

Excitatory and Inhibitory Neurons of the Spinal Cord Superficial Dorsal Horn Diverge in Their Somatosensory Responses and Plasticity *in Vivo*

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The superficial dorsal horn (SDH) of the spinal cord represents the first site of integration between innocuous and noxious somatosensory stimuli. According to gate control theory, diverse populations of excitatory and inhibitory interneurons within the SDH are activated by distinct sensory afferents, and their interplay determines the net nociceptive output projecting to higher pain centers. Although specific SDH cell types are ill defined, numerous classifications schemes find that excitatory and inhibitory neurons fundamentally differ in their morphology, electrophysiology, neuropeptides, and pain-associated plasticity; yet little is known about how these neurons respond over a range of natural innocuous and noxious stimuli. To address this question, we applied an *in vivo* imaging approach in male mice where the genetically encoded calcium indicator GCaMP6s was expressed either in vGluT2-positive excitatory or vIAAT-positive inhibitory neurons. We found that inhibitory neurons were markedly more sensitive to innocuous touch than excitatory neurons but still responded dynamically over a wide range of noxious mechanical stimuli. Inhibitory neurons were also less sensitive to thermal stimuli than their excitatory counterparts. In a capsaicin model of acute pain sensitization, the responses of excitatory neurons were significantly potentiated to innocuous and noxious mechanical stimuli, whereas inhibitory neural responses were only depressed to noxious stimuli. These *in vivo* findings show that excitatory and inhibitory SDH neurons diverge considerably in their somatosensory responses and plasticity, as postulated by gate control theory.

Key words: calcium imaging; dorsal horn; gate control theory; *in vivo*, multiphoton microscopy; neuronal circuits; somatosensory; spinal cord

Significance Statement

Gate control theory posits that opposing spinal excitatory and inhibitory neurons, differently tuned across somatosensory modalities, determine the net nociceptive output to higher pain centers. Little is known about how natural stimuli activate these two neural populations. This study applied an *in vivo* calcium imaging approach to genetically target these neurons and contrast their responses over a range of innocuous and noxious mechanical and thermal stimuli. Compared with excitatory neurons, we found that inhibitory neurons are more sensitive to innocuous touch and far less sensitive to thermal stimuli. An acute model of pain also revealed that these subtypes undergo divergent mechanosensory plasticity. Our data provide important and novel insights for gate-control inspired models of pain processing.

Introduction

Somatosensory afferents specialized to extract distinct features of the internal and external environment converge in the dorsal horn of the spinal cord. Information pertaining to noxious

thermal and mechanical stimuli is primarily carried by small-diameter fibers—with important exceptions (Abraira and Ginty, 2013; Nogi et al., 2019)—which synapse onto neurons residing in laminae I and II, the superficial dorsal horn (SDH). The SDH is interspersed with abundantly diverse excitatory (glutamatergic) and inhibitory (primarily GABAergic) interneurons (Todd and Sullivan, 1990; Yasaka et al., 2010; Zeilhofer et al., 2012; Punnett et al., 2014; Häring et al., 2018; Sathiyamurthy et al., 2018), which are ideally positioned to shape the activity of neighboring glutamatergic projection neurons. It is estimated that <5% of lamina I cells are projection neurons (Spike et al., 2003; Cameron et al., 2015), supporting the notion that significant nociceptive processing occurs within the dorsal horn before information is relayed to higher pain centers. Our understanding of dorsal horn circuitry is hampered by the challenge of

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parsing dorsal interneurons into specific cell types, but such attempts routinely find overarching differences in the morphology (Grudt and Perl, 2002; Heinke et al., 2004; Maxwell et al., 2007; Todd, 2010; Yasaka et al., 2010) and physiology (Heinke et al., 2004; Browne et al., 2020) between excitatory and inhibitory subtypes.

Many models of dorsal horn nociceptive processing, or population coding, incorporate inhibitory neurons recruited by thermal and mechanical stimuli (Ma, 2010; Prescott et al., 2014). One such classical model is Gate Control Theory (GCT), which continues to guide pain research over half a century later (Melzack and Wall, 1965). GCT posits that mechanical activation of innocuous, low-threshold primary afferents recruits downstream spinal inhibitory interneurons, which in turn suppress the activity of nociceptive projection neurons, thus closing the gate on nociceptive signaling from the spinal cord to the brain. In pain sensitization, it is often found that excitatory and inhibitory drives are potentiated and suppressed, respectively, thus opening the gate and giving rise to hyperalgesia and allodynia (Zeilhofer et al., 2012; Kuner, 2015; Kopach et al., 2017; Gong et al., 2019; Gradwell et al., 2020). Modified versions of GCT have since been proposed, which similarly incorporate inhibitory neurons in models of thermal processing and the mechanical cessation of itch (Ma, 2010; Prescott et al., 2014; Bourane et al., 2015), but little is known about how these neurons are engaged by natural stimuli *in vivo*.

In vivo imaging of the spinal cord is particularly challenging because of the abundance of light-scattering myelin and a high degree of motion artifact (Johannssen and Helmchen, 2013), but a number of important *in vivo* calcium imaging studies have emerged characterizing the mechanical and thermal responses of SDH neurons to noxious and non-noxious stimuli (Johannssen and Helmchen, 2010; Nishida et al., 2014; Ran et al., 2016; Sekiguchi et al., 2016; Chen et al., 2018; Ran and Chen, 2019; Chisholm et al., 2021). The vast majority of these studies, however, collected and pooled data from a mixed population of excitatory and inhibitory neurons, and none specifically discerned both subtypes. Therefore, it remains unknown if these populations differ in their encoding of natural somatosensory stimuli *in vivo*, where the cutaneous receptors and descending modulation remain intact.

In this study, we apply an *in vivo* calcium imaging approach to measure the responses of individual vGluT2-positive excitatory and vIAAT-positive inhibitory SDH neurons to a range of innocuous and noxious mechanical and thermal stimuli. These two broad populations of neurons differed considerably; inhibitory neurons were more sensitive to innocuous mechanical stimuli and less sensitive to thermal stimuli than their excitatory counterparts. Nonetheless, both neural subtypes encoded noxious mechanical stimuli. To our knowledge, this is the first direct comparison of the responses of excitatory and inhibitory SDH neurons across natural somatosensory modalities. We also compared how the mechanosensory responses of these two populations changed in an acute model of pain sensitization. Intradermal injection of capsaicin potentiated excitatory neurons to both innocuous and noxious stimuli, whereas the responsiveness of inhibitory neurons to innocuous stimuli was unchanged, but noxious responses were attenuated. Collectively, these findings provide important insights pertaining to GCT models of nociceptive processing and how excitatory and inhibitory SDH neurons are recruited, both in normal and sensitized pain states.

Materials and Methods

Animals. Homozygous floxed GCaMP6s mice (derived from strain Ai96; stock #024106, The Jackson Laboratory) were crossed either with IRES vGluT2-Cre (Slc17a6; stock #016963, The Jackson Laboratory) or IRES vIAAT-Cre mice (Slc32a1; stock #016962, The Jackson Laboratory) to drive GCaMP6s expression in excitatory or inhibitory neurons, respectively (Fan and Sdrulla, 2020). The mice were kept under standard colony conditions, with 12 h day/night cycles and food and water access *ad libitum*. Adult male mice 8 weeks or older were used for all experiments.

***In vivo* imaging chamber.** A spinal *in vivo* imaging chamber was adopted from a previously described method (Farrar et al., 2012). Briefly, mice were anesthetized with isoflurane (4% induction, 2% holding). The back of the animal was shaved and sterilized with alternating topical iodine and alcohol. Mice were injected subcutaneously near the neck with 5 mg/kg ketoprofen and 0.2 mg/kg dexamethasone. As an initial landmark, the T13 vertebra was identified as the peak of the spinal arch. An incision was made in the overlying skin, and the identity of T13 was confirmed by palpating the last (13th) rib (false rib). The surrounding muscle and ligaments were excised, and the underlying vertebra were cleaned and dried with gauze and gel foam. The spine was secured with posts containing two stainless steel side clamps. A T12 and T13 laminectomy was performed, exposing L3–L5 of the spinal cord (Harrison et al., 2013), and the spinal cord was kept free of blood and moistened with saline rinses. A stainless steel plate with an imaging window (expanded on the rostrocaudal axis compared with Farrar et al., 2012) was screwed to secure the side clamps firmly to the spine. A transparent silicon elastomer (Kwik-Sil, World Precision Instruments) and a custom-cut no. 1.5 glass rectangular coverslip was placed over the preparation with caution to limit air bubbles. After the elastomer set, the coverslip and implant were further secured with a mixture of dental cement and cyanoacrylate, and the surrounding skin was mended with Vetbond (3M). Mice typically resumed normal activity within hours of surgery and were given at least 1 d to recover before imaging. In some cases where the window clarity was poor, the mouse was allowed to recover for several days, and the elastomer and coverslip were replaced.

Two-photon imaging. Before imaging, implanted mice were briefly anesthetized with 4% isoflurane and secured with two threaded side bars (Fig. 1A) in a mobile stage that fit under the two-photon microscope. Isoflurane was sustained between 0.9 and 1.2% unless stated otherwise. This low concentration of anesthetic limited the likelihood of large withdrawal reflexes, with only slight changes in muscle tone in response to noxious stimuli. Occasionally, stimuli generated strong reflexive kicks or sustained movement, in which case the trial was discarded. Additional care was taken to orient the animal to minimize movement of the spine with breathing. Body temperature was maintained with a feedback system consisting of a rectal thermal probe and an electric thermal blanket.

A clear region of the imaging window was first identified under an obliquely illuminated 5× air objective (Fig. 1B). Saline was placed over the window for imaging with a Zeiss 20×/1.0 water immersion objective (catalog #421452–9880). Our two-photon imaging system consisted of a Zeiss 7 MP microscope equipped with a femtosecond-pulsed Ti:Sapphire laser (Coherent) and Zen imaging software (Zeiss). A 425 × 425 μm region was scanned with the laser tuned to 940 nm (1.27 μs/pixel dwell time). Images were acquired at ~2.6 frames per second with a photomultiplier tube (gain = 800). Care was taken to shield light from external sources. The hindlimb ipsilateral to the area of imaging was accessed for stimulation from a small opening beneath the imaging stage. To identify superficial neurons for imaging, the ipsilateral hindlimb was manually stimulated by alternating touch and pinch. This was necessary because of the low background fluorescence inherent to GCaMP6s. The objective was then retracted to find the most superficial neurons that were responsive, and from this position, the objective was driven down to capture the widest field of view containing clearly outlined neurons. Because of the curvature of the spinal cord, these neurons likely comprised a mixture of lamina I and II outer neurons.

Image processing and analysis. Before analysis, raw imaging files collected for each stimulus within the same field of view were concatenated into a single time series using ImageJ software (Fiji). To ensure neurons

