activity in the ACC, reduced nociceptive input from the dorsal horn spinal cord would alter cortical activity. Since nitrous oxide partially inhibits the withdrawal response to noxious stimuli [37,144], we hypothesized that nitrous oxide could alter the ascending pain signal. In fact, a number of studies have sought to characterize the effect of nitrous oxide on Fos expression in the laminae of the dorsal horn of the spinal cord. Notably, these studies do not report changes in Fos expression in the projection neuron layer of the dorsal horn spinal cord [39,44,175]. However, the method by which nitrous oxide is administered is not consistent: using sedating general anesthetics to apply noxious stimuli to induce Fos, or not administering nitrous oxide for

the entire duration of noxious stimulus application. As such, these methods make it difficult to isolate the specific actions of nitrous oxide in the spinal cord.

To test the hypothesis that nitrous oxide decreases activity in the dorsal horn of the spinal cord, we performed the capsaicin assay on mice that also received either air or nitrous oxide and quantified Fos expression in the dorsal horn of the spinal cord (SCDH). Plantar injections of capsaicin induces both sensory-discriminative (flinches, shaking, and guarding [176]) and emotional-affective (licking) pain behavior and induces robust ipsilateral Fos in the dorsal horn of the spinal cord [177]. Although previous studies have assessed the behavioral and physiological effects of nitrous oxide on similar tests in rodents [45,46], the assessment of analgesia and the method and duration of nitrous oxide exposure varies widely. Our setup allows for continuous administration of nitrous oxide with an unobstructed view of mouse behavior, and without the necessity of additional anesthetics to administer noxious stimuli (**Figure 3.4.3A, B**). Consistent with previous studies, including our own, we found significant decreases in both reflexive (flinches) and affective motivational behavior (licks). Interestingly, we did not observe a significant difference in either the number of shaking bouts performed or in time spent guarding the affected hindpaw (**Figure 3.4.3C, D**).

A decrease in nociceptive behavior can result from decreased activity in projection neurons of the SCDH. However, nitrous oxide does not alter Fos expression in the SCDH projection neuron layer (**Figure 3.4.4B**), suggesting that nitrous oxide does not decrease activity in SCDH projection neurons. We further confirmed this result by quantifying Fos expression in the lateral parabrachial nucleus (lPBn), which is innervated by over 90% of projections from the SCDH projection neurons [178]. Nitrous oxide did not change Fos expression in the lPBn (**Figure 3.4.4C**), suggesting that nitrous oxide indeed induces analgesia via effects on supraspinal circuits.

Next, we queried whether the vlPAG is necessary for nitrous oxide to induce analgesia. We hypothesized that nitrous oxide would increase Fos expression in the vlPAG, since

activation of opioidergic circuits within the vlPAG have been established as critical to maintaining nitrous oxide-induced analgesia. Surprisingly, we did not observe a change in capsaicin-induced Fos in the vlPAG under nitrous oxide exposure (**Figure 3.4.4D, F**). We therefore conclude that nitrous oxide analgesia is mediated by increased activity of the ACC, specifically in superficial layers 2/3.

Finally, we asked how nitrous oxide changes Fos expression in the ACC in the presence of a noxious stimulus. In our previous work, we demonstrated that while nitrous oxide increased spontaneous activity, the percent of neurons that increased firing in response to a noxious stimulus decreased compared to air [145]. We therefore hypothesized that while nitrous oxide increases Fos expression compared to air under baseline conditions (i.e. without noxious stimuli), we do not expect an increase in Fos in the presence of capsaicin. Indeed, across the ACC broadly, and within layer 2/3, there is no significant increase in Fos expression under nitrous oxide exposure. However, when compared to our previously published work, we find that the fold change in Fos expression under capsaicin compared to no stimulation decreases, mirroring our previous finding that nitrous oxide induces a relative decrease in nociception-induced activity (**Figure 3.4.4E, F**). We observe a slight increase in fold change in layer 2/3 activation, and no change in layers 5-6. This finding therefore confirms our previous finding that activation of ACC layer 2/3 is important to mediating nitrous oxide analgesia.

## 3.3 DISCUSSION

Our previous work demonstrates that nitrous oxide increases activity in excitatory, non-opioid receptor-expressing neurons in superficial layer 2/3 in the ACC to induce analgesia. In this study, we show that the increase in ACC activity is not due to nitrous oxide-mediated activation of upstream regions that also mediate pain perception. We additionally show that nitrous oxide analgesia is not secondary to reduced nociceptive input from the spinal cord, as Fos expression in both the SCDH projection neuron layer and lPBn are unchanged by nitrous oxide exposure.

Surprisingly, nitrous oxide does not alter Fos expression in the vlPAG, previously thought to be necessary for nitrous oxide-mediated analgesia, suggesting that the key to nitrous oxide-mediated analgesia may, instead, be the specific activation of the ACC.

