Title: Kappa opioids inhibit the GABA/glycine terminals of rostral ventromedial medulla projections in the superficial dorsal horn of the spinal cord

Running Title: Opioid inhibition of the RVM to SDH synapse

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Abstract:

Background and Purpose: Descending projections from neurons in the rostral ventromedial medulla (RVM) make synapses within the superficial dorsal horn of the spinal cord that are involved in acute nociception and the development of chronic pain and itch. In addition, this projection plays an important role in mediating the analgesic effects of opioids. However, our knowledge about the spinal synaptic targets of RVM projections and their modulation by opioids is lacking.

Experimental Approach: We used *ex vivo* optogenetic stimulation of RVM descending fibres and whole-cell patch-clamp recordings from superficial dorsal horn (SDH) neurons to identify the target neurons and to investigate their descending synaptic inputs.

Key Results: We demonstrate that SDH neurons are targeted by descending GABA/glycine inhibitory inputs from the RVM, although glycinergic inputs predominate. These SDH neurons had diverse morphological and electrical properties. This inhibitory synapse was presynaptically suppressed by the kappa opioid receptor agonist U69593. By contrast, the mu-opioid receptor agonist DAMGO inhibited only a subset of RVM-SDH synapses, acting both pre- and postsynaptically, while the delta-opioid receptor agonist deltorphin II had little effect.

Conclusion and Implications: Developing reliable and effective alternatives to opioid analgesics requires a detailed, mechanistic understanding of how opioids interact with nociceptive circuits. This study selectively and systematically characterises the synaptic connections between RVM projection neurons and their SDH targets to advance our knowledge of how this descending projection is organised and modulated. In addition, it improves our understanding of how opioids alter spinal pathways involved in the sensations of pain and itch.

Key Words: raphe-spinal tract; descending pain pathway; supraspinal; substantia gelatinosa; opioid; inhibitory neurotransmission

Introduction:

Incoming noxious signals received by the superficial dorsal horn (SDH) are strongly modified by descending fibres that originate in higher brain regions including the brainstem and the cerebral cortex (Millan, 2002). The brainstem rostral ventromedial medulla (RVM), which includes the raphe magnus and gigantocellular reticular nucleus alpha, forms the final common part of descending pathways from these higher centres and have a key role in modulating pain transmission at the spinal level (Basbaum & Fields, 1984; Gautier, Geny, Bourgoin, Bernard & Hamon, 2017; Heinricher, Tavares, Leith & Lumb, 2009; Lau & Vaughan, 2014; Zhuo & Gebhart, 1990).

Anatomical studies have shown that RVM projections to the dorsal horn of the spinal cord release either serotonin or the inhibitory neurotransmitters GABA and/or glycine (Hossaini, Goos, Kohli & Holstege, 2012; Marinelli, Vaughan, Schnell, Wessendorf & Christie, 2002; Pedersen, Vaughan & Christie, 2011; Zhang et al., 2015). Electron microscopy of RVM projection terminals in the SDH suggests that the majority of these terminals release GABA, with some also releasing glycine (Aicher, Hermes, Whittier & Hegarty, 2012; Antal, Petko, Polgar, Heizmann & Storm-Mathisen, 1996; Light & Kavookjian, 1985) and these findings have been supported by *in vivo* electrophysiological studies (Kato et al., 2006). More recent behavioural studies using opto- or chemogenetics to selectively engage inhibitory descending populations, confirms that they modulate nocifensive responses (Cai, Wang, Hou & Pan, 2014; Francois et al., 2017; Zhang et al., 2015). As a result, GABA is considered to be the major neurotransmitter released by RVM projections into the SDH and this inhibitory input alters spinal nociception.

Immunohistochemical studies show that GABAergic, parvalbumin-positive, and non-GABAergic, calbindin-positive (presumably glutamatergic) SDH neurons receive inputs from the RVM (Aicher, Hermes, Whittier & Hegarty, 2012; Antal, Petko, Polgar, Heizmann & Storm-Mathisen, 1996). These SDH target neurons appear to consist of several different morphological types (Kato et al., 2006; Light & Kavookjian, 1985). Furthermore, different populations of inhibitory RVM projections make axo-axonic contacts with primary sensory afferents (Zhang et al., 2015), or preproenkephalin-positive-inhibitory interneurons (Francois et al., 2017).Thus, there

is little consistent data about the SDH neurons targeted by descending inhibitory RVM projections.