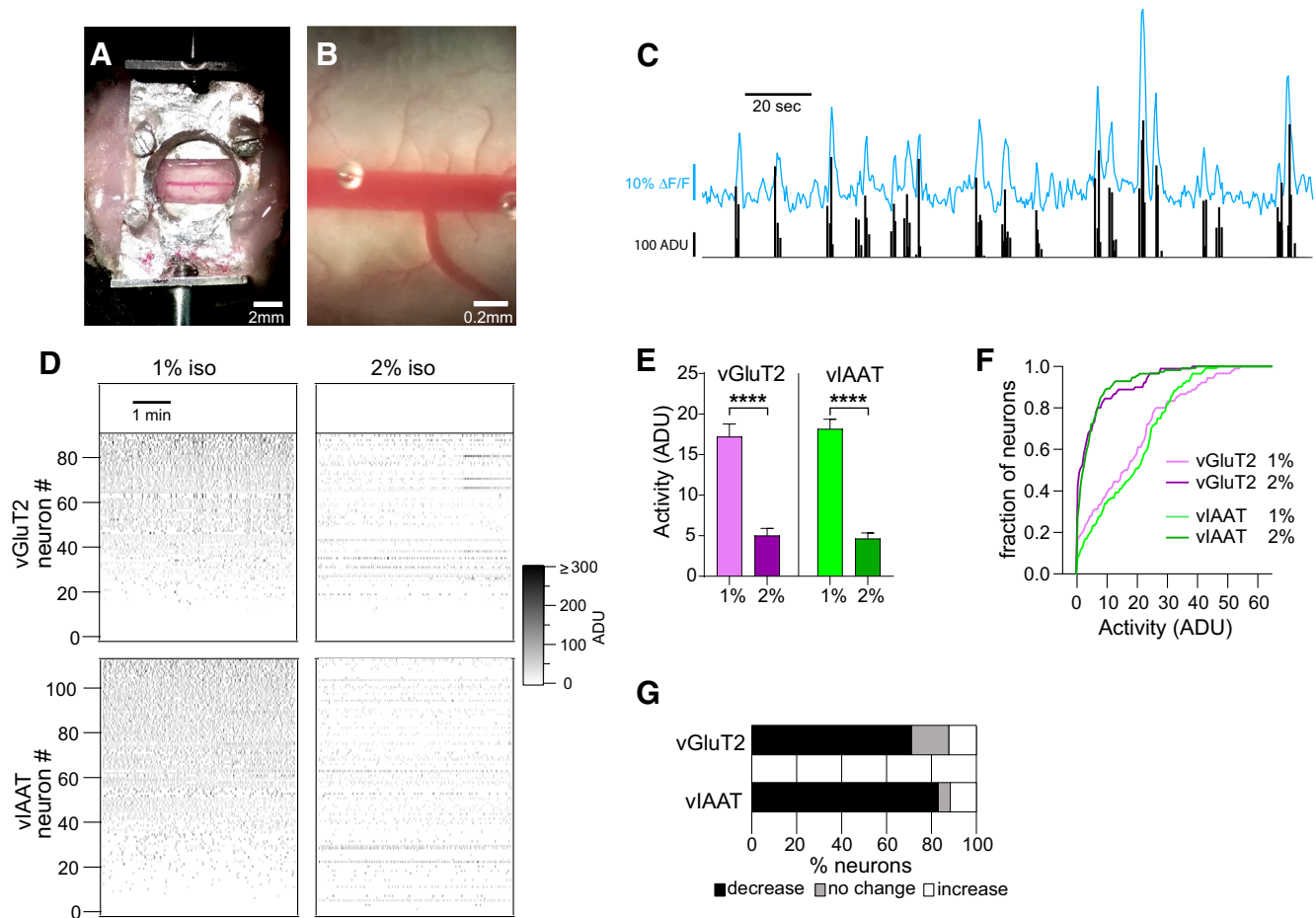


Figure 1. *In vivo* calcium imaging and anesthetic effects on spontaneous activity. **A**, *In vivo* imaging implant revealing the L3–L5 spinal cord beneath a glass coverslip and silicone elastomer. **B**, The spinal cord in **A** magnified with a 5× objective. **C**, Baseline spontaneous calcium activity or events of an example neuron in 1% isoflurane (iso) detected and processed by CalmAn. The $\Delta F/F$ signal (top, blue) and the corresponding deconvolved signal (bottom, black) are shown. **D**, Image plot of the baseline deconvolved activity of each neuron studied (4 mice per genotype; vGluT2, $n = 90$; vIAAT, $n = 112$) in 1% and 2% iso. Grayscale values correspond to the event magnitude within the imaging frame; 0 ADU (white) indicates no activity. Values exceeding 300 ADU are black for contrast enhancement (largest value 1204 ADU). Neurons are ranked lowest to highest based on their mean activity in 1% iso. **E**, The mean baseline activity over a 5 min period at the indicated concentration of iso. **F**, Cumulative probability plots of the mean baseline activity of the individual vGluT2 or vIAAT neuron in **F**. **G**, Summary of the percentage of neurons showing the indicated change after switching from 1 to 2% iso. Plus or minus 20% change in mean baseline activity indicated as increase or decrease, respectively; no change for all other neurons (see Materials and Methods). **** $p < 0.0001$, paired t -test.

with low baseline activity were detected, each experimental stack contained images during mechanical stimulation. For image and movie presentation of stimulus-evoked activity, ΔF images were generated by creating a template image of the mean baseline before stimulation, which was then subtracted from all images in the stack. For images, a maximum projection was then taken from this subtracted stack. Movies were exported from ImageJ software using mp4 coding and MPEG compression. All raw imaging time series used in this study were assessed for quality before analysis. In rare instances where large, uncorrectable motion artifacts and/or drift in the z -axis was observed, the images were excluded.

Quantitative image analysis was performed using CalmAn software (Pneumatikakis et al., 2016; Giovannucci et al., 2019). Briefly, time series image stacks were median filtered and motion corrected using the built-in rigid motion correction algorithm. CalmAn applies a nonnegative factorization method that considers the spatial and temporal components of calcium signals for identifying and separating neurons. Putative neurons are further restricted by a convolutional neural network classifier. We found this automated method yielded comparable results to the initial $\Delta F/F$ values obtained by human drawn regions of interests using ImageJ and, therefore, it was used for all data collection. In a subset of experiments (Fig. 1), the spontaneous activity of each detected neuron was estimated with the CalmAn deconvolution algorithm, with parameters set to match the kinetics of GCaMP6s (Chen et al., 2013).

Isoflurane and spontaneous activity. Animals were allowed to equilibrate to the indicated concentration of isoflurane (1 or 2%) for at least 10 min before recording the spontaneous baseline activity. Values reported in the manuscript represent the mean deconvolved activity (output by CalmAn) measured over a 5 min period. Frames in which no activity occurred were included in averages. In determining the number of cells that decreased, did not change, or increased following a switch to 2% isoflurane, the following criteria were used: (1) Increase was defined as any neurons that started at zero and gained activity or that had spontaneous activity initially, and the level of that activity increased >20% from the initial value. (2) Decrease was any neuron that showed a >20% drop in activity. (3) No change included neurons that started and ended with no activity or neurons with activity that did not increase or decrease by >20%.

Manual mechanical stimulation. Either the paw, distal hindlimb (tibia and fibula), or proximal hindlimb (femur) were touched or firmly pinched with the index finger and thumb to cover the area of interest. For the testing of touch responses, the thumb and index finger were gently moved up and down over the surface of skin in the proximal to distal direction, rotating medial to lateral to cover a large area of the targeted hindlimb region, approximately once every 2 s with an effort to minimize shear force. Under the low concentration of anesthesia used for these experiments, it was common to feel a slight change in muscle tone in response to pinch but not touch stimuli. We focused on

responses of the distal hindlimb because mechanical stimulation of this area recruited the most neurons (100.00% vIAAT, 94.23% vGluT2). This region also permitted the least variable recordings because it was easier to secure than the paw and more easily accessed than the proximal hindlimb, especially in the later presented pressure pinch experiments.

Pressurized air pincher. The medial and lateral surface of the distal hindlimb was pinched with a pair of forceps (catalog #RS-8240, Roboz) driven by pressurized air (see Fig. 3A,B). Our methods were modified from a previous report (Graham et al., 2004) to include a loss-of-resistance syringe (catalog #24-1018-64, Smiths Medical) with minimal friction. The use of a picospritzer (Picospritzer III, Parker) permitted transistor–transistor logic pulse syncing with the imaging software and precise control over the onset, magnitude, and duration of the pinch stimulus. Further control of interstimulus intervals and durations were programmed using a pulse generator (Pulsemaster A300, World Precision Instruments). Once enough pressure was applied to close the forceps, the force (measured at the end of forceps) scaled linearly with pressure (see Fig. 3C). The pounds per square inch (psi) values referenced throughout the text indicate the picospritzer air pressure settings. Values for 4, 5, 6, 7, 8, 9, 10 psi correspond to 8.6, 80.8, 152.9, 225.0, 297.2, 369.3, 441.2 g, respectively (derived from linear fit). These forces were sufficient to secure the hindlimb in place when reflexive increases in muscle tone were presumably occurring within the noxious range. The position of the forceps was periodically inspected to ensure the same region of the hindlimb was stimulated throughout the experiment.

Thermal stimuli. An imaging area containing visible neurons was first identified by alternating touch and pinch stimuli. These responses were used to later compare mechanical and temperature responses. A 50 ml beaker of water was heated or cooled to the desired temperature. Immediately before imaging, the temperature of the water was stirred, measured, and corrected if necessary. After recording baseline activity, the ipsilateral hindlimb was submerged from the paw to approximately midway up the femur for 30 s. The data used to quantify the mean responses of individual neurons are from duplicate trials at a given temperature performed on each animal. The hindlimb was gently patted dry with tissues and given at least 2 min to recover before the next trial. Care was taken to limit mechanical stimulation during temperature trials, and any instances in which the hindlimb was mistakenly touched or submersion triggered a strong reflexive kick or sustained movement were discarded.

Cutaneous temperature measurements. The cutaneous temperature of the mouse hindlimb was measured in similar anesthetic conditions to those used for imaging experiments. Core body temperature was maintained with a thermal blanket, and measurements were performed on the hindlimb after waiting at least 10 min in 1% isoflurane. A fine microprobe thermometer (BAT-12, Physitemp) was adhered to the midpoint of the plantar surface of the paw, the tibia/fibula (distal hindlimb), and the distal region of the femur (proximal hindlimb). Temperatures were recorded at each location 1 min after positioning the probe.

Capsaicin experiments. A region of the distal hindlimb generating reliable responses to manual mechanical stimuli (touching and mild pinching) was identified. Before injection, neurons were imaged during touch (~40 s duration) and a pressure-driven pinch (8 psi, 1 s duration). Intradermal capsaicin (30 μ l of 500 μ M; 10 mM stock in DMSO diluted 1:20 in saline) or vehicle control (DMSO diluted 1:20 in saline) was then injected within this region and, following a 3 min wait, the same touch and pressure pinch protocol was repeated.

Data analysis and statistics. Data were analyzed and plotted using MATLAB, Prism, Excel, and Igor Pro (WaveMetrics) with Neuromatic (<http://www.neuromatic.thinkrandom.com/>). Unless stated otherwise, fluorescence data are expressed as the percentage change from baseline as follows: $\frac{F - F_0}{F_0} \times 100 = \% \Delta F/F$. Neurons with a >20% change in peak fluorescence from baseline following the stimulus were considered responsive. This method yielded similar results to those manually classified by an observer on data subsets. Area under the curve (AUC) measurements ($\% \Delta F/F \times \text{sec}$) were derived for each neuron by subtracting its mean $\% \Delta F/F$ at baseline from the $\% \Delta F/F$ measured at each frame following the stimulus; these values were then summated to integrate the response.

The pressure–response relationship, within each neuron or averaged over neurons, was fit with linear or sigmoid functions, and the R^2 values were derived from the residuals. The following sigmoid function was used:

$$\text{base} + \frac{\text{max}}{1 + e^{\frac{\text{xhalf} - x}{\text{rate}}}}$$

where *base* and *max* are the minimum and maximum response values, respectively; *rate* is the rise time; and *xhalf* is the estimated pressure at which 50% of the response was reached.

All data are expressed as the mean $\pm$ SEM, unless stated otherwise. Omnibus between-group comparisons were performed using either one-way or two-way ANOVAs as indicated in the text. Two-tailed *t* tests were used to determine significance between means within cells (paired) or between cell types (unpaired). Cutaneous temperature measurements were tested for a significant difference from 32°C using a *z* test. Comparisons between fractions of neurons belonging to two groups were performed using χ^2 tests. For multiple comparisons, *p* values were corrected with the Holm–Bonferroni method. Unless stated otherwise, *p* value significance is expressed as follows: *p* > 0.05 not significant (ns), *p* < 0.05 *, *p* < 0.01 **, *p* < 0.001 ***, *p* < 0.0001 ****.

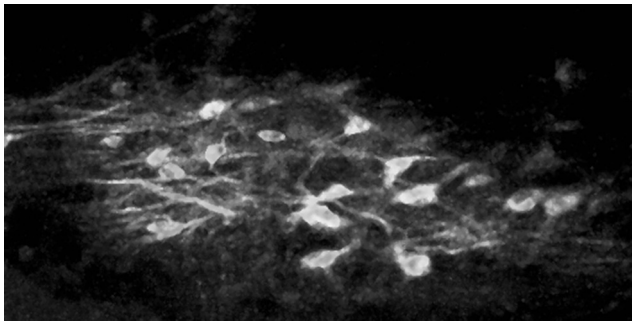
Results

Effects of anesthesia on the spontaneous calcium activity of excitatory and inhibitory SDH neurons

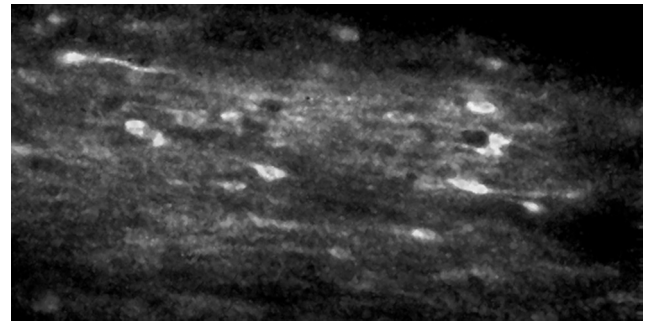
To compare the *in vivo* activity of excitatory and inhibitory SDH neurons, two mouse lines were generated expressing the genetically encoded calcium indicator GCaMP6s, either in vGluT2- or vIAAT-positive neurons, respectively. Dorsal horn neurons expressing the vesicular transporters for glutamate (vGluT2) or inhibitory amino acids (vIAAT) represent two broad, nonoverlapping populations (Punnakkal et al., 2014; Häring et al., 2018; Das Gupta et al., 2021). Within the SDH, the vast majority of inhibitory neurons are GABAergic, with glycinergic neurons primarily occupying deeper lamina (Punnakkal et al., 2014; Peirs et al., 2020). A previous *in vitro* study from our group confirmed that the inhibitory marker Pax2 coexpressed with GCaMP6s in SDH neurons from the vIAAT but not the vGluT2 mouse line (Fan and Sdrulla, 2020).