### 3.3.1 Nitrous oxide-induced Fos activation in SCDH neurons

Our findings confirm that nitrous oxide does not alter Fos expression in the projection neuron layer, suggesting that ascending nociceptive input to the brain persists. Interestingly, previous studies reported that nitrous oxide increases Fos expression in laminae III-V [39,44,175], layers that contain wide-dynamic range neurons that respond to innocuous and noxious mechanical stimulation [179]. Whether these are projection neurons was not determined. Reduced spinal cord-mediated reflexes may instead result from direct action of nitrous oxide on the spinal cord, specifically in the deeper laminae. For example, Orii et al. demonstrated that nitrous oxide activates GABAergic neurons in deeper spinal cord laminae [180], in which GABAergic neurons can influence high-threshold mechanosensory inputs onto lamina I projection neurons [181]. Additionally, the general anesthetics halothane and sevoflurane have been shown to inhibit the monosynaptic stretch reflex in a dose-dependent manner [182,183], which involves the activation of motor neurons in the ventral horn of the spinal cord [184]. It could therefore be the case that nitrous oxide similarly inhibits reflexes via action on the ventral horn; however, nitrous oxide analgesia likely requires cortical mechanisms since spinal cord transections reverse nitrous oxide-induced analgesia [35].

### 3.3.2 Nitrous oxide-induced Fos activation in supraspinal regions

Although we demonstrated that nitrous oxide-induced analgesia can, at least partially, be explained by decreasing the relative increase in noxious stimulus-induced activity in the ACC, additional circuit mechanisms involving descending inhibition are unclear. Previous studies point to the necessity of the vlPAG to nitrous oxide analgesia; however, we observed that nitrous

oxide did not change Fos expression compared to air. The discrepancy between our work and others could be explained by the fact that previous studies focused on reflexive, rather than affective-motivational, behavior. Even though the molecular identity of nitrous oxide-activated neurons in the vlPAG is unknown, since Fos expression in the SCDH is unchanged, our present conclusion is that he vlPAG is not as involved in nitrous oxide-mediated analgesia as previously thought.

If the vlPAG is not involved, where else might nitrous oxide act to influence the affective-emotional aspect of pain? Nitrous oxide did not change Fos expression in BLA neurons or in projection neurons to the ACC, although we have yet to examine if, and to what extent, nitrous oxide influences noxious stimulus-evoked Fos in the BLA. We also observed no change in Fos expression in the LHb. As the LHb is known to reduce pain processing via opioid receptors, the lack of nitrous oxide-induced activation in this region could help to explain the conflicting evidence of whether nitrous oxide-mediated analgesia depends on endogenous opioids at all [185]. We did observe significant increases in nitrous oxide-induced Fos expression in both the MD and ILn, which receive nociceptive input and, especially in the case of the MD, mediate affective-motivational responses. However, the lack of activation in neurons of these regions projecting to the ACC suggest that the increased ACC activity is due to a direct action of nitrous oxide on the ACC. If so, the extent to which nitrous oxide-induced ACC activity influences downstream regions that mediate analgesia is an important question. Combining anterograde projections from the ACC or retrograde from the MD and ILn with Fos immunostaining would help to address the mechanism by which nitrous oxide increases activity of ACC neurons and produces analgesia.