Despite their many drawbacks, opioids are still the gold standard for treating pain. Spinal and descending circuits contribute to the analgesic actions of opioids (Corder, Castro, Bruchas & Scherrer, 2018; Zorman, Belcher, Adams & Fields, 1982), and intrathecal injection of mu (μ), kappa (κ) and delta (δ) opioid receptor agonists are typically analgesic (Bailey et al., 1993; Borgbjerg, Frigast, Madsen & Mikkelsen, 1996; Goodchild & Gent, 1992; Morgan, Heinricher & Fields, 1992; Schmauss, 1987), although the data for κ-agonists is controversial (Danzebrink, Green Sa Fau - Gebhart & Gebhart, 1995; Kim, Seol, Lee, Yaksh & Jun, 2011). However, no study has directly determined if the RVM-SDH synapse plays a role in opioid-mediated spinal analgesia. It is well known that opioids inhibit excitatory synaptic currents (EPSCs) within the SDH, particularly from primary sensory afferents (Gerhold, Drdla-Schutting, Honsek, Forsthuber & Sandkuhler, 2015; Glaum, Miller & Hammond, 1994; Hori, Endo & Takahashi, 1992; Kim, Shim, Kim & Kim, 2018; Kohno, Kumamoto, Higashi, Shimoji & Yoshimura, 1999; Randic, Cheng & Kojic, 1995; Snyder et al., 2018). Conversely, μ-opioid modulation of inhibitory synaptic currents (IPSCs) has not been consistently observed, and κ- and δ-opioid agonists appear to have no effect (Grudt & Henderson, 1998; Kerchner & Zhuo, 2002; Kohno, Kumamoto, Higashi, Shimoji & Yoshimura, 1999). These findings suggest that opioids appear to target excitatory transmission to a greater extent than inhibitory transmission within the SDH.

Here, we use optogenetics in combination with *ex vivo* electrophysiology to selectively stimulate RVM projections and investigate their synaptic properties and targets within the SDH. Then, to better understand how opioids modulate this descending input, we determined the effects of μ-, κ- and δ-opioid receptor agonists on these inhibitory synaptic inputs from the RVM.

Methods:

Experiments were carried out on male and female Sprague Dawley rats in accordance with guidelines set by the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes. All experiments were approved by the Northern Local Health District Animal Ethics Committee (Approval no. RESP18-208). Pregnant female rats were obtained from the Animal Resources Centre (Canning Vale, Australia) and were housed in the Kolling Institute Facility. After weaning, animals of the same gender were housed in groups of two to three in individually ventilated cages under controlled light (12-hr light–dark cycles) and temperature (23 ± 1°C, 70% humidity) with ad libitum access to water and food pellets. Cages were enriched with a house igloo, tissues for nesting, and straws or paddle pop sticks on alternate weeks. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology.*

Stereotaxic injection

Stereotaxic injections were performed on 3-4 weeks old rats weighting 40-90 g under 1.5 - 3 % isoflurane anesthesia using a stereotactic apparatus (Kopf Instruments, Tujunga, CA). An AAV8.Syn.ChR2(H134R).GFP (3.3×10^{13} genomic copies/ml, a kind gift from Edward Boyden; Addgene #58880) or AAV5.hSyn.hChR2(H134R).mCherry.WPRE.pA (5.8×10^{12} genomic copies/ml, a kind gift from Karl Deisseroth; UNC vectorcore av4320d) was injected through a glass capillary pipette (40-70 µm diameter) in the rostral ventromedial medulla (RVM) at an angle of 10^{o} posterior to the target area to avoid the transverse venous sinus. The following coordinates were used: Bregma ML: 0.0 mm; AP, -8.3-10.1 mm; DV, -7.9-9.7 mm, scaled by the ratio of the measured bregma – lambda distance (6.8 - 8.3 mm) to the adult distance of 9 mm. Injections were delivered at a rate of 15 nl min^{-1} for a total volume of around 120 nL (Nanoject II, Drummond) and following the injection the pipette was left in place for 5 minutes, then slightly lifted (50 µm) and held in place for additional 10 min before complete removal. Experiments were performed 7-10 weeks after viral injection.