Adult vGluT2/GCaMP6s and vIAAT/GCaMP6s mice were implanted with a spinal cord window (Farrar et al., 2012; Sekiguchi et al., 2016; Fig. 1A,B), which permitted *in vivo* two-photon calcium imaging of L3–L5 SDH neurons under the volatile anesthetic isoflurane. Excitatory and inhibitory SDH neurons generally differ from each other in their action potential thresholds and firing rates (Heinke et al., 2004; Punnakkal et al., 2014); therefore, we tested whether the spontaneous baseline calcium activity differed between these two populations of neurons. To quantify baseline activity, we used the built-in deconvolution feature of CalmAn software (Fig. 1C), which exploits a priori information about calcium indicator kinetics to improve event detection (Pnevmatikakis et al., 2016; Giovannucci et al., 2019). In 1% isoflurane, there was no difference in the mean baseline activity over a 5 min imaging period between the two populations [Fig. 1D–F; vGluT2 = 17.23 ± 1.54 arbitrary deconvolved units (ADU), *n* = 90 neurons; vIAAT = 18.20 ± 1.17 ADU, *n* = 112; *p* = 0.62; four mice per genotype].

A previous *in vivo* study found that the activity of a mixed population of excitatory and inhibitory SDH neurons was attenuated by isoflurane (Sekiguchi et al., 2016). We, therefore, increased the concentration of isoflurane to gauge the effects of anesthetic dose on these two populations and to simultaneously validate our deconvolution method for measuring baseline



Movie 1. Example touch and pinch responses of vGluT2 neurons. ΔF movie from the example experiments in Figure 2A,C showing the responses of vGluT2 neurons to touching and pinching different regions of the hindlimb (100 frames per stimulus with brief gaps in acquisition between). Playback speed is approximately 10-fold real time. See labels for time, location, and type of stimulus. [View online]



Movie 2. Example touch and pinch responses of vIAAT neurons. ΔF movie from the example experiments in Figure 2B,D showing the responses of vIAAT neurons to touching and pinching different regions of the hindlimb (100 frames per stimulus with brief gaps in acquisition between). Playback speed is approximately 10-fold real time. [View online]

activity. Following a 15 min exposure to 2% isoflurane, the activity of both excitatory and inhibitory neurons was reduced by more than threefold (Fig. 1D–F; vGluT2 = 5.05 ± 0.85 ADU, $p < 0.0001$; vIAAT = 4.68 ± 0.66 ADU, $p < 0.0001$). Similar to 1% isoflurane, no difference between excitatory and inhibitory neurons was observed at this higher concentration of anesthetic ($p = 0.73$). Comparing the effects of increasing isoflurane on individual neurons (Fig. 1G), the vast majority of either subtype showed a decrease in baseline activity (percentage of neurons showing decrease: vGluT2 = 71.11%, vIAAT = 83.04%; no change: vGluT2 = 16.67%, vIAAT = 5.36%; increase: vGluT2 = 12.22% and vIAAT = 11.61%; see above, Materials and Methods). Collectively, these findings suggest that the baseline activity levels are comparable between excitatory and inhibitory SDH neurons and that both populations are symmetrically attenuated by isoflurane. Therefore, for the remaining experiments, we use the minimal amount of isoflurane required to limit reflexive movement of the hindlimb during stimulation (0.9–1.2%) to maximally preserve neural activity.

Divergent mechanosensory tuning of excitatory and inhibitory neurons

To compare the receptive fields and mechanosensory tuning of excitatory and inhibitory SDH neurons, we delivered a series of innocuous gentle touches or noxious pinches lasting 10–15 s, covering one of three regions of the hindlimb—the paw, distal hindlimb (tibia/fibula), or proximal hindlimb (femur; Movies 1, 2; Fig. 2A–D). Inhibitory neurons were highly responsive to touching of the hindlimb, displaying brisk time-locked calcium signals that were comparable to pinching. The responses of excitatory neurons to touch were relatively diminished, yet these neurons still displayed robust pinch responses.

It was previously shown that individual unclassified dorsal horn neurons are broadly tuned to the location of mechanical stimuli (Nishida et al., 2014). To explore this in excitatory and inhibitory neurons, we detected individual neurons responsive to a given form of mechanical stimulation ($>20\%\Delta F/F$; see above, Materials and Methods) and examined the percentage that was activated by one, two, all three, or no region of the hindlimb. For touch stimuli, 36.54% of vGluT2 neurons remained inactive over all three regions (Fig. 2E, none), whereas no vIAAT neurons of this type were observed; all responded to at least one region (four mice for each genotype, vGluT2, $n = 104$ neurons; vIAAT, $n = 81$ neurons). For pinch stimuli, all vGluT2 and vIAAT neurons were activated by at least one region, and the vast majority were activated by two or more hindlimb regions (94.23% vGluT2,

87.65% vIAAT; Fig. 2E, sum of two and three). We also examined across the touch and pinch responses of individual neurons to gain an overall sense of the mechanosensory receptive fields. Quantifying neurons responsive to at least one form of mechanical stimulation (either touch or pinch) at each hindlimb region revealed that the majority of individual vGluT2 and vIAAT neurons had wide receptive fields; 75.00% of vGluT2 and 58.02% of vIAAT neurons responded to all three locations, whereas nearly all neurons responded to at least two regions (100.00% vIAAT and 95.19% vGluT2). This suggests that many excitatory and inhibitory SDH neurons have spatially broad mechanosensory receptive fields, but these findings do not exclude the potential presence of neurons with smaller receptive fields that may be uncovered using finer stimuli.

For the remaining experiments, we focus on responses of the distal hindlimb because this region was the easiest to access, permitting replicable stimulation with minimal motion artifact (see above, Materials and Methods). The peak change in fluorescence from baseline ($\%\Delta F/F$) elicited by touch was markedly higher in inhibitory than in excitatory neurons (vIAAT = 96.36 ± 7.35 , vGluT2 = $35.42 \pm 3.82\%\Delta F/F$, $p < 0.0001$), whereas no difference was observed in pinch responses (vIAAT = 104.05 ± 8.46 , vGluT2 = $112.82 \pm 8.08\%\Delta F/F$, $p = 0.45$; Fig. 2F,G). Within vGluT2 neurons, responses to pinch were substantially greater than to touch ($p < 0.0001$), whereas the same comparison in vIAAT neurons showed no difference ($p = 0.23$).

Our approach allowed us to compare the responses to touch and pinch within individual neurons (Fig. 2H). A substantially greater fraction of vIAAT neurons were responsive to touch (91.36% vs 48.08% of vGluT2; Fig. 2I, sum of touch only and both). In both populations, very few neurons responded exclusively to touch (touch only, 0% of vGluT2, 12.35% of vIAAT), with many more responding to both pinch and touch (both, 48.08% of vGluT2, 79.01% of vIAAT neurons). A greater fraction of vGluT2 neurons responded only to pinch (pinch only, 46.15% vs 8.64% of vIAAT, $p < 0.0001$). Together, these findings indicate that inhibitory neurons are more sensitive to innocuous mechanical stimuli than excitatory neurons, yet the majority of individual neurons in either population were responsive to noxious mechanical stimuli.

Pinch responses of excitatory and inhibitory neurons to a range of forces and durations

Our observation that inhibitory neurons were also responsive to a noxious pinch stimulus (Fig. 2) did not rule out that non-noxious, low-threshold, mechanosensitive fibers, likely activated

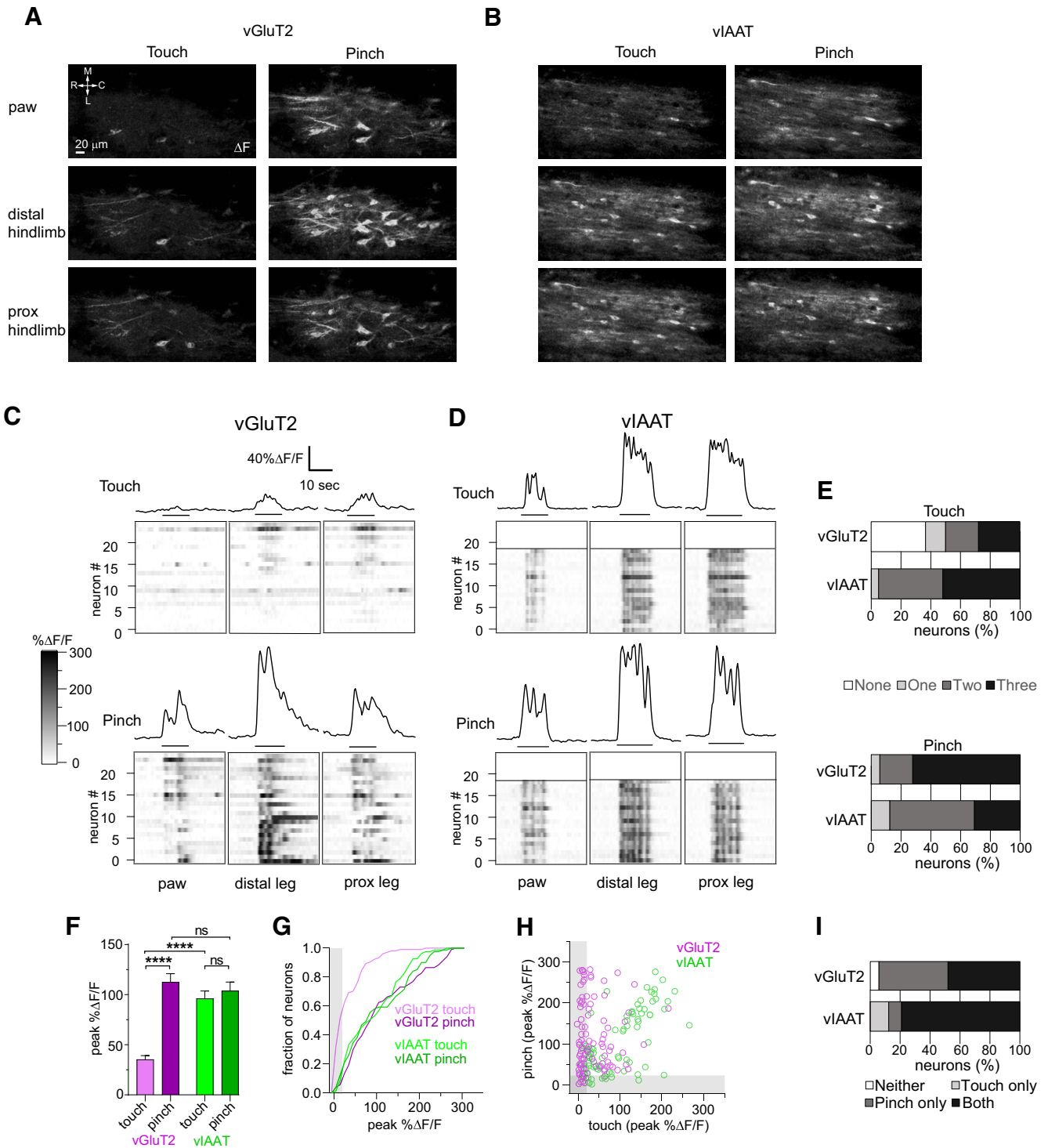


Figure 2. Touch and pinch responses of vGluT2 and vIAAT neurons. **A**, Example experiment showing the *in vivo* responses of vGluT2 neurons to touching or pinching different regions of the hindlimb (paw, distal hindlimb, and proximal hindlimb). ΔF images show the change in fluorescence induced by the stimulus (maximum projection of ~ 30 -s-long time series after subtracting mean baseline). Arrows indicate the orientation of the mouse, medial (M), lateral (L), rostral (R), caudal (C). **B**, As in **A** but for vIAAT neurons. **C**, Image plot showing the % $\Delta F/F$ of the individual vGluT2 neurons ($n = 25$ neurons) detected from the same mouse in **A**. Traces represent the mean % $\Delta F/F$ of all neurons. **D**, As in **C** but for the vIAAT neurons ($n = 18$ neurons). **E**, The percentage of neurons (4 mice of each genotype) responsive to one, two, or all three regions of the hindlimb stimulated by touch (top) or pinch (bottom). **F**, Summary of the mean peak % $\Delta F/F$ elicited by touch or pinch in vGluT2 ($n = 104$) and vIAAT ($n = 81$) neurons. **G**, Cumulative probability plot of the peak responses of the individual neurons in **F**. Vertical light gray bar indicates unresponsive neurons ($< 20\% \Delta F/F$). **H**, Comparison of the peak responses to touch and pinch; each neuron represents one point. Horizontal and vertical light gray bars (20% $\Delta F/F$ cutoff) indicate neurons that were responsive only to touch or only to pinch, respectively; intersecting dark gray, responsive to neither; all other neurons, responsive to both. **I**, Summary of the percentage of vGluT2 and vIAAT neurons from **H** only responsive to the indicated stimulus or both stimuli (touch and pinch). **** $p < 0.0001$, paired and unpaired *t*-test.