## 3.4 FIGURES

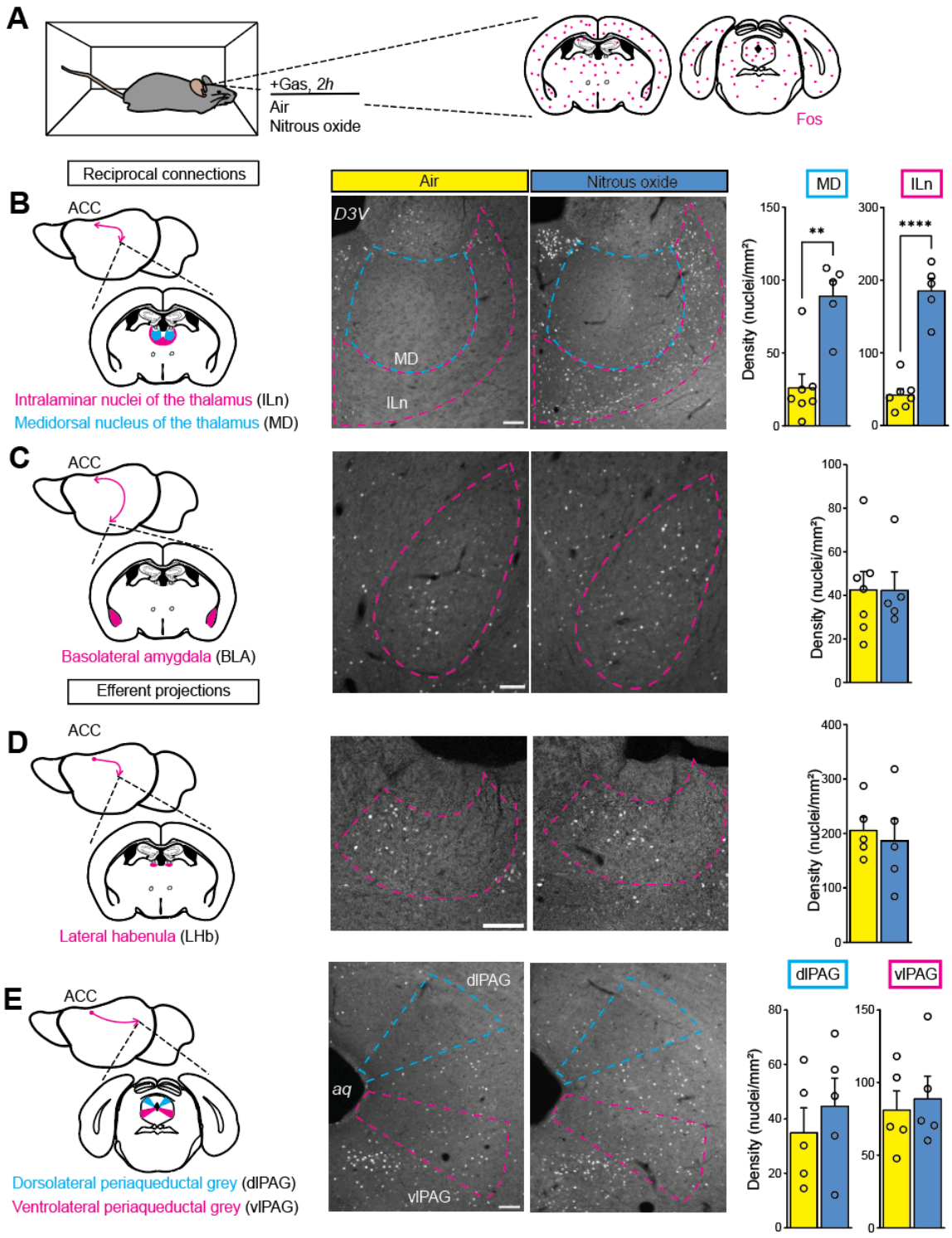


**Figure 3.4.1 Nitrous oxide activates some, but not all, regions in the brain that process pain aversion.**
(*Figure caption continued on the next page.*)

(*Figure caption continued from the previous page.*)
Fos expression (white; middle column), a correlate of neuronal activity, after exposure to air or nitrous oxide (60%), in the mediodorsal thalamus and intralaminar nucleus (A; Student's t-test, $p \leq 0.0012$ and 0.0001, respectively), lateral habenula (B: Student's t-test, $p \leq 0.6933$), basolateral amygdala (C; Student's t-test, $p \leq 0.9379$), and dorsolateral and ventrolateral periaqueductal gray (D; Student's t-test, $p \leq 0.5010$ and 0.7076, respectively). Schematics of region locations, adapted from the Allen Brain Atlas, are depicted in the left column, additionally labeled by the direction of connections between regions and the ACC. Scale bar = 100 μm. All data are depicted as mean ± SEM.