Electrophysiology

Rats were deeply anesthetised with isoflurane (3%, assessed by rate of breathing, lack of righting reflexes, and lack of response to paw squeeze) and transcardially perfused with ice-cold N-methyl-D-glucamine solution (NMDG) containing (in mM): 93 NMDG, 30 $NaHCO_3$, 25 glucose, 2 thiourea, 3 Na-pyruvate, 2.5 KCl, 1.2NaH_2PO_4, 20 HEPES, 10 $MgSO_4$, 0.5 $CaCl_2$, 5 sodium ascorbate, 2 kynuenic acid (~300 mOsm), equilibrated with 95% O_2-5% CO_2. Parasagittal spinal cord slices (280mm) of the lumbar enlargement were prepared with a vibratome (Leica VT1200S) in the same ice-cold NMDG solution and then maintained in this solution for 10 minutes at 34°C. After, they were transferred into artificial cerebrospinal fluid (ACSF) (in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$, equilibrated with 95% O_2 and 5% CO_2) and kept at room temperature (rt) until use. For recording, slices were individually transferred to a chamber on an upright fluorescence microscope (Olympus BX51) and superfused continuously with ACSF (33°C, flow rate 2.5 ml·min^{-1}). Spinal cord neurons were visualised with a 40X water-immersion objective using Dodt gradient contrast optics. Whole-cell patch-clamp recordings from spinal cord neurons were performed in current-clamp or voltage-clamp configuration. Patch pipettes (3-5 MΩ) were filled with an intracellular solution composed of (mM): 136 K-gluconate, 4 NaCl, 5 EGTA, 0.5 $CaCl_2$, 10 HEPES, 5 Na-phosphocreatine, 5 MgATP, 0.3 NaGTP, and 0.1% biocytin, pH 7.3 with KOH (290–295 mOsm). The chloride reversal potential using these solutions is ~-87 mV. Series resistance (< 25 MΩ) was compensated by 65% and continuously monitored during experiments. The liquid junction potential was not corrected. A brief blue laser light (0.5-1 ms, 473 nm; OptoDuet Laser, IkeCool, Los Angeles, CA) was delivered at 20-30 s intervals through the objective lens (x40) of the microscope to illuminate the superficial laminae of the dorsal horn. Inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated in the presence of the AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) or 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 5 μM) and the NMDA receptor antagonist, d-2-Amino-5-phosphonopentanoic acid (D-AP5, 25 μM).
The $GABA_A$ receptor antagonist, SR95531 (3 μM) and the glycine receptor antagonist, strychnine (0.5 μM) were added to isolate the glycine- and $GABA_A$-receptors mediate currents. All recordings were filtered (4-10 kHz low pass filter)

with Multiclamp 700B amplifier (Molecular Device) and digitised at a sampling rate of 10 kHz with an A/D converter (NI USB-6251, National Instruments), and stored using a data acquisition program (AxographX, Axograph Scientific Software). Off-line analysis was performed using Clampfit10 (Molecular Device) and Igor Pro 6 (WaveMetrics).

Immunohistochemistry and confocal imaging

Rats were deeply anesthetized by isoflurane or intraperitoneal injection of pentobarbital sodium 80 mg.kg−1) and perfused transcardially with NMDG solution or 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS). Brains and spinal cords were removed and post-fixed in 4% PFA for 48 hours at 4°C. Coronal sections of the brainstem (40-80 µm thick) and parasagittal sections of the lumbar spinal cord (40 - 280 µm thick) were cut with a vibratome (Leica VT100S). Free-floating sections were rinsed in PBS, incubated in 50 % ethanol for 30 min, and then for 3h in PBS supplemented with 0.3% Triton X-100 and 10% horse serum. They were then incubated for 48h at 4°C with goat polyclonal CGRP antibody (1:1000, Abcam), biotinylated lection IB4 (1:500, Invitrogen) and chicken polyclonal GFP antibody (1:1000, Aves lab) diluted in PBS supplemented with 0.1% Triton X-100 and 10% horse serum. They were then rinsed in PBS and incubated for 3h at rt with donkey anti-goat Alexa-568 (1:1000, Abcam), streptavidin Alexa-647 (1:1000, Abcam) and donkey anti-chicken Alexa-488 (1:1000, Jackson Immuno Res). To visualise biocytin-filled cells following electrophysiological recording, spinal cord slices were fixed in 10 % formalin solution (Sigma) and incubated with streptavidin Alexa-647 (1:1000, Abcam). Sections and slices were rinsed in PB, mounted with Mowiol/Dabco (25 mg.ml^{-1}) and stored at 4°C. All sections were imaged in a Leica TCS SP5 confocal microscope using a 20X (NA 0.7) or a 40X (NA 1.25) objective and Ar/Kr laser set at 488, 561 and 633 nm for excitation of Alexa-488, Alexa-568 and Alexa-647, respectively. Stacks of optical sections were acquired at a pixel resolution of 0.12 µm and a z-step ~1 µm, and images were processed using ImageJ software (NIH). SDH cell morphology was classified based on previously described criteria (Yasaka, Tiong, Hughes, Riddell & Todd, 2010). Briefly, islet cells have an extensive rostrocaudal (RC) dendritic tree spanning > 400 mm and small dorsoventral (DV) dendritic trees. Central cells have a similar form, but their RC dendritic trees are shorter. Radial cells have similar length

dendrites in RC and DV directions and the largest soma to dorsal (SD) dendritic tree length. Vertical cells have dominant ventral dendrites and short dorsal dendrites, resulting in a distinct SV/SD ratio (Table 1).

Experimental design and data analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Given an expected opioid receptor effect size of approximately 1.5 (Kohno, Kumamoto, Higashi, Shimoji & Yoshimura, 1999), a minimum of eight successful recordings from individual neurons (for two-tailed tests, paired t tests with α and β errors of .05) are required. In addition, our pilot data and previous data using electrophysiological recording from spinal slices from our laboratory (Winters, Jeong & Vaughan, 2020) found that slice viability was higher in tissue from adult animals (>P28). This impacted the ability to obtain whole cell recordings with giga-ohm seals and electrical cells parameters that were stable for the duration of experiments. We are able to obtain an average of 1.5 successful neuron recordings per animal. In longer experiments investigating opiate receptor agonists effects (Figures 5 & 6) this was reduced and in the majority of these experiments data was only obtained in one slice per animal (> 80 %). Depending on neuron viability, between 1 - 3 different drugs were tested in each neuron. The experimenter randomly rotated the order of drug application between different recordings to reduce potential bias resulting from the order of drug testing. This also aided in spreading specific drug experiments across different animals. Both male and female animals were used in all experimental groups and additional animals were added to several experiments where the analysis was based on neuronal type, as this could only be determined following post-hoc visualisation. The researchers were not blinded to the drug during electrophysiology experiments or to spinal cord neuron type during analysis.