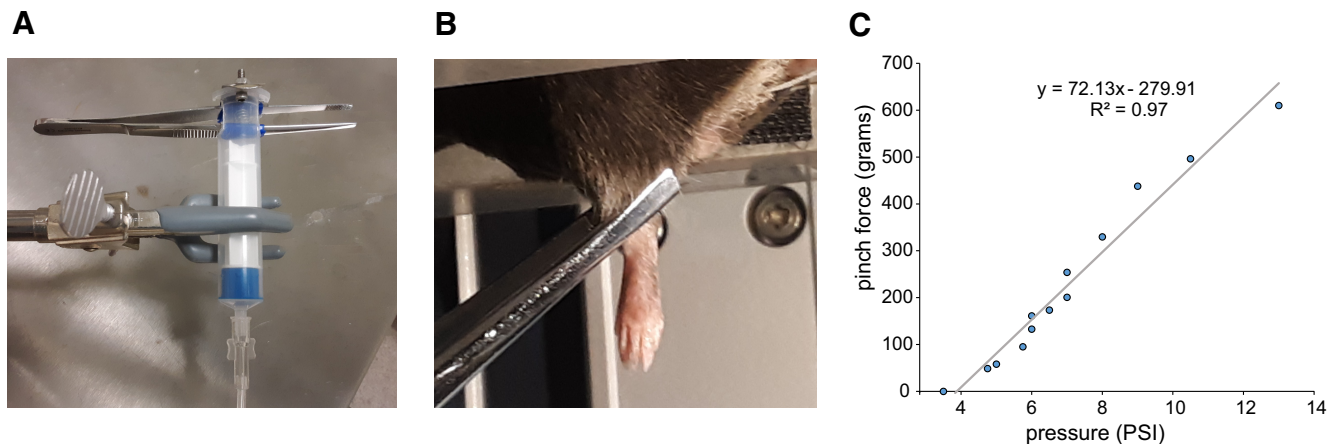


Figure 3. Pressure pincher. **A**, Loss-of-resistance syringe modified to accommodate forceps. **B**, Positioning of forceps over the medial-lateral distal hindlimb. **C**, Pressure-force relationship showing the linear fit used to estimate force.

while pinching, were solely driving these neurons. In contrast, if vIAAT neurons are also activated by nociceptive afferents, their responses should be elevated when high-threshold fibers are recruited by escalating forces. To test the ability of vIAAT and vGluT2 neurons to encode over a range of mechanical stimuli, we used a method where the distal hindlimb was pinched by a pair of forceps controlled by pressurized air, allowing for finer control of the force and duration (Graham et al., 2004). Once enough pressure (measured in psi, at the air source) was applied to close the forceps, the force generated scaled linearly (Fig. 3C). We first measured the calcium responses of these neurons to a 1 s pinch, with air pressures between 4 and 10 psi (9 and 440 g, respectively; see above Materials and Methods for values), incrementing 1 psi with at least 1 min between stimuli (Fig. 4A,B). No difference was observed in the peak $\Delta F/F$ between vGluT2 ($n = 118$, five mice) and vIAAT ($n = 101$, four mice) neurons at lower forces (4, 5, 6, or 7 psi); however, somewhat surprisingly, the responses of vIAAT neurons were greater than vGluT2 neurons at higher forces (8, 9, and 10 psi; Table 1, Fig. 4D). The mean peak vIAAT response scaled linearly across the forces tested (linear fit $R^2 = 0.99$), suggesting that vIAAT neurons as a population are dynamic within this range of stimuli. In contrast, the mean peak vGluT2 response plateaued as the force increased and was described better by a sigmoid fit (sigmoid fit, $R^2 = 0.98$; linear fit, $R^2 = 0.80$).

A previous calcium imaging study in a mixed population of SDH neurons found that many individual cells responded nonlinearly over a similar range of forces (Sekiguchi et al., 2016). Consistent with these findings, we observed that the individual cells of both populations were better described by a sigmoid dose–response function (mean R^2 of fit of each neuron: linear, vGluT2 = 0.48, vIAAT = 0.69; sigmoid, vGluT2 = 0.75, vIAAT = 0.86). Together with the average fit data, this suggests that individual vIAAT neurons, nonlinearly tuned to different pinch forces, may integrate at the population level to dynamically cover a wider range of stimuli. Individual vIAAT neurons also appeared to cover higher pinch forces as the mean half-maximum pressure values (EC_{50}), extracted from individual fits were significantly higher than in vGluT2 neurons (vIAAT, 6.43 ± 0.38 psi; vGluT2, 4.97 ± 0.33 psi, $p < 0.01$).

The lowest pinch force tested (4 psi) activated more vIAAT than vGluT2 neurons (64.36 vs 37.29%, respectively; Table 1; Fig. 4D). The next incremental increase of force (5 psi) recruited the majority of vIAAT and vGluT2 neurons (80.20 and 71.19%, respectively). As expected, the maximum number of responders

for both cell types was reached at the highest force tested (10 psi; vIAAT = 98.02% and vGluT2 = 87.29%). Despite the recruitment of more vIAAT neurons to low pinch force, they tended to remain well below their maximum response when compared with vGluT2 neurons. The cumulative percentage of neurons reaching 90% or more of their maximum peak response at or below a given pinch force revealed that at 8 psi only approximately one-third of vIAAT neurons (33.66%) had neared their maximum, whereas approximately two-thirds (66.95%) of vGluT2 neurons had ($p < 0.0001$; Table 1; Fig. 4E).

These data suggest that despite vIAAT neurons displaying greater sensitivity to innocuous stimuli than vGluT2 neurons, their evoked peak activity was still capable of encoding noxious stimuli—perhaps covering an even wider dynamic range. However, it was apparent in the average traces of vGluT2 neurons that the tails of the calcium signal became larger as the pinch force increased (Fig. 4A,B). To account for this sustained activity, we quantified the integrated calcium signal, AUC. Interestingly, the AUC of both neural types displayed a linear pinch–force response relationship (Fig. 4F; linear fit, R^2 : vGluT2 = 0.95, vIAAT = 0.99), in contrast to the nonlinear peak responses for vGluT2 neurons, suggesting that the sustained activity of excitatory neurons might convey information pertaining to the magnitude of noxious stimuli.

This discrepancy between our peak and area data could arise if excitatory and inhibitory neurons adapt differently to sustained mechanical stimuli. To test this, we measured calcium responses to an 8 psi pinch of varying durations (0.1, 0.2, 0.5, 1, 2, 5, and 10 s; Fig. 4A,B). As the pinch duration increased, the responses of both populations broadened and exhibited mild adaptation. The peak amplitude, percentage of cells responding, percentage of cells reaching 90% of maximum, and the AUC were all similar between vGluT2 and vIAAT neurons, only showing minor differences across the durations tested (Table 1; Fig. 4G–J). The mean peak amplitude reached its maximum by 2 s (Fig. 4G), and the area increased with pinch duration sublinearly (Fig. 4J). The majority of cells were activated by a duration as low as 0.2 s (vGluT2 = 58.47%; vIAAT = 78.22%; Fig. 4H). Together these data suggest that vIAAT and vGluT2 populations comparably encode the duration of noxious stimuli.

Excitatory neurons are more responsive to thermal stimuli than inhibitory neurons

We next compared the thermal responses of vGluT2-positive excitatory and vIAAT-positive inhibitory SDH neurons over a

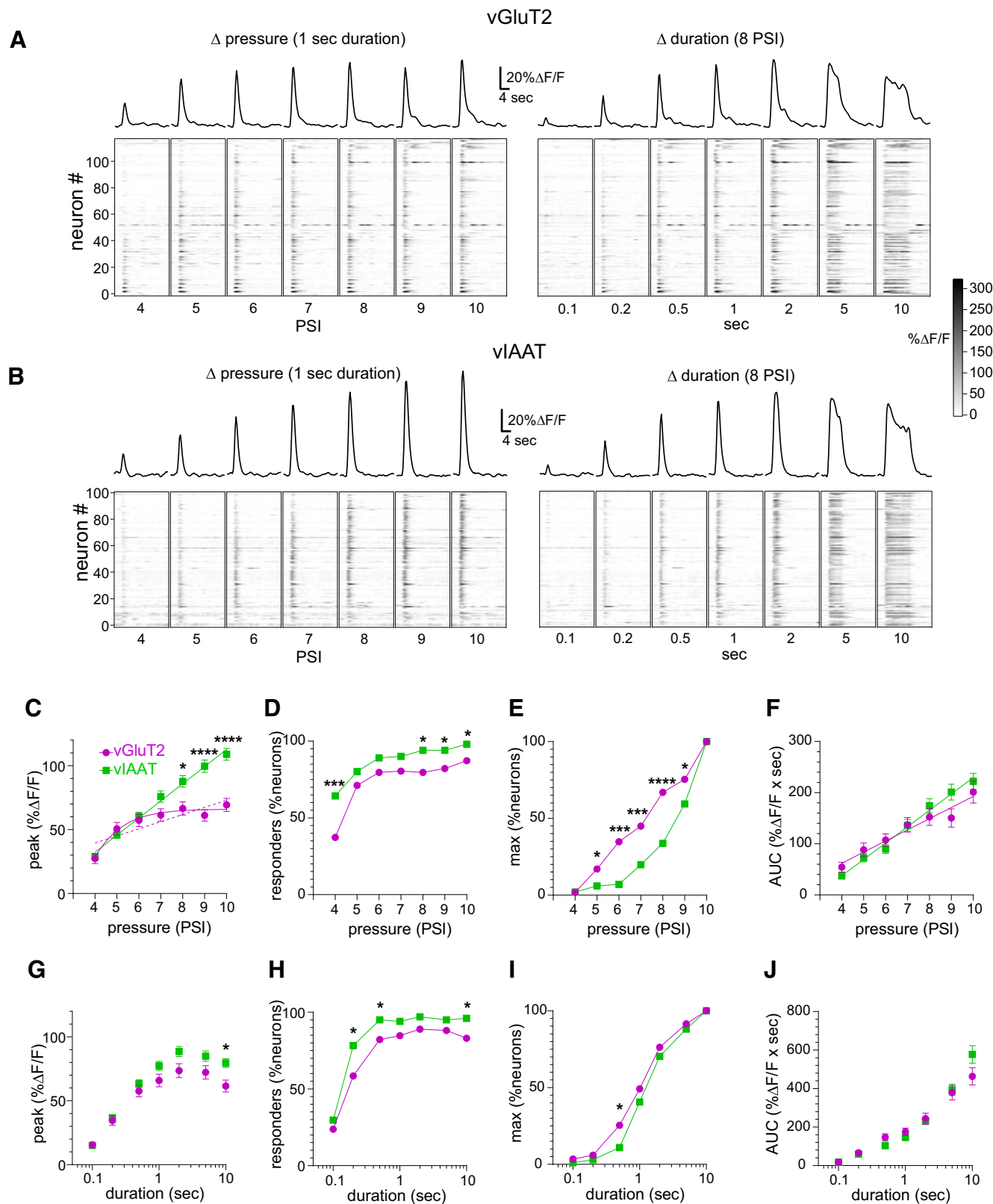


Figure 4. Neural responses to graded pinch force and duration. **A**, Image plot of the % Δ F/F of all detected vGluT2 neurons ($n = 118$, from 5 mice) in response to a 1 s pinch of varying force (4–10 psi, left) and to an 8 psi pinch of varying duration (0.1–10 s, right). Neurons are ordered lowest to highest according to their estimated half-maximum pressure (EC_{50}). Top, Traces are the mean % Δ F/F of all neurons; psi values indicate the air pressure used to drive the forceps and correlate to the force exerted (see above, Materials and Methods; Fig. 3). **B**, As in **A** but for vIAAT neurons ($n = 101$, from 4 mice). **C**, The mean peak response of all vGluT2 and vIAAT neurons at the indicated pinch pressure (1 s duration). Solid line indicates linear fit for vIAAT and sigmoid fit for vGluT2 neurons. Dashed line indicates linear fit for vGluT2. **D**, The percentage of neurons responding at the indicated pressure. **E**, The percentage of neurons reaching their maximum at or below the indicated pressure. **F**, Integrated area of calcium responses (AUC) at different pressures. **G–J**, As in **C–F** but for varying pinch duration (log scale; 8 psi pressure). Asterisk indicates significant difference between genotypes for the same stimulus. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; unpaired t -test and χ^2 .