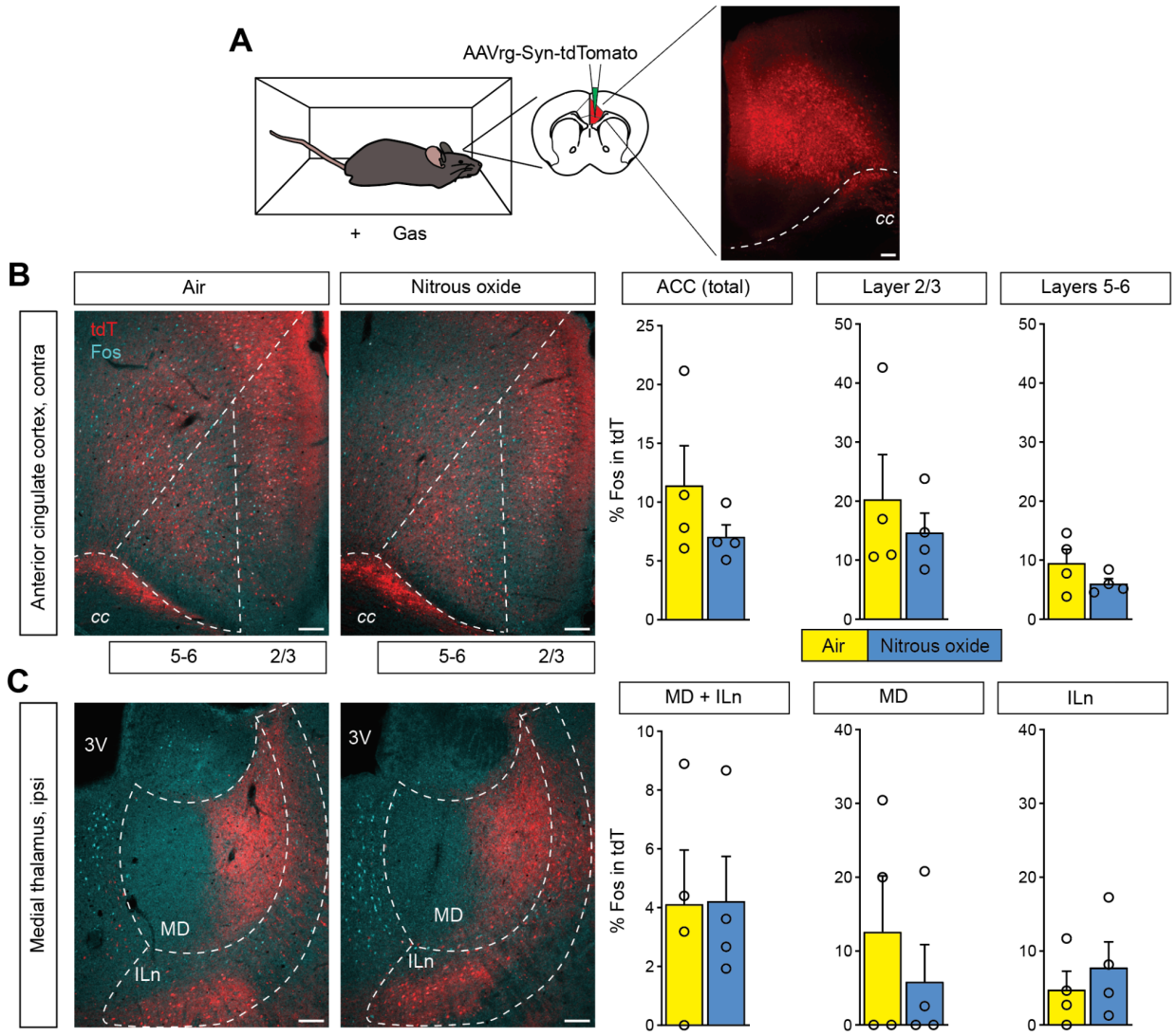


**Figure 3.4.2 Nitrous oxide does not preferentially activate upstream regions of the ACC.** (**A**) Fos expression patterns induced by nitrous oxide in regions that project to the ACC. Regions identified by unilaterally injecting a retrograde viral tracer into the ACC. Layers 2/3 and 5-6 are delineated by white dashed lines. (**B-C**) Left: Retrograde labeling of projection neurons (red) and Fos immunofluorescence (cyan) in the contralateral ACC, (**B**) the MD, and ILn (delineated by white dashed lines). (**C**). Right: Quantification of the percent of projection neurons expressing air- or nitrous oxide-induced Fos (**B**: Student's t-test $p \leq 0.4099$ (total), $p \leq 0.5241$ (layer 2/3), $p \leq 0.2133$ (layers 5-6), **C**: $p \leq 0.9766$ (MD + ILn), $p \leq 0.4857$ (MD), $p \leq 0.5086$ (ILn). Corpus callosum marked as *cc*; 3V: third ventricle. Scale bar = 100 µm.