Neuron electrical properties: resting membrane potential (RMP) was monitored in the current-clamp configuration (I=0 mode) immediately after entering whole-cell mode. Action potential (AP) discharge patterns were obtained by injecting 20 pA-step currents of 1 s duration ranging from -100 pA to 300 pA at 5 s interval. Membrane resistance (Rm) was calculated from responses in the linear region of current-voltage relationship around RMP. AP discharge patterns were classified based on

previously described criteria (Abraira et al., 2017; Grudt & Perl, 2002; Santos, Melnick & Safronov, 2004). Briefly, Delayed-firing (DF) neurons showed a ramped and delayed potential preceding AP discharge: Tonic-firing (TF) neurons showed a continuous firing pattern; Phasic-firing (PF) neurons showed similar characteristics to TF neurons, except they displayed burst firing (≥ 2 AP, 30-150Hz) at rheobase potential; Adapted-firing (AF) neurons showed several APs during the early period in the depolarisation step. Gap-firing (GF) neurons showed a clear gap between bursts of APs. When possible, the classification of AP discharge was determined within 5 min after whole-cell mode since changes in intracellular Ca2+ concentration can affect after hyperpolarizations controlling AP frequency and adaptation (Prescott & De Koninck, 2002). In fact, the RMP became more hyperpolarised, Rm decreased, and APs discharge changed >10 min after whole-cell mode in some cells. In 9/25 neurons that initially had a TF pattern, changed to AF after >10 mins whereas the remaining 16/25 neurons maintained their TF discharge pattern. In contrast, the firing patterns of PF (7/7) and DF (17/18) neurons remained relatively stable over the recording period. As we could not monitor the AP discharge in the early period after initiation of whole-cell mode of every cell, we grouped TF and AF neurons together as non-DF neurons.

oIPSCs and drug applications: Patch clamped SDH neurons were monitored in voltage clamp mode at a membrane potential of -50 mV. If a current of more than 10 pA was detected in response to a brief pulse of blue light, the laser power or duration (max 1 ms) was increased until a 50 –1200 pA oIPSC current was stimulated (291 ± 224 pA, n = 146). After the baseline stabalised, (> 5 mins) experiments to investigate drug effects were commenced. Drugs were applied 5–10 min and a minimum of 6 traces (> 2 min period) were averaged to measure the amplitude, decay time constant and PPR of oIPSCs in the absence or presence of drugs. Drug effects on oIPSCs were assessed at fixed time points: over the last 2 min prior to drug application, during drug application and then during washout or antagonist application.

The glycinergic contribution to oIPSCs was measured as the amplitude of the oIPSC remaining following application of SR95531, or as the decrease in oIPSC amplitude after strychnine application.

The % effects of opioid agonists on oIPSC parameters were calculated as the value measured during opioid agonist application, divided by the average of the pre-

agonist and antagonist values. Thus, in cells where sequential opioid agonist/antagonist combination were tested the pre-agonist value were measured in solution with other opioid related agonists and their specific antagonists present. Cells inhibited by opioid agonist were define as those with a significant decrease in the oIPSC after the agonist was applied (Mann Whitney test) and which partially or fully recovered in the presence of its specific antagonist. Cells where no significant decrease was detected were defined as non-inhibited. The order in which opioid agonists were applied was sequentially rotated between neuron recordings for each experiment to avoid bias.

Decay time constants were measured by fitting a bi-exponential function to the averaged oIPSC, and a weighted decay time constant was calculated using the function in pClamp 10.

Paired photostimulation (50 ms interval) was used to calculate the paired-pulse ratio (PPR), the ratio of the second oIPSC peak amplitude over the first peak amplitude. We verified that PPR changes reflect pre- and post- synaptic drug effects at this synapse by measuring the PPR effects of antagonists of postsynaptic $GABA_A$ or glycine receptors, or the agonist of presynaptically expressed $GABA_B$ receptors (Yang, Ma, Feng, Dong & Li, 2002) (Supp. Fig. 2E-G).

Holding currents: the holding current was measured by averaging the entire recording periods (5-20 s) outside of each photostimulation episode. Postsynaptic currents in response to opioid agonists application were filtered offline (50 Hz low-pass filter) for analysis. A neuron was considered to have a holding current response to an agonist if it produced an outward current of >6 pA during continuous agonist superfusion that reversed upon application of the appropriate antagonist (Pedersen, Vaughan & Christie, 2011).