Table 1. Summary of pressure pinch results

		Pressure (psi)						
		4	5	6	7	8	9	10
vGluT2 (118)	Peak (% $\Delta F/F$)	27.35 $\pm$ 3.93	50.58 $\pm$ 5.04	57.3 $\pm$ 4.84	61.33 $\pm$ 4.96	66.52 $\pm$ 5.19*	61.13 $\pm$ 4.51****	69.32 $\pm$ 5.11****
	Responders (%)	37.29***	71.19	79.66	80.51	79.66*	82.20*	87.29*
	Neurons $\geq$ 90% of max peak (%)	1.69	16.95*	34.75***	44.92***	66.95****	75.42*	100.00
	Area (% $\Delta F/F \times s$)	54.22 $\pm$ 9.64	88.19 $\pm$ 13.44	107.27 $\pm$ 12.09	137.1 $\pm$ 14.28	152.42 $\pm$ 16.31	150.52 $\pm$ 17.94	201.1 $\pm$ 21.53
vIAAT (101)	Peak (% $\Delta F/F$)	28.81 $\pm$ 1.96	45.94 $\pm$ 2.72	60.27 $\pm$ 3.45	75.82 $\pm$ 4.44	87.7 $\pm$ 4.52*	99.59 $\pm$ 4.87****	108.88 $\pm$ 4.59****
	Responders (%)	64.36***	80.20	89.11	90.10	94.06*	94.06*	98.02*
	Neurons $\geq$ 90% of max peak (%)	1.98	5.94*	6.93***	19.80***	33.66****	59.41*	100.00
	Area (% $\Delta F/F \times s$)	37.22 $\pm$ 4.2	72.75 $\pm$ 8.08	89.95 $\pm$ 8.76	135.97 $\pm$ 11.64	174.3 $\pm$ 13.86	200.94 $\pm$ 15.47	222.28 $\pm$ 15.85
		Duration (s)						
		0.1	0.2	0.5	1	2	5	10
vGluT2 (118)	Peak (% $\Delta F/F$)	15.57 $\pm$ 1.59	34.53 $\pm$ 3.64	57.57 $\pm$ 4.52	65.82 $\pm$ 4.94	73.54 $\pm$ 5.45	72.20 $\pm$ 5.23	61.50 $\pm$ 4.66*
	Responders (%)	23.73	58.47*	82.20*	84.75	88.98	88.14	83.05*
	Neurons $\geq$ 90% of max peak (%)	3.39	5.93	25.42*	49.15	76.27	91.53	100.00
	Area (% $\Delta F/F \times s$)	17.74 $\pm$ 3.60	66.06 $\pm$ 9.80	146.69 $\pm$ 18.80	175.07 $\pm$ 19.19	243.61 $\pm$ 28.70	378.99 $\pm$ 38.46	463.46 $\pm$ 45.00
vIAAT (101)	Peak (% $\Delta F/F$)	14.99 $\pm$ 1.34	36.64 $\pm$ 2.58	63.30 $\pm$ 3.02	77.26 $\pm$ 3.56	88.53 $\pm$ 3.90	84.86 $\pm$ 4.13	79.51 $\pm$ 3.43*
	Responders (%)	29.70	78.22*	95.05*	94.06	97.03	95.05	96.04*
	Neurons $\geq$ 90% of max peak (%)	0.99	2.97	10.89*	40.59	70.30	88.12	100.00
	Area (% $\Delta F/F \times s$)	17.56 $\pm$ 3.04	60.18 $\pm$ 8.97	103.05 $\pm$ 9.17	145.86 $\pm$ 10.56	230.83 $\pm$ 15.83	394.01 $\pm$ 27.85	577.12 $\pm$ 45.27

Response values to varying pinch force and duration compared between vGluT2 and vIAAT neurons. Max indicates the percentage of neurons exceeding 90% of their maximum response at or below the indicated stimulus. Number of neurons in parentheses. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; unpaired t -test and χ^2 .

range of innocuous and noxious temperatures. Mice were imaged while their hindlimb was submerged for 30 s in a beaker of water at the indicated temperature: 4°C cold, 15°C cool, 32°C neutral, 37°C warm, and 45°C hot (Fig. 5A,B). Thermal responses were markedly smaller in vIAAT neurons compared with vGluT2 neurons; the mean peak, AUC, and percentage of neurons responding were all significantly lower at each temperature tested (Table 2; Fig. 5C–F; four animals per genotype). vGluT2 neurons were preferentially tuned to thermal stimuli within the nociceptive range (cold and hot), although they also responded to cool and warm temperatures.

As expected, exposure to 32°C, which is commonly used to approximate physiological skin temperature (Ran et al., 2016), yielded the smallest thermal responses within vGluT2 and vIAAT neurons (Fig. 5A–F). The mean peak, AUC, and percentage of neurons responding were significantly lower than all other temperatures tested (Table 2), with one exception, that is, the percentage of vIAAT neurons responding to 32°C and 15°C was comparably low (5.36% vs 10.71%, respectively; $p = 0.14$). A fraction of vGluT2 neurons were notably responsive to 32°C (27.27%). We reasoned that this could be because of a reduction in peripheral skin temperature below 32°C by isoflurane (Xu et al., 2021) and therefore measured the cutaneous temperature at different regions of the hindlimb under anesthetic and animal heating methods similar to those used in our imaging studies (see above, Materials and Methods). The temperature measured at the paw, distal hindlimb, and proximal hindlimb ($28.78 \pm 0.21^\circ\text{C}$, $30.16 \pm 0.33^\circ\text{C}$, and $31.44 \pm 0.51^\circ\text{C}$, respectively; five animals) significantly differed (one-way ANOVA, $p < 0.001$). Cutaneous temperatures at the paw and distal hindlimb but not the proximal hindlimb were significantly below 32°C (z test, $p < 0.0001$, $p < 0.05$, and $p = 0.62$, respectively), suggesting that neurons responsive to 32°C might be detecting slight changes in temperature.

Our approach allowed us to compare the ability of individual neurons to respond across the different temperatures tested. A substantially greater percentage of vGluT2 neurons were thermal

sensitive (neurons activated by one or more temperatures; vGluT2 = 93.64%, $n = 110$; vIAAT = 40.18%, $n = 112$; $p < 0.0001$). Many vGluT2 neurons responded to all temperatures tested (32.73%, excluding 32°C); conversely, vIAAT neurons of this type were relatively scarce (4.46%; vs vGluT2 $p < 0.0001$). The majority of vGluT2 neurons were responsive to both noxious temperatures (hot and cold, 63.64%), whereas far fewer vIAAT neurons exhibited this property (9.82%; vs vGluT2 $p < 0.0001$; Fig. 5G,I). A similarly low percentage of vGluT2 and vIAAT neurons responded to hot but not cold (10.00 vs 8.04%, respectively, $p = 0.61$) or cold but not hot (19.10 vs 20.54%, $p = 0.79$). The same comparison between non-noxious warm and cool temperatures showed that more vGluT2 neurons were responsive to both (32.73% vs vIAAT = 5.36%, $p < 0.0001$; Fig. 5H,J). A comparable percentage of cells were responsive to warm but not cool (vGluT2 = 21.82% vs vIAAT = 19.64%, $p = 0.69$), whereas significantly more vGluT2 than vIAAT neurons were responsive to cool but not warm (29.10 vs 5.36%, $p < 0.0001$; additional statistical comparisons in Table 2).

All neurons imaged in these temperature experiments were also tested for their responsiveness to mechanical stimuli—light touch and pinch delivered to the same region of the hindlimb. The vast majority of neurons from both genotypes were responsive to at least one form of mechanical stimulation (vIAAT = 100%, vGluT2 82.73%). Many individual vGluT2 neurons exhibited robust responses to both thermal and mechanical stimuli, whereas other neurons were relatively skewed in their tuning, preferring one of either modality (Fig. 6A–E). Nonetheless, these findings are consistent with other reports that many SDH neurons are polymodal with respect to thermal and mechanical stimuli.

Capsaicin differentially alters the mechanosensory drive of excitatory and inhibitory neurons

Numerous studies have found pain sensitization to correlate with changes in the balance of excitation and inhibition within the SDH *in vitro* (Kuner, 2015; Gong et al., 2019; Gradwell et al.,

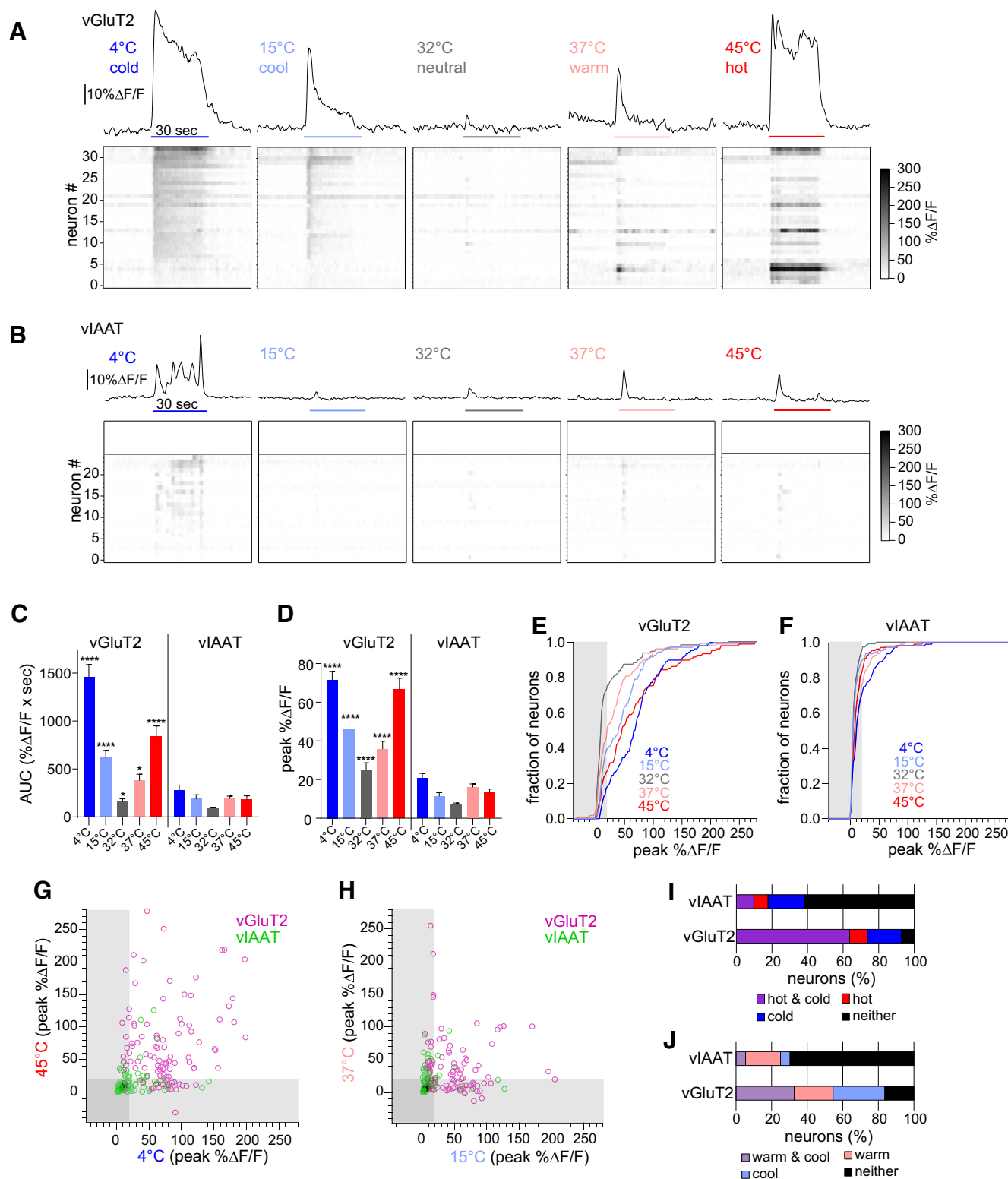


Figure 5. Temperature responses of vGluT2 and vIAAT neurons. **A**, Example mouse. Image plot of vGluT2 neurons ($n = 33$) during a 30 s submersion of the hindlimb in water at the indicated temperature. Neurons are sorted from lowest to highest according to their peak response to 4°C. Top, Traces represent the mean %ΔF/F of the neurons displayed. **B**, As in **A** but vIAAT neurons ($n = 25$). **C**, The mean integrated area of the calcium response (AUC) of all vGluT2 (4 mice, $n = 110$) and vIAAT neurons (4 mice, $n = 112$) elicited by the indicated thermal stimulus. Asterisk indicates significantly different from vIAAT neurons at the same temperature. **D**, As in **C** but for peak %ΔF/F. **E**, Cumulative probability plot of the peak %ΔF/F of the individual vGluT2 neurons in **C**. Vertical light gray bar indicates unresponsive neurons (<20%ΔF/F). **F**, As in **E** but vIAAT neurons. **G**, Comparison of the peak %ΔF/F induced by 4°C and 45°C in all individual vGluT2 and vIAAT neurons. Horizontal and vertical light gray bars (20%ΔF/F cutoff) indicate neurons that were responsive to only 4°C or only 45°C, respectively; dark gray, responsive to neither. **H**, As in **G** but comparing 15°C and 37°C. **I**, Summary of the percentage of neurons responsive to the temperatures indicated in **G**. **J**, As in **I** but for the warm (light red) and cool (light blue) responses shown in **H**. Data in **C–J** generated from duplicate trials (see Materials and Methods). * $p < 0.05$, **** $p < 0.0001$, unpaired t -test.

Table 2. Summary of temperature results

		Temperature				
		4°C	15°C	32°C	37°C	45°C
vGluT2 (110)	Peak (%ΔF/F)	71.56 ± 4.42*##	46.02 ± 3.77*##	24.92 ± 3.69*	35.77 ± 3.99*#S	66.52 ± 5.86*#S
	responders (%)	82.73*##	61.82*##	27.27*	54.55*#S	73.64*#S
	Area (%ΔF/F × s)	1458.39 ± 130.67*##†	617.03 ± 77.53*##‡	161.56 ± 27.82*	380.36 ± 65.72*#S‡	844.09 ± 104.80*#S†
vIAAT (112)	Peak (%ΔF/F)	20.86 ± 2.37*##†	11.42 ± 1.83*##	7.35 ± 0.62*	16.04 ± 1.61*#	13.26 ± 1.75*#†
	responders (%)	30.36*##	10.71*##	5.36*	25.00*#	17.86*#
	Area (%ΔF/F × s)	283.15 ± 48.59*##	189.48 ± 41.81*##	92.69 ± 10.18*	193.27 ± 23.81*#	186.33 ± 34.43*#

Response values over the range of temperatures tested. Number of neurons in parentheses. Symbols correspond to $p < 0.05$ for the following comparison between cell subtypes at the same temperature or between temperatures within subtypes: vGluT2 versus vIAAT (*), 4°C versus 32°C (#), 37°C versus 45°C (S), 45°C versus 4°C (†), 4°C versus 15°C (‡), and 37°C versus 15°C (§). Paired and unpaired t -test; χ^2 .