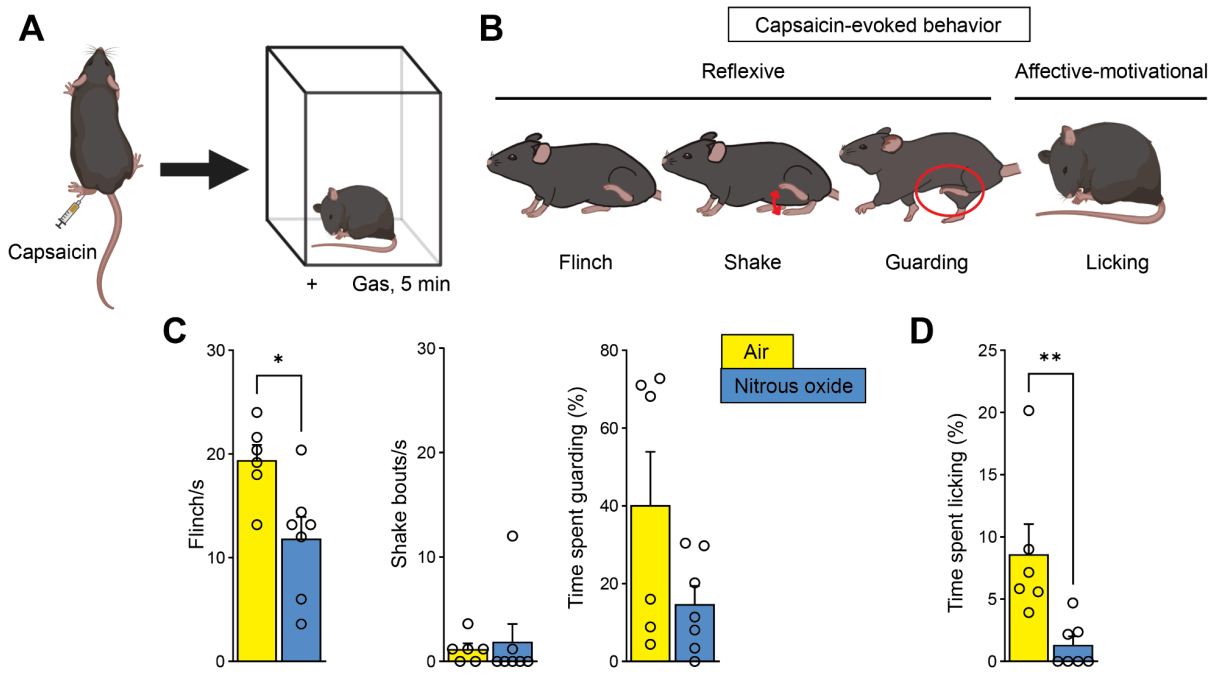


**Figure 3.4.3 Nitrous oxide decreases reflexive and affective-motivational behavior evoked by intraplantar capsaicin.**

(**A**) Mice are placed in an induction box for 5 min and breathe either air or nitrous oxide (60%) immediately following an intraplantar injection of capsaicin (3 µg/10 µL) into the left hind paw. (**B**) Behavioral responses are classified as either reflexive or affect-motivational. (**C, D**) Quantification of behavior: Flinches per second (Student's t-test, $p \leq 0.0161$), shake bouts per second (Mann-Whitney test, $p \leq 0.3963$), percent of time spent guarding (Student's t-test, $\leq 0.0874$), and percent time spent licking (Student's t-test, $p \leq 0.1282$).

Schematics for injection and licking in (**A**) and (**B**) were created using BioRender.