Statistics: All data are expressed as means ± SD (error bars). Statistical analysis was undertaken only for studies where each group size ≥5 with IBM SPSS statistics version 26. Normality was assessed with Shapiro-Wilk test and no significant variance inhomogeneity was confirmed with Levene test or Mauchly's test. We used parametric statistics and post hoc tests (two tailed Student's t-test, one-way ANOVA with Tukey or repeated measures ANOVA with Bonferroni) if the data distribution was normal and variability homogenous. Post hoc tests were conducted only if F achieved $P < 0.05$. In cases where these requirements were not met, non-parametric statistics and post hoc tests were used (Friedman test with Dunn-

Bonferroni, Kruskal-Wallis test with Dunn-Bonferroni, Wilcoxon signed-rank tests and Mann-Whitney test). Differences were considered significant when $P < 0.05$.

Drugs and reagents

All reagents were obtained from Sigma-Aldrich, Abcam, and Tocris Bioscience. Opioid related drugs were as follows; [5-Methionine] Enkephalin (10 µM; sigma), [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO, 1 µM; abcam) and antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP, 1 µM; abcam), U69593 (0.3 µM; abcam) and antagonist nor-binaltorphimine (nor-BNI, 0.3 µM; abcam), [D-Ala2]-deltrophin II (0.3 µM; abcam) and antagonist ICI174864 (1 µM; Tocris). Stock solutions of neurochemicals were made in distilled water, or DMSO, aliquoted, and then frozen. During recordings, stock drugs were diluted to working concentrations in ACSF (≤1:3,000 drug solvent: ACSF) immediately before use and applied by bath superfusion.

Results:

RVM projections to the spinal cord dorsal horn terminate primarily in LI-LIIo and are inhibitory.

To examine synaptic transmission from the rostral ventromedial medulla (RVM) onto neurons in the superficial dorsal horn (SDH), we injected an adeno-associated viral vector (AAV) encoding channelrhodopsin-2 (ChR2) fused to green fluorescence protein (GFP) into the RVM (Figure 1C). ChR2/GFP positive (ChR2+/GFP+) fibers were detected in superficial and deep laminae of the spinal dorsal horn (Fig. 1A). In the SDH, the fibers were predominantly present in lamina I (LI) and outer lamina II (LIIo) as identified by calcitonin gene related peptide (CGRP) expression, but less so in inner lamina II (LIIi) as identified by isolectin B4 (IB4) expression (Fig. 1A, B; n = 7). This innervation pattern is consistent with previous reports (Antal, Petko, Polgar, Heizmann & Storm-Mathisen, 1996).

In parasagittal spinal cord slices, SDH neurons were patch clamped in whole-cell voltage clamp mode at a holding potential of -50 mV (Cl^- equilibrium potential = -87 mV) and synaptic currents were monitored when $ChR2^+/GFP^+$ fibers were excited with blue light. Optical stimulation evoked outward currents (Fig 2A), and unlike spontaneous inward synaptic currents, the optically evoked postsynaptic currents were unaffected by the AMPA and NMDA receptors antagonists NBQX and APV (Fig. 2A, B, n = 10). However, they were blocked by co-application of the $GABA_A$ and glycine receptor antagonists SR95531 and strychnine, demonstrating they are inhibitory postsynaptic currents (oIPSCs; Fig. 2A, B, n = 10). Further, oIPSCs were abolished by the voltage gated sodium channel blocker TTX and recovered by addition of the potassium channel blocker 4-AP (Supp. Fig. 1). Together, these observations indicate SDH neurons receive an inhibitory, GABAergic and glycinergic, monosynaptic connection from RVM projection fibers.

Properties of SDH neurons targeted by the inhibitory RVM input

SDH neurons have been classified by their electrophysiological and morphological properties in rodents (Grudt & Perl, 2002; Yasaka, Tiong, Hughes, Riddell & Todd, 2010) but see (Browne et al., 2020). To characterise SDH neurons receiving oIPSCs (n = 77), we analysed their AP firing pattern in response to depolarising currents,

membrane properties (input resistance (Rm) and resting membrane potential (RMP)), plus their morphological features (post-hoc biocytin staining). SDH neurons which received inhibitory RVM inputs were primarily delayed- (DF; 44 %) and tonic-firing (TF; 38 %) neurons, although phasic- (PF), adapted- (AF) and gap- (GF) action potential firing patterns were also observed (Fig. 2C, D). Membrane properties were similar amongst these cell types, except for PF neurons which had a more depolarised RMP (Fig. 2E).