2020). To test for these changes *in vivo*, we adopted an acute model of pain sensitization induced by the TRPV1 agonist capsaicin (Torebjörk et al., 1992; Gilchrist et al., 1996; O’Neill et al., 2012). We compared the calcium responses to touch (~40 s duration) and noxious pinch (8 psi, 1 s duration) before and shortly after an intradermal injection (30 μ l, hindlimb) of either vehicle or 500 μ M capsaicin (Fig. 7A,B). We found that mechanical responses were not substantially altered by vehicle injection in either population (Fig. 7A, C; vGluT2, $n = 67$, three mice; vIAAT, $n = 84$, four mice). A two-way ANOVA showed no interaction of genotype and injection for vehicle ($p = 0.29$) but a significant interaction for capsaicin treatment ($p < 0.0001$). Capsaicin markedly increased the peak response to touch by twofold in vGluT2 neurons (Fig. 7B, D; $n = 89$, three mice, 2.00 ± 0.16 , $p < 0.0001$; within neuron values normalized to preinjection control). In stark contrast, no change was observed in the touch responses of vIAAT neurons ($n = 73$, three mice, 1.05 ± 0.13 , $p = 0.53$). Capsaicin injection also significantly potentiated pinch responses in vGluT2 neurons (1.36 ± 0.11 , $p < 0.0001$); vIAAT neurons, conversely, exhibited a decreased peak pinch response (0.61 ± 0.06 , $p < 0.001$).

The precise timing of the pressurized pinching method permitted us to look at the integrated activity (AUC) of the pinch-evoked responses. Similar to their peak responses (Fig. 7D), vGluT2 neurons showed a significant increase in the AUC of pinch responses following capsaicin injection, undergoing an approximately twofold change (2.10 ± 0.27 , $p < 0.001$). However, vIAAT neurons showed no change in the AUC of pinch responses (0.83 ± 0.14 , $p = 0.45$), in contrast to the suppression observed in their peak.

Capsaicin treatment recruited additional mechanosensitive vGluT2 neurons, as evident in the drop of nonresponsive neurons (preinjection 15.73%, postinjection 3.37%, $p = 0.005$; Fig. 7E). No recruitment of vIAAT neurons was observed (preinjection 10.96%, postinjection 8.22%, $p = 0.57$). Capsaicin also reduced the percentage of vGluT2 neurons only responsive to pinch (preinjection 28.09%,

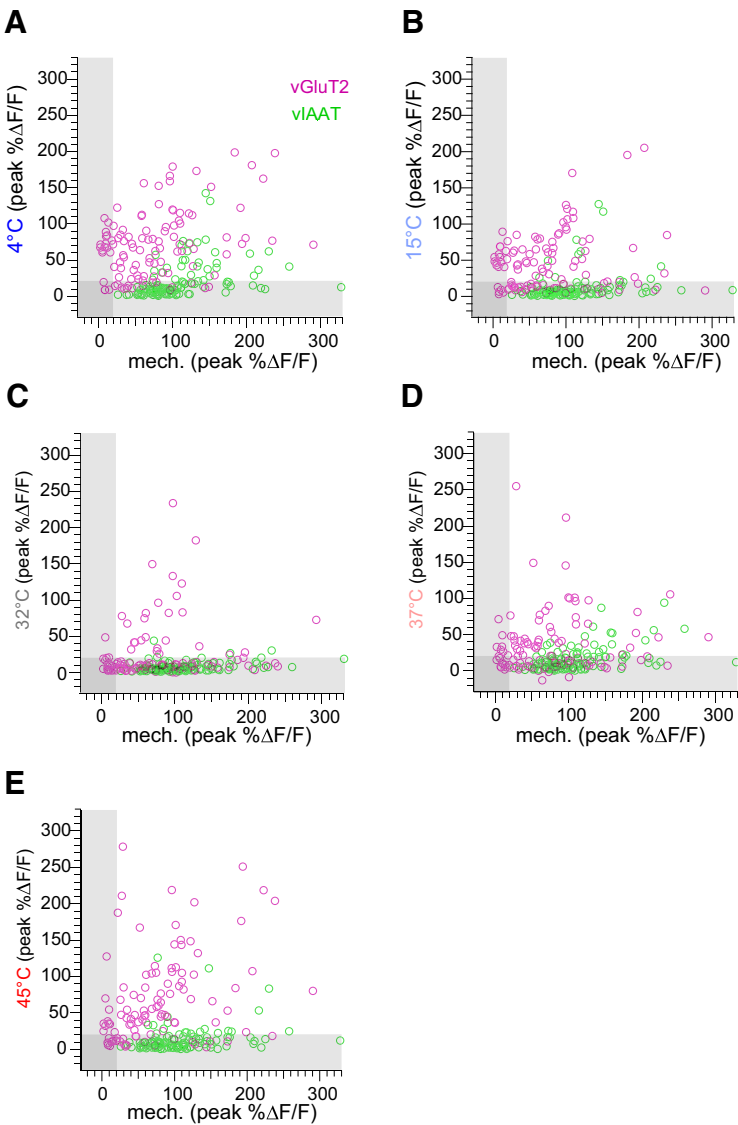


Figure 6. Comparison of mechanical and temperature responses. **A**, The peak mechanical (mech.) response (%ΔF/F) induced in vGluT2 ($n = 110$) and vIAAT neurons ($n = 112$) by alternating touches and pinches over the hindlimb versus the peak response induced by a 4°C thermal stimulus. **B**, As in **A** but for 15°C. **C**, As in **A** but for 32°C. **D**, As in **A** but for 37°C. **E**, As in **A** but for 45°C.

postinjection 4.49%, $p < 0.0001$) and expanded the population responsive to both touch and pinch (preinjection 50.56%, postinjection 86.52%, $p < 0.0001$). Among vIAAT neurons, capsaicin increased the percentage of touch-only neurons (preinjection 9.59%, postinjection 27.40%, $p < 0.01$), consistent with the drop in mean pinch responses (Fig. 7D). Together, these findings

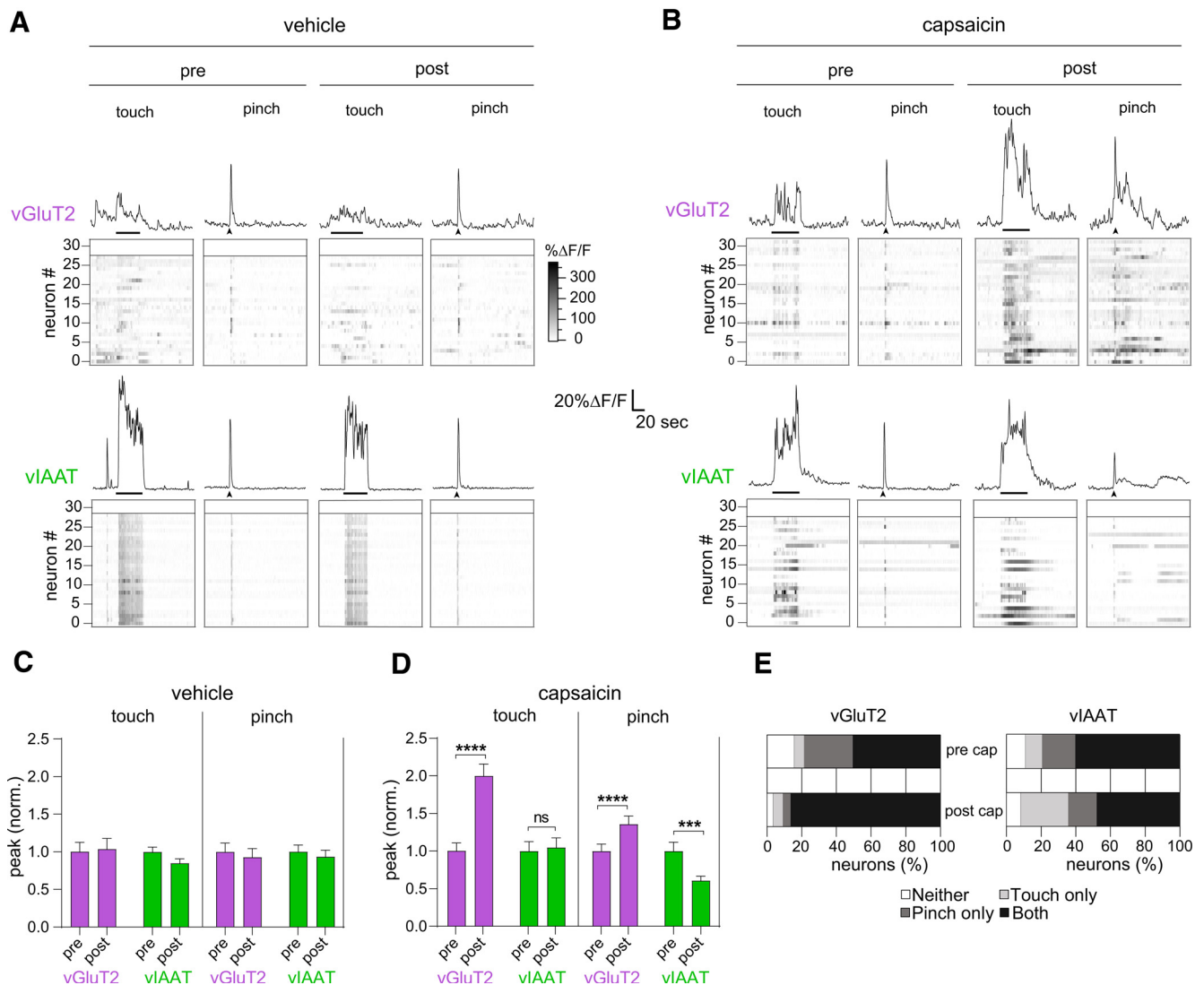


Figure 7. Neural responses to mechanical stimuli following capsaicin treatment. **A**, Image plots of example experiments showing responses to touch (~ 40 s duration) and pinch (force = 8 psi, duration = 1 s, arrowhead) of the distal hindlimb measured before (pre) and after (post) vehicle injection (vGluT2, $n = 28$; vIAT, $n = 29$). Top, Traces represent the mean $\% \Delta F/F$ of the neurons displayed. Arrowheads indicate the onset of the pinch stimulus. **B**, As in **A** but following capsaicin injection (vGluT2, $n = 32$; vIAT, $n = 28$). **C**, The mean of peak responses (normalized to preinjection control values) of all neurons to touch and pinch, before and after vehicle injection (vGluT2, $n = 67$, 3 mice; vIAT, $n = 84$, 4 mice). **D**, As in **C** but following capsaicin injection (vGluT2, $n = 89$, 3 mice; vIAT, $n = 73$, 3 mice). **E**, Summary of the percentage of vGluT2 and vIAT neurons responsive (only to touch or pinch, both, or neither) before (pre cap) and after (post cap) capsaicin injection. n.s., not significant, $***p < 0.001$, $****p < 0.0001$; paired t -test.

show that SDH mechanosensory responses are asymmetrically altered by capsaicin, facilitating excitatory neurons and, to a lesser extent, suppressing inhibitory neurons.

Discussion

In this study, we applied an *in vivo* calcium imaging approach to characterize the activity of two broadly inclusive classes of genetically targeted excitatory and inhibitory SDH neurons. The non-invasiveness and stability of the two-photon imaging window permitted us to compare the spontaneous activity and natural somatosensory responses of these neural populations under minimal anesthetic and with limited motion artifact (Movies 1, 2). We found that inhibitory neurons are more sensitive to innocuous mechanical stimuli, faithfully encode into the noxious range of pinch forces, and are less sensitive to thermal stimuli than excitatory neurons. Capsaicin elicited opposing plasticity of mechanosensory responses, that is, potentiating excitatory neurons and suppressing inhibitory neurons.

SDH cell types

Recently developed molecular tools for identifying and manipulating dorsal horn neurons and their somatosensory afferents have revealed the circuits behind nociceptive processing to be highly complex (Prescott et al., 2014; Peirs and Seal, 2016; Harding et al., 2020b). Therefore, the vGluT2 and vIAT neural populations imaged in these studies each likely represent a heterogeneous mix of cell types with uniquely specialized circuit functions. However, to date, no agreed-on criteria exists for identifying a single dorsal horn cell type with an archetypical molecular, morphologic, and physiological profile (Browne et al., 2020; Harding et al., 2020b). Unbiased multidimensional RNA-sequencing methods suggest a wide degree of diversity, with at least 15 excitatory and 15 inhibitory neurons within the dorsal horn, many of which are present in the SDH (Häring et al., 2018; Sathiyamurthy et al., 2018). Nonetheless, overarching differences are routinely observed between excitatory and inhibitory SDH neurons, including their morphology (Grudt and Perl, 2002;

Heinke et al., 2004; Maxwell et al., 2007; Todd, 2010; Yasaka et al., 2010), excitability (Heinke et al., 2004; Browne et al., 2020), synaptic receptors (Kerr et al., 1998; Engelman et al., 1999; Chen et al., 2016; Sullivan et al., 2017), and neuropeptide composition (Das Gupta et al., 2021). The dichotomy of cellular attributes existing between these two populations may explain why we observed large-scale differences in their somatosensory tuning to thermal and mechanical stimuli. The added dimension of how SDH neurons respond over a breadth of natural stimuli may aid in their classification, as has been essential in our understanding of the retinal cell types behind visual processing (Seung and Sümbül, 2014).