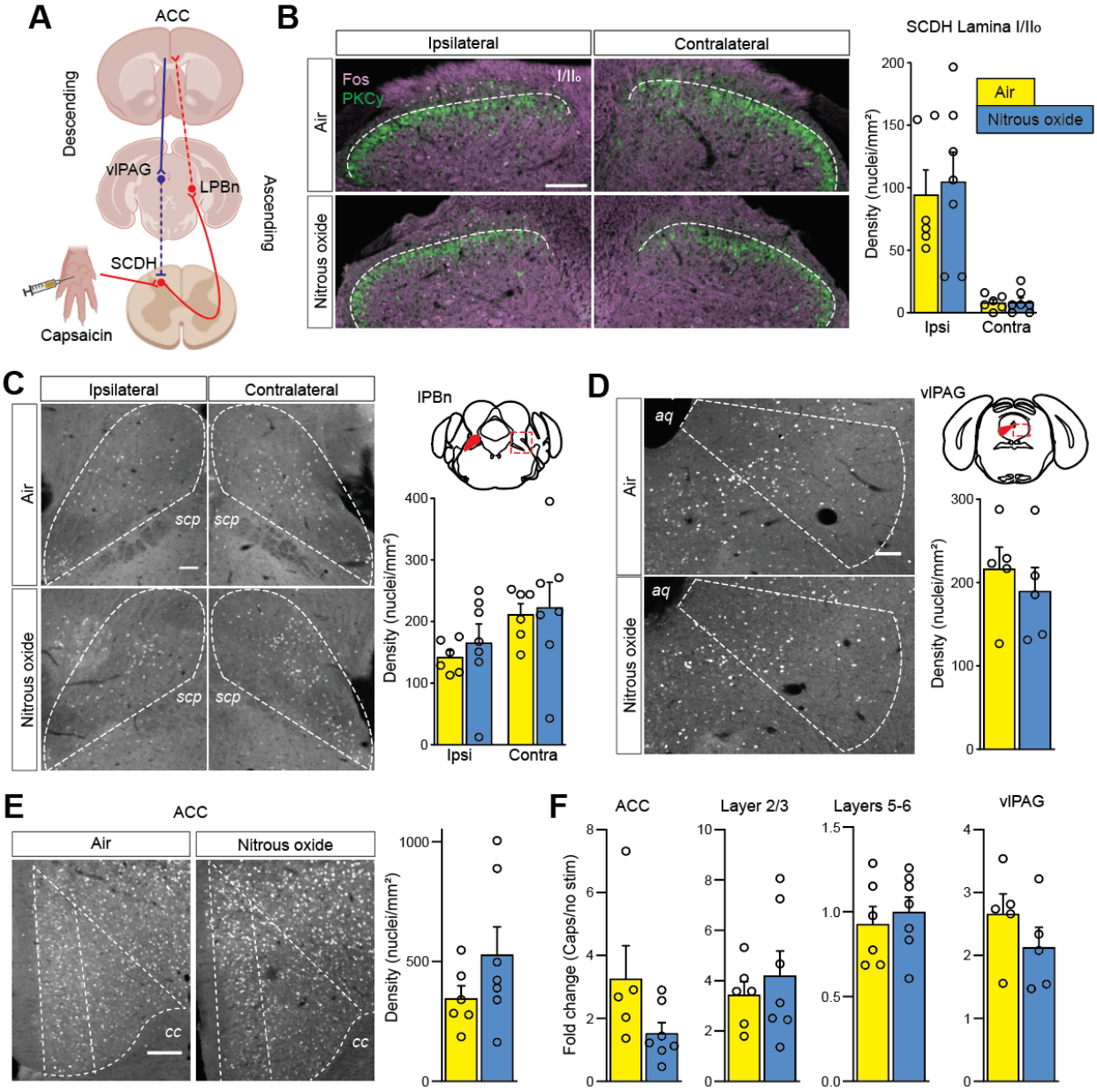


**Figure 3.4.4 Nitrous oxide analgesia does not change Fos expression in ascending or descending pain pathways.**
(A) Quantifying Fos immunoreactivity in the ascending and descending pain pathways, simplified, after intraplantar injection of capsaicin (3.0 µg/10 µL) (B) Left: Fos immunofluorescence (pink) in the superficial dorsal horn (dorsal to PKCγ, green) ipsi- and contralateral to the injected paw. Right: quantification of Fos density induced by nitrous oxide compared to air (two-way ANOVA, FDR corrected). (C-E) Left: Fos immunofluorescence (white) in (C) the lPBn (depicted right, top; two-way ANOVA, FDR corrected), (D) the vlPAG (depicted right, top; Student's t-test, $p \leq 0.5032$), and (E) the ACC (Student's t-test, $p \leq 0.1991$). (F) Quantification of the fold change in Fos expression under air or nitrous oxide, with or without capsaicin, in the ACC (Mann-Whitney test, $p \leq 0.0732$), ACC layer 2/3 (Student's t-test, $p \leq 0.5243$), (*Figure caption continued on the next page.*)

(*Figure caption continued from the previous page.*)
ACC layers 5-6 (Student's t-test, $p \leq 0.9697$), and vlPAG (Student's t-test, $p \leq 0.2690$).
Schematic in (**A**) was created in BioRender. Laminae I and outer II are marked as $I/II_o$; *scp*: superior cerebellar peduncle; *aq*: cerebral aqueduct; *cc:* corpus callosum. Scale bar = 100 μm.

## 3.4 METHODS

### Animal husbandry

Adult C56Bl/6 mice were used and maintained in accordance with the Institutional Animal Care and Use Committee of the University of California San Francisco (UCSF; protocol AN199730).

*Viral injection*

We unilaterally injected 300 nL of pAAV-CAG-tdTomato (codon diversified, gift from Edward Boyden, Addgene plasmid # 59462 ; http://n2t.net/addgene:59462 ; RRID:Addgene_59462) into the left ACC of 9 mice under 2% isoflurane anesthesia.

### Behavioral apparatus and anesthesia delivery

*Fos induction by nitrous oxide*

We performed Fos induction experiments as in Weinrich and Liu et al., 2023. In brief, mice received continuous exposure to 60% nitrous oxide or air in an induction chamber (2L/min, 7L, VetEquip) for 2 hours. Mice were then immediately anesthetized with Avertin and transcardially perfused with saline and 4% formaldehyde, after which the brain was removed for immunohistochemistry. Brains were postfixed in formalin for 4 hours at 4°C, cryoprotected in 30% w/v sucrose in PBS for 48 hours at 4°C, and frozen in OCT at -80°C.

*Fos induction by capsaicin*

Mice received a unilateral intraplantar injection (3 µg in 10 µL) of capsaicin in buffer (v/v 10% ethanol, 10% Tween80, 80% saline) immediately prior to a 5 minute exposure to medical air or 60% nitrous oxide in an induction chamber (2L/min, 2L, VetEquip). Mice were returned to the home cage for a further 85 minutes and then anesthetized with Avertin and euthanized as described above.

*Behavior scoring*

Videos were played at 1/5x speed and the first 10 seconds of each minute was manually scored by a blind observer. The behaviors assessed were classified as reflexive or

affective-motivational. Reflexive behaviors included flinches (a withdrawal and replacement of the affected paw from the chamber floor), shakes (a rapid oscillation of the affected paw, raised, and hinged at the ankle), and guarding (the affected paw did not maintain contact with the floor). Affective-motivational behaviors were licks of the affected paw. Flinches and shake bouts were individually reported as behavior/second, while guarding and licking were scored as the percent of time performed in the observed period.

**Tissue processing and analysis**

*Fos immunohistochemistry*

Coronal sections were taken at 30 µm and collected at 120 µm intervals (Fos only) or 50 µm at 150 µm (Fos and tdTomato) on Superfrost Plus microscopy slides. Fos immunohistochemistry was performed as in Weinrich and Liu et al., 2023. Briefly, slides were incubated in 10% normal goat serum in PBS with 0.3% Triton-X for 2 hours, primary antibody solution containing 1% NGS overnight (1:1000 Cell Signaling Technologies rabbit anti-Fos; 1:500 Invitrogen biotinylated antibody (Figure 3.3.2 only); 1:5000 Invitrogen guinea pig anti-PKCγ (Figure 3.3.4 only)) and secondary antibody solution containing 1% NGS for 1 hour (1:1000 Invitrogen goat anti-rabbit 594, 1:1000 streptavidin fluorophore 405, 1:1000 Invitrogen goat anti-guinea pig 647) before coverslipping with Fluoromount G. Slides were washed 3 x 10 min with 1X PBS prior to incubation steps, between antibody incubations, and before coverslipping. All steps were performed at room temperature and slides were incubated in the dark. Tissue containing tdTomato was additionally incubated with biotinylated antibody after the initial primary antibody incubation and coverslipped without DAPI.