Morphological analysis, based on 4 defined dendritic tree shapes (islet, vertical, radial and central) (Browne et al., 2020; Grudt & Perl, 2002; Yasaka, Tiong, Hughes, Riddell & Todd, 2010) was conducted in 42 SDH target neurons. Two-thirds of SDH target neurons were classified into one of four defined types (n = 28, Table 1 & Fig. 3A-J) and the remaining one-third were unclassifiable (n = 14; Fig. 3K-M). The morphologies of the two major postsynaptic target cells of RVM projections, the DF and TF neurons, were investigated in more depth. All morphological types were found in the TF neuronal population (Figure 3). DF neurons had all morphologies except islet cells and there were more radial DF- than TF- neurons. However, no significant difference in morphological properties between TF and DF neurons were detected.

Optically evoked IPSCs have both GABAergic and glycinergic components

Stimulation of the RVM projection can induce GABAergic and/or glycinergic responses in SDH neurons (Hossaini, Goos, Kohli & Holstege, 2012; Kato et al., 2006). We assessed the relative contribution of GABA and glycine to oIPSCs by sequentially applying strychnine and SR95531. Each antagonist reduced the amplitude (Fig. 2B,4A) and changed the decay kinetics of oIPSCs (Fig. 4A,B). The decay kinetics of the SR95531-insensitive glycinergic oIPSCs were faster than strychnine-insensitive GABAergic oIPSCs (Fig.4B), consistent with previous reports (Anderson, Graham, Beveridge, Tooney, Brichta & Callister, 2009; Labrakakis, Rudolph & De Koninck, 2014). Moreover, we found that oIPSCs with faster decay kinetics had a larger glycinergic component (Fig. 4C). The decay kinetics of oIPSCs in DF neurons were consistently faster compared to other neuron types (Non-DF neurons = TF+ PF+ AF + GF neurons; Fig. 4D-E). Furthermore, the glycine

component of oIPSCs was greater in DF versus non-DF cells (Figure 4C; glycinergic component of DF cells (green circles) = 69.1 ± 23 %, n = 14 vs non-DF (orange circles) = 50.6 ± 20 %, n = 21, $p < 0.05$).

Presynaptic modulation of RVM-SDH inhibitory transmission by opioid agonists

While there is much evidence that opioids modulate spinal nociceptive circuits by modifying excitatory neurotransmission (Hori, Endo & Takahashi, 1992; Kohno, Moore, Baba & Woolf, 2003; Kumamoto, Mizuta & Fujita, 2011; Yaksh, 1987), less is known about their actions on inhibitory transmission. We therefore compared the effect of μ-, κ- and δ-opioid receptor agonists on oIPSCs. It was observed that oIPSCs in most SDH neurons were inhibited by the κ-agonist U69593, some were inhibited by the μ-agonist DAMGO, and none were altered by the δ-agonist deltorphin II (Figure 5AB, E, n = 34/41, 17/38, 1/20). The effects of U69593 and DAMGO were selectively reversed by the μ-, κ- receptor antagonists CTAP and nor-BNI, respectively, while the δ-antagonist ICI174864 had no effect (Figure 5A-C,D).

The effect of opioid receptor agonists on the short-term plasticity characteristics of the RVM-SDH synapse was assessed by measuring the paired-pulse ratio (PPR) before and after drug application and during drug reversal with receptor specific antagonist (Figure 5A-C,F). Responses to U69593 and DAMGO were accompanied by a change in PPR (Figure 5F,G, Supp. Fig. 2A-D), indicating κ- and μ- receptor agonists have a pre-synaptic mode of action (Xu-Friedman & Regehr, 2004). On the other hand, the PPR did not change in the presence of deltorphin II.

We also compared the effects of opioid receptor agonists at DF and non-DF neurons. oIPSCs were inhibited in a greater portion of DF-neurons by U69593 compared to DAMGO (81 %, n = 13/16 for U69593; 23 %, n = 3/13 for DAMGO, $p < 0.05$ Fischer's exact test; Fig 5DE and Supp. Fig. 2AC). By contrast, U69593 and DAMGO inhibited oIPSCs in similar proportions of non-DF neurons (84 %, n = 21/25 for U69593; 56 %, n = 14/25 for DAMGO, $p = 0.06$ Fischer's exact test; Fig 5DE and Supp. Fig. 2BD).

A subset of target SDH neurons have opioid induced postsynaptic responses.

Under our recording conditions, DAMGO, deltorphin-II and U69593 induced outward currents in some SDH target neurons (Figure 5A-C and 6). These outward currents, likely opioid-induced potassium channel currents, were reversed by CTAP, ICI174864 and nor-BNI, respectively (Fig. 5A,B,C & 6 (Eckert & Light, 2002; Kim, Shim, Kim & Kim, 2018; Santos, Melnick & Safronov, 2004; Wang et al., 2018)). Postsynaptic currents in response to DAMGO (33/56 neurons), and deltorphin II (10/26) were commonly detected, while U69593 stimulated currents were measured in a minority of SDH neurons (9/49 neurons; Fig 6A-D). When we assessed the postsynaptic opioid effects at DF and non-DF subgroups of neurons, we found that DAMGO induced larger and more frequent currents in non-DF neurons than DF neurons (Figure 6C-D). Similar holding current results were observed when the endogenous μ/δ- opioid receptor agonist, met-enkephalin was applied (DF: 5.3 ± 1 pA, n = 2; non-DF: 21.0 ± 13 pA, n = 4, not shown).