Inhibition in gate control

Numerous population coding theories for dorsal horn nociceptive processing rely on the recruitment of inhibitory interneurons, but very little is known about how these neurons behave *in vivo*. To the best of our knowledge, these are the first *in vivo* recordings of the mechanosensory responses in genetically identified inhibitory dorsal horn neurons that do not rely solely on physiological parameters for *post hoc* classification (Lee et al., 2019). We found inhibitory neurons were considerably more responsive to innocuous mechanical stimuli than excitatory neurons. Consistent with our findings, inhibitory SDH neurons *in vitro* are readily activated by electrical stimulation of the dorsal root at intensities subthreshold for pain fibers (Daniele and MacDermott, 2009). Additionally, lamina I projection neurons—a likely subset of the excitatory neurons imaged in these studies—are minimally responsive to innocuous mechanical stimuli in normal conditions (Torsney and MacDermott, 2006; Allard, 2019; Chisholm et al., 2021). The sensitivity of inhibitory SDH neurons to innocuous stimuli supports that they act as nociceptive gatekeepers to adjacent projection neurons. A subset of superficial dynorphin-expressing inhibitory neurons are indeed activated by low-threshold inputs in acute slices, and their ablation produces mechanical allodynia (Duan et al., 2014). How these superficial inhibitory neurons compare with the deeper inhibitory neurons, such as the parvalbumin-positive cells also involved in gate control (Petitjean et al., 2015; Boyle et al., 2019), remains to be determined.

Activation of SDH inhibitory neurons via low-threshold afferents is predominantly polysynaptic (Daniele and MacDermott, 2009). The superficial excitatory neurons imaged in our studies were minimally responsive to touch. Thus, it is likely that the excitatory interneurons residing in deeper dorsal horn (Duan et al., 2014; Abaira et al., 2017; Cheng et al., 2017; Zhang et al., 2018), where low-threshold inputs are more abundant (Abaira and Ginty, 2013), are intermediaries for driving the robust touch responses we observed in superficial inhibitory neurons. The majority of these inhibitory neurons were also responsive to pinch, raising the question of whether they could faithfully encode over a range of noxious mechanical stimuli. Somewhat surprisingly, we found that the peak calcium responses of inhibitory neurons were more dynamic than excitatory neurons in the nociceptive range of pinch forces tested (Fig. 4). Many inhibitory SDH neurons additionally receive direct monosynaptic input from high-threshold fibers (Heinke et al., 2004; Leitner et al., 2013), and the graded recruitment of these fibers may account for their ability to encode dynamically over the nociceptive range. Nonetheless, given their dual sensitivity to innocuous touch and noxious pinch, it is conceivably possible to drive inhibitory neurons to the same extent with fundamentally different mechanical stimuli, perhaps explaining the similar responses to touch and pinch that we observed (Fig. 2F).

Despite the plateau in the peak calcium responses observed in excitatory neurons at higher pinch forces, the integrated calcium response (AUC) was nonetheless dynamic over the range of pinch forces tested. This discrepancy in the peak and integrated calcium signal may result methodologically from the improved ability of calcium indicators to detect the total number of action potentials over the frequency (Harding et al., 2020a) or, alternatively, from the fundamentally different firing properties of these two populations. Inhibitory neurons require less depolarizing current to reach threshold, firing tonically at rates proportional to the stimulus (Heinke et al., 2004; Browne et al., 2020). In contrast, many excitatory neurons display spiking patterns that are delayed and irregular (Yasaka et al., 2010; Punnakkal et al., 2014), in part because of an A-type potassium conductance (Zhang et al., 2018; Browne et al., 2020). It is, therefore, possible that the maximum spiking rate of excitatory neurons is less relevant than the total number of spikes, which would be better reflected in the integrated calcium response. Future imaging studies in identified neural subpopulations, combined with ground truth electrophysiology, are required to determine whether excitatory and inhibitory neurons spike-code natural stimuli differently.

Temperature

We found that SDH excitatory neurons were markedly more sensitive to thermal stimuli than inhibitory neurons (Fig. 5). A previous *in vivo* imaging study reported that somatostatin or calbindin-positive neurons together account for most temperature-sensitive SDH neurons (Ran and Chen, 2019). These lineages are minimally overlapping and predominantly excitatory within the SDH (Antal et al., 1991; Proudlock et al., 1993; Albuquerque et al., 1999; Duan et al., 2014; Gutierrez-Mecinas et al., 2016), which is consistent with our findings. It has been hypothesized that inhibitory neurons activated downstream of thermal stimuli (particularly warm temperatures) act to disinhibit pain neurons, giving rise to the thermal grill illusion (Ma, 2010; Prescott et al., 2014). Temperature-sensitive inhibitory neurons were present in our experiments and detected in a recent study (Ran et al., 2021), but these neurons were rare in both cases, and we found their responses were small compared with excitatory neurons, raising questions about the role of inhibition in thermal population coding. However, these findings do not preclude the possibility of thermal-sensitive inhibitory neurons residing outside our imaging plane.

Most excitatory neurons in our study responded robustly to both hot and cold temperatures, consistent with previous findings in unidentified neurons (Ran et al., 2016). Lamina I projection neurons, a subset of the vGluT2-positive neurons we studied, respond to both hot and cold stimuli (Chisholm et al., 2021). It is also known that many superficial cells respond to both thermal and mechanical stimuli (Nishida et al., 2014; Chisholm et al., 2021). Congruently, nearly all the excitatory and inhibitory neurons identified in our temperature studies were also mechanosensitive. This multimodality of SDH neurons argues against strict labeled lines in somatosensory coding (Ma, 2010; Prescott et al., 2014) but in turn raises interesting questions about how population coding can convey distinct qualities of somatosensation and differentiate innocuous and noxious stimuli, especially as information must pass through broadly tuned projection neurons before reaching higher pain centers (Agashkov et al., 2019).

Capsaicin-induced plasticity

Changes in the balance of excitation and inhibition within the SDH contribute to the onset and maintenance of neuropathic

and inflammatory pain (Zeilhofer et al., 2012; Kuner, 2015; Gradwell et al., 2020). In chronic pain models, insult induces opposing synaptic plasticity in excitatory and inhibitory neurons, expected to augment or attenuate their activity, respectively (Leitner et al., 2013; Kopach et al., 2017; Gong et al., 2019). In our study, we used a model involving intradermal capsaicin, which acutely promotes allodynia and hyperalgesia (Torebjörk et al., 1992; Gilchrist et al., 1996). We found that it markedly enhanced the responses of excitatory neurons to innocuous touch (many neurons gaining sensitivity) and noxious pinch stimuli. Inhibitory neurons, in contrast, showed no change to touch while exhibiting a mild suppression to pinch. Of note, the peak responses to pinch but not the AUC were attenuated in inhibitory neurons. A potential explanation for this discrepancy is that the maximum rate of firing of inhibitory neurons may decline following capsaicin, but the total number of action potentials generated remains the same. If true, this could alter the timing of inhibitory weights and open the gate, resulting in hyperalgesia.

It may at first seem surprising that the touch responses of inhibitory SDH neurons were unchanged by capsaicin, as disinhibition is proposed by GCT to play an integral role in mechanical allodynia (Zeilhofer et al., 2012; Gradwell et al., 2020). It is possible that plasticity of inhibitory neurons occurs on slower time scales than excitatory neurons and therefore did not manifest in the acute capsaicin model. Alternatively, such changes may occur in inhibitory neurons of deeper laminae (Petitjean et al., 2015; Boyle et al., 2019). It is also important to note that disinhibition can result either from reduced activity of inhibitory neurons or changes in the synaptic efficacy of inhibition; our studies do not resolve the latter. For example, chloride dysregulation, a major contributor of disinhibition (Price and Prescott, 2015), may disproportionately affect excitatory neurons (Lee et al., 2019). Alternatively, the heightened responsiveness of excitatory neurons could occur independently of inhibition via presynaptic or postsynaptic potentiation (Ikeda et al., 2006; Kuner, 2015) and/or alterations in intrinsic excitability. Indeed, capsaicin treatment reduces A-type potassium currents (I_A) in excitatory neurons, permitting $A\beta$ inputs to exceed threshold before the arrival of inhibition (Zhang et al., 2018).

The chronic *in vivo* imaging methods we used allowed us to image large populations of spatially and genetically resolved neurons. However, the low temporal resolution of our imaging precluded investigations into the timing of these responses, which is undoubtedly essential in understanding how fast excitatory and inhibitory transmission integrate in gate control. Furthermore, despite the fact that action potentials map fairly reliably onto calcium indicators used in brain (Chen et al., 2013) and spinal cord (Harding et al., 2020a), electrophysiology outperforms calcium imaging in reconstructing *in vivo* stimuli from neural recordings (Wei et al., 2020). Therefore, demand remains for improved optical voltage sensors (Knöpfel and Song, 2019), imaging methods (Sekiguchi et al., 2016; Harding et al., 2020b), and high throughput electrical recordings (Lee et al., 2019), to parse out dorsal horn somatosensory circuits *in vivo*.

The use of anesthetic is another important consideration for *in vivo* studies. We found that the dose of isoflurane has an impact on the spontaneous activity of dorsal horn neurons (Fig. 1). A seminal study comparing anesthetized and awake behaving animals showed that isoflurane reduced both spontaneous and evoked activity of SDH neurons (Sekiguchi et al., 2016). Thus, awake behaving experiments are paramount in determining how spinal nociceptive processing integrates with motor (Koch et al.,

2017) and descending (Heinricher et al., 2009) control, but, for the same reason, interpreting this data will prove challenging. For example, the somatosensory system of the animal will be variably activated by unpredictable locomotion or arousal states.

Intersectional genetic approaches recently revealed distinct channels of excitatory interneurons for driving somatosensory reflexes to pain, touch, temperature, and itch (Gatto et al., 2021). Our *in vivo* studies in anesthetized animals suggest that excitatory and inhibitory SDH neurons are activated by fundamentally different stimuli, and therefore inhibition may serve to contrast between somatosensory channels rather than sharpen the resolution within, which is contrary to what is typically observed in other sensory systems (Isaacson and Scanziani, 2011). Future *in vivo* studies characterizing more refined neural subtypes and their responses to natural stimuli will aid in identifying dorsal horn cell types based on their function, in addition to their variety of forms.

References

- Abraira VE, Ginty DD (2013) The sensory neurons of touch. *Neuron* 79:618–639.
- Abraira VE, Kuehn ED, Chirila AM, Springel MW, Toliver AA, Zimmerman AL, Orefice LL, Boyle KA, Bai L, Song BJ, Bashista KA, O'Neill TG, Zhuo J, Tsan C, Hoynoski J, Rutlin M, Kus L, Niederkofer V, Watanabe M, Dymecki SM, et al. (2017) The cellular and synaptic architecture of the mechanosensory dorsal horn. *Cell* 168:295–310.e19.
- Agashkov K, Krotov V, Krasniakova M, Shevchuk D, Andrianov Y, Zabenko Y, Safronov BV, Voitenko N, Belan P (2019) Distinct mechanisms of signal processing by lamina I spino-parabrachial neurons. *Sci Rep* 9:19231.
- Albuquerque C, Lee CJ, Jackson AC, MacDermott AB (1999) Subpopulations of GABAergic and non-GABAergic rat dorsal horn neurons express Ca^{2+} -permeable AMPA receptors. *Eur J Neurosci* 11:2758–2766.
- Allard J (2019) Physiological properties of the lamina I spinoparabrachial neurons in the mouse. *J Physiol* 597:2097–2113.
- Antal M, Polgár E, Chalmers J, Minson JB, Llewellyn-Smith I, Heizmann CW, Somogyi P (1991) Different populations of parvalbumin- and calbindin-D28k-immunoreactive neurons contain GABA and accumulate 3H-D-aspartate in the dorsal horn of the rat spinal cord. *J Comp Neurol* 314:114–124.
- Bourane S, Duan B, Koch SC, Dalet A, Britz O, Garcia-Campmany L, Kim E, Cheng L, Ghosh A, Ma Q, Goulding M (2015) Gate control of mechanical itch by a subpopulation of spinal cord interneurons. *Science* 350:550–554.
- Boyle KA, Gradwell MA, Yasaka T, Dickie AC, Polgár E, Ganley RP, Orr DPH, Watanabe M, Abraira VE, Kuehn ED, Zimmerman AL, Ginty DD, Callister RJ, Graham BA, Hughes DI (2019) Defining a spinal microcircuit that gates myelinated afferent input: implications for tactile allodynia. *Cell Rep* 28:526–540.e6.
- Browne TJ, Gradwell MA, Iredale JA, Maden JF, Callister RJ, Hughes DI, Dayas CV, Graham BA (2020) Transgenic cross-referencing of inhibitory and excitatory interneuron populations to dissect neuronal heterogeneity in the dorsal horn. *Front Mol Neurosci* 13:32.
- Cameron D, Polgár E, Gutierrez-Mecinas M, Gomez-Lima M, Watanabe M, Todd AJ (2015) The organisation of spinoparabrachial neurons in the mouse. *Pain* 156:2061–2071.
- Chen T, Taniguchi W, Chen QY, Tozaki-Saitoh H, Song Q, Liu RH, Koga K, Matsuda T, Kaito-Sugimura Y, Wang J, Li ZH, Lu YC, Inoue K, Tsuda M, Li YQ, Nakatsuka T, Zhuo M (2018) Top-down descending facilitation of spinal sensory excitatory transmission from the anterior cingulate cortex. *Nat Commun* 9:1886.
- Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreier ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499:295–300.
- Chen Y, Derkach VA, Smith PA (2016) Loss of Ca^{2+} -permeable AMPA receptors in synapses of tonic firing substantia gelatinosa neurons in the chronic constriction injury model of neuropathic pain. *Exp Neurol* 279:168–177.