*Confocal imaging and image analysis*

Images were acquired with a 10X objective on an Olympus FV3000 confocal laser scanning microscope. A blind experimenter quantified Fos and tdTomato expression. Fold change was calculated as (capsaicin Fos density / average no stim Fos density). Data from Weinrich et al.,

2023 was used to calculate the area for layers 2/3 (*Cux2* expression) and the average no stim Fos density for ACC (total), layer 2/3, and layers 5-6. Data from Figure 1 was used to calculate the average no stim Fos density for the vlPAG.

**Statistical analysis**

Statistical tests were performed with GraphPad Prism. The threshold for significance for all tests was set at $p \leq 0.05$. The levels of significance were indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, and **** = $p \leq 0.0001$.

# CHAPTER 4

# DISCUSSION

## 4.1 SUMMARY

We discovered that nitrous oxide, a general anesthetic with analgesic properties, paradoxically increases spontaneous ACC activity. Since neuronal activity of the ACC is often correlated with perceived pain intensity, we sought to characterize the change in ACC activity evoked by nitrous oxide exposure and identify the circuits involved in mediating nitrous oxide-induced analgesia. In Chapter 2, we show that the increased activity is driven by excitatory, non-opioid receptor-expressing neurons in superficial layer 2/3 of the ACC. We further demonstrate that, while the maximum neuronal activity evoked by noxious stimuli is unchanged, the *relative* increase in evoked activity is decreased under nitrous oxide. We posit that the relative decrease in noxious stimulus-evoked ACC activity is critical to inducing and maintaining nitrous oxide-analgesia, and moreover, can be used as a brain activity-based biomarker for ensuring sufficient analgesia under general anesthesia. In Chapter 3, we suggest that nitrous oxide oxide-induced analgesia originates in the ACC, as nitrous oxide does not change Fos expression in projection neurons in the SCDH. Additionally, the MD and ILn, major sources of nociceptive information to the ACC, exhibit profound nitrous oxide-induced Fos expression, but not in neurons projecting to the ACC. We also show that nitrous oxide does not affect Fos expression in the vlPAG despite reducing affective-motivational behaviors to noxious stimuli, suggesting that the vlPAG may be less integral to nitrous oxide-mediated analgesia than previously hypothesized.

## 4.2 FUTURE DIRECTIONS

Two major questions remain regarding the relationship between nitrous oxide-mediated activation of the ACC and analgesia: 1) which circuits, originating from the ACC, are involved,

and 2) is the increase in spontaneous activity in the ACC sufficient to induce analgesia? To address the first, a transsynaptic anterograde viral tracer [186] injected into the ACC would label neurons that are directly innervated by the ACC. Labeled cells in downstream regions that express nitrous oxide-induced Fos would help to identify pathways of interest. Viral expression could be restricted to layer 2/3 [187] or to molecularly-distinct subgroups of neurons (e.g. opioidergic neurons [188]) to elucidate the relative contributions of neuron classes to nitrous oxide-induced analgesia.

The second question is more difficult to answer, as methods commonly used in neuroscience - optogenetics and chemogenetics - to address necessity and sufficiency of circuit activity *in vivo* have been criticized, among other things, for their lack of physiological relevance in simulating neuronal activity [189,190]. Indeed, the idea that ACC activity correlates to the intensity of perceived pain largely comes from studies in rodent models that use opto- or chemogenetics to maximally stimulate, or completely eliminate, neuronal activity in the ACC. Yet, our studies have demonstrated that analgesia is present even when ACC activity is *increased above baseline*. It is possible that using lower stimulation frequencies or duration in optogenetic protocols, or using a lower ligand concentration in chemogenetic experiments, could recapitulate the effects of nitrous oxide on ACC spontaneous activity.

However, it is also possible that the complex pharmacology of nitrous oxide means that using techniques that activate or inhibit neuronal signaling through a single signaling pathway obscures the microcircuit and polypharmacological interactions at play. Indeed, while nitrous oxide is primarily known as a $GABA_A$ receptor agonist and NMDA receptor antagonist [191], nitrous oxide also exerts effects at sodium, glycine, acetylcholine, serotonin, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and kainate receptors. How these receptors interact to mediate nitrous oxide analgesia is unknown, as is the necessity of opioid receptors [144,185]. To further complicate matters, the profile of receptors engaged by nitrous oxide would suggest that nitrous oxide, like most other anesthetics, suppresses neuronal

activity; however, NMDA receptor hypofunction in the prefrontal cortex has been shown to disinhibit pyramidal neurons [121] and indeed, our work and others [60,192] have instead observed robust *activation* of neuronal activity under nitrous oxide exposure. Our Fos immunohistochemistry and *in situ* experiments suggest that activation of layer 2/3 neurons could be integral to the changes in neuronal activity induced by nitrous oxide, although this hypothesis would have to be confirmed using calcium imaging or electrophysiological techniques.