Recently, a small population of excitatory lamina II SDH neurons (10%) have been shown to co-express μ- and δ -opioid receptors (Wang et al., 2018). We analysed 20 neurons that had both deltorphin II and DAMGO applied. Postsynaptic DAMGO responses were measure in the majority of SDH target cells that responded to deltorphin II (6/7 neurons; Figure 6FG).

As the function of the descending pain pathway and response to opioids have significant sex differences (Custodio-Patsey, Donahue, Fu, Lambert, Smith & Taylor, 2020; Loyd & Murphy, 2014; Mogil, 2020; Yu et al., 2021) we compared the data derived from male and female spinal slices. The opioid effects we described were similar in spinal tissue from female and male rats (Supp. Fig. 3).

Discussion:

While descending inputs into the spinal cord are important modulators of spinal nociceptive processing, little is known about the spinal neurons they target and their modulation by analgesic drugs (Lau & Vaughan, 2014; Ossipov, Morimura & Porreca, 2014; WeiWei, WenDi, Mengru, Tuo & Chen, 2021). Data addressing these questions are incomplete, mainly because the roles and organisation of both the spinal circuit and the descending inputs, are very complex and involve many different neuronal subtypes (Gautier, Geny, Bourgoin, Bernard & Hamon, 2017; Haring et al., 2018; Todd, 2017). In addition, the tools needed to selectively activate specific inputs are relatively new, and the adult spinal slice preparation is notoriously tricky to work with.

We have characterised the spinal synapse of a key descending pain modulatory pathway, between RVM projection neurons and those in the superficial dorsal horn. First, we have used optogenetics to show that RVM-SDH synapses are inhibitory and involve both of the neurotransmitters, GABA and glycine. Then, we demonstrate that SDH target neurons have diverse electrical and morphologies properties, and are therefore a likely a highly heterogeneous population of interneurons. Next, we define a subgroup of DF neurons that receive oIPSC with a dominant glycinergic phenotype and finally, we unexpectedly found that the RVM-SDH synaptic transmission is presynaptically inhibited by κ– opioid agonists and, in a subpopulation of primarily non DF-neurons, also modulated by μ-opioid receptor agonists. Together, these data improve our understanding of the SDH targets of descending RVM inputs and demonstrates that endogenous κ-opioid agonists are likely to play a critical role in modulating this synapse's strength.

SDH neurons targeted by the RVM.

SDH neuronal populations have been differentiated by their electrical, morphological, and neurochemical characteristics (Light & Kavookjian, 1985; Yasaka, Tiong, Hughes, Riddell & Todd, 2010). Recently, attempts to integrate these findings using non-biased characterisations of dorsal horn neurons have shown that none of the currently employed methods can be reliably linked to function (Browne et al., 2020; Merighi, 2018; Punnakkal, von Schoultz, Haenraets, Wildner & Zeilhofer, 2014; Smith et al.,

2015; Todd, 2017). Despite this, there is a reasonable tendency for TF neurons, and neurons with an islet morphology, to be GABAergic and many DF neurons, especially large ones with a vertical neurite morphology, are glutamatergic (Browne et al., 2020; Merighi, 2018; Punnakkal, von Schoultz, Haenraets, Wildner & Zeilhofer, 2014; Yasaka, Tiong, Hughes, Riddell & Todd, 2010). However, it is unlikely that all TF neurons are inhibitory, or that all DF neurons are excitatory. The SDH target neurons we sampled had a variety of firing patterns (DF>TF>PF>>AF>GF) and morphologies (non-classifiable≈vertical>radial≈ central>islet) that indicating that they are made up of both inhibitory and excitatory interneurons. This finding is consistent with previous anatomical work (Aicher, Hermes, Whittier & Hegarty, 2012; Antal, Petko, Polgar, Heizmann & Storm-Mathisen, 1996) and suggests that the outcome of activation of inhibitory RVM inputs will depend on the function of their SDH target and role its in the nociceptive circuit.

The role of inhibitory RVM inputs into the SDH

RVM inputs to the dorsal horn of the spinal cord have long been known to both facilitate and inhibit spinal nociceptive transmission (Zhou & Gebhart, 1990; Zhuo, 2017). Optogenetic activation and inhibition of the GABAergic inhibitory RVM input into the dorsal horn of the spinal cord has recently been shown to facilitate and reduce withdrawal behaviours in response to mechanical stimulation (Francois et al., 2017), suggesting that bidirectional control can be achieved by altering the activity of inhibitory RVM inputs in the spinal cord. Although this finding was interpreted in the context of these inputs making monosynaptic contacts with spinal tonic-firing-enkephalin^{+}-GABAergic interneurons (Francois et al., 2017), our data highlighting that RVM inputs interact with multiple types of SDH interneurons, including TF neurons, suggests that the behavioural outcome of RVM signalling in the SDH is likely to be even more complex. Previous data suggests that different cohorts of RVM projection neurons will be activated during different behavioural states (Gautier, Geny, Bourgoin, Bernard & Hamon, 2017; Gebhart, 2004; Heinricher, Tavares, Leith & Lumb, 2009). Therefore, it seems likely that the behavioural outcomes of activating descending RVM inputs will depend on the spinal connectivity of the specific RVM cohorts engaged, in concert with the activity of the spinal nociceptive circuit. In a study that used *in vivo* electrophysiology to record GABA- and glycine-mediated IPSCs in the SDH of