- Cheng L, Duan B, Huang T, Zhang Y, Chen Y, Britz O, Garcia-Campmany L, Ren X, Vong L, Lowell BB, Goulding M, Wang Y, Ma Q (2017) Identification of spinal circuits involved in touch-evoked dynamic mechanical pain. *Nat Neurosci* 20:804–814.
- Chisholm KI, Lo Re L, Polgár E, Gutierrez-Mecinas M, Todd AJ, McMahon SB (2021) The encoding of cutaneous stimuli by lamina I projection neurons. *Pain* 162:2405–2417.
- Daniele CA, MacDermott AB (2009) Low-threshold primary afferent drive onto GABAergic interneurons in the superficial dorsal horn of the mouse. *J Neurosci* 29:686–695.
- Das Gupta RR, Scheurer L, Pelczar P, Wildner H, Zeilhofer HU (2021) Neuron-specific spinal cord translatomes reveal a neuropeptide code for mouse dorsal horn excitatory neurons. *Sci Rep* 11:5232.
- Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X, Ross S, Lowell BB, Wang Y, Goulding M, Ma Q (2014) Identification of spinal circuits transmitting and gating mechanical pain. *Cell* 159:1417–1432.
- Engelman HS, Allen TB, MacDermott AB (1999) The distribution of neurons expressing calcium-permeable AMPA receptors in the superficial laminae of the spinal cord dorsal horn. *J Neurosci* 19:2081–2089.
- Fan W, Sdrulla AD (2020) Differential modulation of excitatory and inhibitory populations of superficial dorsal horn neurons in lumbar spinal cord by A β -fiber electrical stimulation. *Pain* 161:1650–1660.
- Farrar MJ, Bernstein IM, Schlafer DH, Cleland TA, Fetcho JR, Schaffer CB (2012) Chronic *in vivo* imaging in the mouse spinal cord using an implanted chamber. *Nat Methods* 9:297–302.
- Gatto G, Bourane S, Ren XD, Costanzo S, Fenton PK, Halder P, Seal RP, Goulding MD (2021) A functional topographic map for spinal sensorimotor reflexes. *Neuron* 109:91–104.e5.
- Gilchrist HD, Allard BL, Simone DA (1996) Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. *Pain* 67:179–188.
- Giovannucci A, Friedrich J, Gunn P, Kalfon J, Brown BL, Koay SA, Taxis J, Najafi F, Gauthier JL, Zhou P, Khakh BS, Tank DW, Chklovskii DB, Pnevmatikakis EA (2019) CalmAn: an open source tool for scalable calcium imaging data analysis. *Elife* 8:e38173.
- Gong N, Hagopian G, Holmes TC, Luo ZD, Xu X (2019) Functional reorganization of local circuit connectivity in superficial spinal dorsal horn with neuropathic pain states. *eNeuro* 6:ENEURO.0272-19.2019.
- Gradwell MA, Callister RJ, Graham BA (2020) Reviewing the case for compromised spinal inhibition in neuropathic pain. *J Neural Transm (Vienna)* 127:481–503.
- Graham BA, Brichta AM, Callister RJ (2004) An *in vivo* mouse spinal cord preparation for patch-clamp analysis of nociceptive processing. *J Neurosci Methods* 136:221–228.
- Grudt TJ, Perl ER (2002) Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J Physiol* 540:189–207.
- Gutierrez-Mecinas M, Furuta T, Watanabe M, Todd AJ (2016) A quantitative study of neurochemically defined excitatory interneuron populations in laminae I–III of the mouse spinal cord. *Mol Pain* 12:174480691662906.
- Harding EK, Boivin B, Salter MW (2020a) Intracellular calcium responses encode action potential firing in spinal cord lamina I neurons. *J Neurosci* 40:4439–4456.
- Harding EK, Fung SW, Bonin RP (2020b) Insights into spinal dorsal horn circuit function and dysfunction using optical approaches. *Front Neural Circuits* 14:31.
- Häring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lönnerberg P, La Manno G, Sharma N, Borgius L, Kiehn O, Lagerström MC, Linnarsson S, Ernfrors P (2018) Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. *Nat Neurosci* 21:869–880.
- Harrison M, O'Brien A, Adams L, Cowin G, Ruitenberg MJ, Sengul G, Watson C (2013) Vertebral landmarks for the identification of spinal cord segments in the mouse. *Neuroimage* 68:22–29.
- Heinke B, Ruscheweyh R, Forsthuber L, Wunderbaldinger G, Sandkühler J (2004) Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice. *J Physiol* 560:249–266.
- Heinricher MM, Tavares I, Leith JL, Lumb BM (2009) Descending control of nociception: specificity, recruitment and plasticity. *Brain Res Rev* 60:214–225.
- Ikedo H, Stark J, Fischer H, Wagner M, Drdlá R, Jäger T, Sandkühler J (2006) Synaptic amplifier of inflammatory pain in the spinal dorsal horn. *Science* 312:1659–1662.
- Isaacson JS, Scanziani M (2011) How inhibition shapes cortical activity. *Neuron* 72:231–243.
- Johannsen HC, Helmchen F (2010) *In vivo* Ca²⁺ imaging of dorsal horn neuronal populations in mouse spinal cord. *J Physiol* 588:3397–3402.
- Johannsen HC, Helmchen F (2013) Two-photon imaging of spinal cord cellular networks. *Exp Neurol* 242:18–26.
- Kerr RC, Maxwell DJ, Todd AJ (1998) GluR1 and GluR2/3 subunits of the AMPA-type glutamate receptor are associated with particular types of neurone in laminae I–III of the spinal dorsal horn of the rat. *Eur J Neurosci* 10:324–333.
- Knöpfel T, Song C (2019) Optical voltage imaging in neurons: moving from technology development to practical tool. *Nat Rev Neurosci* 20:719–727.
- Koch SC, Del Barrio MG, Dalet A, Gatto G, Günther T, Zhang J, Seidler B, Saur D, Schüle R, Goulding M (2017) ROR β spinal interneurons gate sensory transmission during locomotion to secure a fluid walking gait. *Neuron* 96:1419–1431.e5.
- Kopach O, Medvediev V, Krotov V, Borisjuk A, Tsymbaliuk V, Voitenko N (2017) Opposite, bidirectional shifts in excitation and inhibition in specific types of dorsal horn interneurons are associated with spasticity and pain post-SCI. *Sci Rep* 7:5884.
- Kuner R (2015) Spinal excitatory mechanisms of pathological pain. *Pain* 156 Suppl 1:S11–S17.
- Lee KY, Ratté S, Prescott SA (2019) Excitatory neurons are more disinhibited than inhibitory neurons by chloride dysregulation in the spinal dorsal horn. *Elife* 8:e49753.
- Leitner J, Westerholz S, Heinke B, Forsthuber L, Wunderbaldinger G, Jäger T, Gruber-Schoffnegger D, Braun K, Sandkühler J (2013) Impaired excitatory drive to spinal GABAergic neurons of neuropathic mice. *PLoS One* 8:e73370.
- Ma Q (2010) Labeled lines meet and talk: population coding of somatic sensations. *J Clin Invest* 120:3773–3778.
- Maxwell DJ, Belle MD, Cheunsuang O, Stewart A, Morris R (2007) Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *J Physiol* 584:521–533.
- Melzack R, Wall PD (1965) Pain mechanisms: a new theory. *Science* 150:971–979.
- Nagi SS, Marshall AG, Makdani A, Jarocka E, Liljencrantz J, Ridderstrom M, Shaikh S, O'Neill F, Saade D, Donkervoort S, Foley AR, Minde J, Trulsson M, Cole J, Bonnemann CG, Chesler AT, Bushnell MC, McGlone F, Olsson H (2019) An ultrafast system for signaling mechanical pain in human skin. *Sci Adv* 5:eaaw1297.
- Nishida K, Matsumura S, Taniguchi W, Uta D, Furue H, Ito S (2014) Three-dimensional distribution of sensory stimulation-evoked neuronal activity of spinal dorsal horn neurons analyzed by *in vivo* calcium imaging. *PLoS One* 9:e103321.
- O'Neill J, Brock C, Olesen AE, Andresen T, Nilsson M, Dickenson AH (2012) Unravelling the mystery of capsaicin: a tool to understand and treat pain. *Pharmacol Rev* 64:939–971.
- Peirs C, Seal RP (2016) Neural circuits for pain: recent advances and current views. *Science* 354:578–584.
- Peirs C, Dallel R, Todd AJ (2020) Recent advances in our understanding of the organization of dorsal horn neuron populations and their contribution to cutaneous mechanical allodynia. *J Neural Transm (Vienna)* 127:505–525.
- Petitjean H, Pawlowski SA, Fraine SL, Sharif B, Hamad D, Fatima T, Berg J, Brown CM, Jan LY, Ribeiro-da-Silva A, Braz JM, Basbaum AI, Sharif-Naeini R (2015) Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury. *Cell Rep* 13:1246–1257.
- Pnevmatikakis EA, Soudry D, Gao Y, Machado TA, Merel J, Pfau D, Reardon T, Mu Y, Lacefield C, Yang W, Ahrens M, Bruno R, Jessell TM, Peterka DS, Yuste R, Paninski L (2016) Simultaneous denoising, deconvolution, and demixing of calcium imaging data. *Neuron* 89:285–299.
- Prescott SA, Ma Q, De Koninck Y (2014) Normal and abnormal coding of somatosensory stimuli causing pain. *Nat Neurosci* 17:183–191.
- Price TJ, Prescott SA (2015) Inhibitory regulation of the pain gate and how its failure causes pathological pain. *Pain* 156:789–792.
- Proudlock F, Spike RC, Todd AJ (1993) Immunocytochemical study of somatostatin, neurotensin, GABA, and glycine in rat spinal dorsal horn. *J Comp Neurol* 327:289–297.

- Punnakkal P, von Schoultz C, Haenraets K, Wildner H, Zeilhofer HU (2014) Morphological, biophysical and synaptic properties of glutamatergic neurons of the mouse spinal dorsal horn. *J Physiol* 592:759–776.
- Ran C, Chen X (2019) Probing the coding logic of thermosensation using spinal cord calcium imaging. *Exp Neurol* 318:42–49.
- Ran C, Hoon MA, Chen X (2016) The coding of cutaneous temperature in the spinal cord. *Nat Neurosci* 19:1201–1209.
- Ran C, Kamalani GNA, Chen X (2021) Modality specific modulation of temperature representations in the spinal cord after injury. *J Neurosci* 41:8210–8219.
- Sathyamurthy A, Johnson KR, Matson KJE, Dobrott CI, Li L, Ryba AR, Bergman TB, Kelly MC, Kelley MW, Levine AJ (2018) Massively parallel single nucleus transcriptional profiling defines spinal cord neurons and their activity during behavior. *Cell Rep* 22:2216–2225.
- Sekiguchi KJ, Shekhtmeyster P, Merten K, Arena A, Cook D, Hoffman E, Ngo A, Nimmerjahn A (2016) Imaging large-scale cellular activity in spinal cord of freely behaving mice. *Nat Commun* 7:11450.
- Seung HS, Sömböl U (2014) Neuronal cell types and connectivity: lessons from the retina. *Neuron* 83:1262–1272.
- Spike RC, Puskár Z, Andrew D, Todd AJ (2003) A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur J Neurosci* 18:2433–2448.
- Sullivan SJ, Farrant M, Cull-Candy SG (2017) TARP γ -2 is required for inflammation-associated AMPA receptor plasticity within lamina II of the spinal cord dorsal horn. *J Neurosci* 37:6007–6020.
- Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* 11:823–836.
- Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J Comp Neurol* 296:496–505.
- Torebjörk HE, Lundberg LE, LaMotte RH (1992) Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol* 448:765–780.
- Torsney C, MacDermott AB (2006) Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord. *J Neurosci* 26:1833–1843.
- Wei Z, Lin BJ, Chen TW, Daie K, Svoboda K, Druckmann S (2020) A comparison of neuronal population dynamics measured with calcium imaging and electrophysiology. *PLoS Comput Biol* 16:e1008198.
- Xu Z, Agbigbe O, Nigro N, Yakobi G, Shapiro J, Ginosar Y (2021) Use of high-resolution thermography as a validation measure to confirm epidural anesthesia in mice: a cross-over study. *Int J Obstet Anesth* 46:102981.
- Yasaka T, Tiong SYX, Hughes DI, Riddell JS, Todd AJ (2010) Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* 151:475–488.
- Zeilhofer HU, Wildner H, Yévenes GE (2012) Fast synaptic inhibition in spinal sensory processing and pain control. *Physiol Rev* 92:193–235.
- Zhang Y, Liu S, Zhang YQ, Goulding M, Wang YQ, Ma Q (2018) Timing mechanisms underlying gate control by feedforward inhibition. *Neuron* 99:941–955.e4.