### 4.2.1 Nitrous oxide for depression

Nitrous oxide has recently been studied as a potential treatment for depression, in part due to the selective and potent effects of nitrous oxide on the affective-motivational component of the pain experience. Single exposures to nitrous oxide prevent rats from developing long-term anxiety- and depression-like behaviors following well-established injury models [193,194]. In a 2015 clinical trial, Nagele and colleagues reported a rapid and sustained decrease in depression and anxiety scores in subjects with treatment-resistant depression following a single dose of nitrous oxide [195]. Nitrous oxide is only one of several NMDA receptor modulators that are being explored as novel treatments for depression, since conventional treatment options are limited, take time, and do not work for many individuals [196]. However, the mechanism of action by which NMDA receptor modulators can alleviate mood disorders is still unknown: ketamine, another NMDA receptor antagonist with high treatment potential [197], also acts on other receptors [198].

Could the antidepressant and analgesic effects of nitrous oxide share a mechanism, and if so, what is the implication for how nitrous oxide mediates pain? Pain, stress, and mood disorders are often comorbid [199], and the prefrontal cortex (PFC) - including the ACC - exhibits many functional and structural changes in major depressive disorder [200]. The ACC in particular has been implicated in pain-induced depression [86], although the use of optogenetics

in this study potentially obscures how relative changes in activity mediate observed behavior. Interestingly, one study specifically implicates pyramidal (excitatory) neurons in layer 2/3 of the PFC: when the gene *Wfs1*, a known cause of major depressive disorder in Wolfram syndrome patients, is selectively deleted from layer 2/3 pyramidal neurons of the PFC in mice, mice develop stress-induced depression-like behaviors [201]. Stress is a known modulator of the pain experience; in mice, acute stress can induce analgesia while chronic stress can cause hyperalgesia [202,203]. In humans, anxiety can exacerbate pain-related symptoms like guarding [204], and the presence of depression-linked brain activity can predict whether an individual's pain persists or resolves [205]. Experiences of both nitrous oxide analgesia and anterior cingulotomy highlight their anxiolytic effects [26,27,51,206]. We did not test for anxiety- or depression-like behaviors, but our results, demonstrating that nitrous oxide exposure 1) selectively activates ACC layer 2/3 excitatory neurons, 2) does not reduce ascending noxious stimulus from projection neurons in the SCDH, and 3) does not appear to engage descending inhibitory controls via the vlPAG, suggest that supraspinal circuits are key to mediating nitrous oxide-induced analgesia. Therefore, relief from stress and anxiety, rather than noxious stimuli, may be key to nitrous oxide's analgesic properties. The extent to which activation of ACC layer 2/3 excitatory neurons contributes to nitrous oxide anxiolysis, and therefore analgesia, is an intriguing question. Moreover, exploring how nitrous oxide alleviates pain-associated stress and mood disorders could help in improving general anesthetic regimens that reduce trauma in cases of intraoperative awareness.

### 4.2.2 The future of nitrous oxide in general anesthesia

Discovered in 1799, nitrous oxide is the oldest general anesthetic that is still in widespread use today due to its low cost, ease of use, relative safety, and the rapid onset of potent analgesia

[66]. However, the continued utility of nitrous oxide has long been debated due to its notoriety as a party drug and as a greenhouse gas.

For decades, nitrous oxide has been a drug of choice for partygoers for its famous euphoria-inducing effects [61]. The many studies cited in this work use single, controlled exposures of nitrous oxide; however, recreational users often take multiple doses to sustain the euphoric effects. High doses can cause potentially deadly side effects like disorientation, nausea, and hypoxia [207,208]. Prolonged use causes severe vitamin B12 deficiency [209] that inhibits DNA synthesis [210] which can, in turn, cause neurodegeneration [211]. Despite the health risks of nitrous oxide misuse, however, nitrous oxide *is* safe and causes minimal side effects when administered in a clinical setting, and deleterious side effects resolve when misuse is ceased. Furthermore, the pharmacological effects of nitrous oxide are advantageous when net inhibition is contraindicated, for example, when identifying epileptic seizure loci [212].

In addition to the dangers of nitrous oxide misuse, growing concern over the greenhouse effect of nitrous oxide has led to many people reassessing continued clinical use [213,214]. One study estimated that the peak amount of nitrous oxide emitted by a German university hospital from 2019-2022 was equivalent to the average amount of carbon dioxide emissions of 188 German people [215]. As the goal of the healthcare industry is to provide care, it is imperative to find or develop alternatives to nitrous oxide that are equally safe and effective outside of the clinic. Our findings, that changes in ACC neuronal activity may have utility as a biomarker of pain perception in an anesthetized patient [145], may be key to identifying such alternatives that reduce, or eliminate, instances of connected consciousness, and ultimately improve patient health.

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