anaesthetised rats (Kato et al., 2006), pinch induced action potentials were suppressed when the RVM was stimulated, suggesting that activation of descending inhibitory RVM neurons is anti-nociceptive (eg. analgesic) (Kato et al., 2006). Like this study and that of Francois et. al. (2017), the stimulation of the RVM was not physiological. Thus, the opposite role for inhibitory RVM inputs into the cord is not necessarily inconsistent, but instead very likely reflects different experimental setups interrogating separate parts of a highly complex circuit that can integrate multiple information streams to respond dynamically and appropriately to a threatening situation as highlighter in (Gebhart, 2004). One approach that may lead to a clearer understanding of the role this circuit plays in spinal nociceptive control is to first identifying cohorts of RVM projection neurons that fire in response to a particular noxious modality, and then carry out behavioural experiments and selectively activate using physiological firing patterns (DeNardo & Luo, 2017; Dugue et al., 2014).

Our observation that oIPSCs onto DF neurons have a dominant glycinergic phenotype indicates they are likely to be inhibited over a shorter timecourse and have altered modulatory responses compared to non-DF neurons (Anderson, Graham, Beveridge, Tooney, Brichta & Callister, 2009; Smith et al., 2016). This finding demonstrates that the quality of inhibition at RVM-SDH synapses is also heterogenous and this too may contribute to the role it plays in spinal nociceptive control.

Presynaptic modulation of the RVM-SDH synapse

This is the first study to directly assess opioid modulation of RVM inputs in the SDH. We found that κ -opioid and to a lesser extent μ -opioid agonists presynaptically reduced oIPSCs in the SDH, a surprising result because presynaptic κ-effects have never been reported at any other central synapses in the dorsal horn of the cord (Hori, Endo & Takahashi, 1992; Kohno, Kumamoto, Higashi, Shimoji & Yoshimura, 1999) while a δ-opioid agonist had no effect. Postsynaptic responses to μ, δ, and κ opioid receptor agonists have been recorded from the soma of RVM projections neurons in medulla brain slices (Kiefel, Rossi & Bodnar, 1993; Marinelli, Vaughan, Schnell, Wessendorf & Christie, 2002; Pan, Tershner & Fields, 1997; Pedersen, Vaughan & Christie, 2011; Winkler, Hermes, Chavkin, Drake, Morrison & Aicher, 2006). In addition, μ-opioid receptors are expressed at the soma of most inhibitory

RVM projection neurons (Francois et al., 2017). Therefore, our data suggests that opioid receptors may be differentially expressed in the somatic and terminal compartments of RVM-projection neurons. However, as we employed viral vectors to express channelrhodopsins in RVM projection neurons, alterations in circuit organisation, synaptic transmission properties and axonal morphology due to the use of these technologies must also be considered (Jackman, Beneduce, Drew & Regehr, 2014; Miyashita, Shao, Chung, Pourzia & Feldman, 2013).

Opioids are important modulators of the descending RVM projection and spinal nociceptive circuits (Bagley & Ingram, 2020; Chen & Heinricher, 2019; Trang, Al-Hasani, Salvemini, Salter, Gutstein & Cahill, 2015; Winters, Christie & Vaughan, 2019). Our finding that inhibition of the RVM inhibitory input into the SDH is reliably achieved with kappa receptor agonists, indicates that neurotransmitter release at the RVM-SDH synapses will be directly and consistently achieved by agonists of this opioid receptor. In contrast, μ-agonist effects at this synapse are more complicated and less universal. Given the scarcity of presynaptic kappa effects in the SDH, kappa agonists effects on spinal or supraspinal evoked IPSCs, which are accompanied by a PPR change are suggestive that the SDH neuron being recorded receives descending inputs from the RVM. As the endogenous kappa receptor agonist dynorphin is elevated in the spinal cord when chronic pain develops (Burgess et al., 2002; Luo, Chen, Ossipov, Rankin, Porreca & Lai, 2008; Wang et al., 2001) and dynorphin releasing neurons in the SDH are required for normal itch sensation, (Kardon et al., 2014), the RVM-SDH synapse may be an important regulator of both pain and itch.

This data helps build a comprehensive understanding of how descending inhibition to the SDH contributes to the processing of spinal sensory signals.

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