Distinct brainstem to spinal cord noradrenergic pathways inversely regulate spinal neuronal activity

Mateusz W. Kucharczyk, Francesca Di Domenico and Kirsty Bannister

Central Modulation of Pain, Institute of Psychiatry, Psychology and Neuroscience, King’s College London, London, SE1 1UL, UK

Correspondence to: Mateusz W. Kucharczyk

Institute of Psychiatry, Psychology and Neuroscience, Wolfson CARD, Guy’s Campus, King’s College London, London, London, SE1 1UL. UK

E-mail: mateusz.kucharczyk@kcl.ac.uk

Running title: Noradrenergic modulation of nociception
Abstract

Brainstem to spinal cord noradrenergic pathways include a locus coeruleus origin projection and diffuse noxious inhibitory controls. While both pathways are traditionally viewed as exerting an inhibitory effect on spinal neuronal activity, the locus coeruleus was previously shown to have a facilitatory influence on thermal nociception according to the subpopulation of coerulean neurons activated. Coupled with knowledge of its functional modular organisation and the fact that diffuse noxious inhibitory controls are not expressed in varied animal models of chronicity, we hypothesised a regulatory role for the locus coeruleus on non-coerulean, discrete noradrenergic cell group(s).

We implemented locus coeruleus targeting strategies by microinjecting canine adenovirus encoding for channelrhodopsin-2 under a noradrenaline-specific promoter in the spinal cord (retrogradely labelling a coeruleospinal module) or the locus coeruleus itself (labelling the entire coerulean module). Coeruleospinal module optoactivation abolished diffuse noxious inhibitory controls (Two-Way ANOVA, P<0.0001), which were still expressed following locus coeruleus neuronal ablation.

We propose that the cerulean system interacts with, but does not directly govern, diffuse noxious inhibitory controls. This mechanism may underlie the role of the locus coeruleus as a ‘chronic pain generator’. Pinpointing the functionality of discrete top-down pathways is crucial for understanding sensorimotor modulation in health and disease.

Keywords: noradrenaline; locus coeruleus; descending modulation; diffuse noxious inhibitory controls; spinal wide dynamic range neurons

Abbreviations: AR = adrenoceptor; CAV = canine adenovirus; ChR2 = channelrhodopsin-2; DNIC = diffuse noxious inhibitory controls; DPMS = descending pain modulatory system; DSP4 = coerulean noradrenergic neurotoxin, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride; LC = locus coeruleus; LC:LC = coerulean module; LC:SC = coeruleospinal module; PRS = catecholaminergic-specific synthetic promoter PRSx8; WDR = wide dynamic range
Introduction

The descending pain modulatory system (DPMS) encompasses noradrenergic projections that underpin a tonic pathway from the locus coeruleus (LC) to the dorsal horn of the spinal cord and diffuse noxious inhibitory controls (DNIC). Previously, upon 1) activation of the DNIC pathway or 2) chemogenetic activation of descending noradrenergic controls following spinal microinjection of canine adenovirus (CAV), wide dynamic range (WDR) neuronal activity was inhibited in a manner that was reversed by spinal application of α₂-AR antagonist atipamezole. Meanwhile, channelrhodopsin-2 (ChR2)-mediated activation of the LC following direct LC lentivirus microinjection (thus labelling a LC:LC module) caused inhibition of the spinal reflex only when the optic fibre was placed ventrally, whereupon atipamezole no longer reversed the inhibitory effect. Intriguingly, in the same study, optoactivation of the dorsal LC noradrenergic neuron population had a pain potentiating effect.

Tying the knowledge from these studies together we hypothesised that either a separate coerulean module operates, via α₂-ARs, to mediate DNIC, or that the LC might have a regulatory function on non-coerulean, discrete noradrenergic cell group(s), for example those from where DNIC originates, according to its modular organisation.

Thus, we aimed to dissect the effects of optogenetic activation of selected LC modules on the mechanically evoked activity of spinal WDR neurons and DNIC expression, while investigating the subserving pharmacology. By using CAV-PRS-ChR2 to label an LC:SC or LC:LC circuit, we have shown that 1) the LC-SC circuit operates via spinal α₁-ARs to cause neuronal inhibition and 2) DNIC expression is abolished upon its optogenetic activation. Thus, we propose that LC:SC and DNIC pathways are functionally distinct, which has implications for the pain, as well as broader sensorimotor, field.

Materials and methods

Animals

Male Sprague-Dawley rats were used for experiments. All procedures described were approved by the Home Office and adhered to the Animals (Scientific Procedures) Act 1986, International Association for Study of Pain and ARRIVE ethical guidelines.


All experiments were designed to contain minimum of 6 rats per group, based on G-power predictions from previous experiments. Animals were randomly assigned to experimental groups. From 60 rats designated for this study, 7 failed to provide stable WDR neuronal recordings, 3 rats developed vestibular problems reaching humane endpoint within 4 days after LC virus microinjection, and 1 animal died 24 hours after DSP4 administration. In total 49 rats were used as follows: 24 rats were used for mixed opto-pharmacology experiments (6 rats per group: LC:LC atipamezole, LC:LC prazosin, LC:SC atipamezole, LC:SC prazosin). Additional 3 rats were used for WDR baseline characterisation with optogenetics (2 for LC:SC, 1 for LC:LC) followed by LC optoelectrical recordings of transduced neurons. In the latter no pharmacology was performed. Further 6 rats were used for DSP4 group, and 15 rats were used as naïve controls, which included 6 rats used for lidocaine microinjection experiment.

DSP4 injections

For ablation of the coerulean noradrenergic fibres across the neuroaxis, 50 mg/kg of selective neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) (Sigma, Dorset, UK) was injected intraperitoneally\textsuperscript{14-16}.

Coerulean neuron transduction

To transduce catecholaminergic coerulean neurons, ipsilateral LC stereotaxic injections of CAV carrying channelrhodopsin 2 under the control of catecholamine-specific synthetic promoter (sPRS)\textsuperscript{11} (CAV-sPRS-hChR2(H134R)-mCherry, titer \(>3\times10^{10}\) TU/ml, PVM, Montpellier, a gift from Professor Anthony Pickering, University of Bristol\textsuperscript{5,6}) were made (Kopf Instruments, UK) analogously to described in detail earlier\textsuperscript{5}. To transduce spinally projecting catecholaminergic brainstem neurons, the same virus was injected in the lumbar spinal cord.\textsuperscript{17}

Spinal cord \textit{in vivo} electrophysiology

\textit{In vivo} electrophysiology was performed on animals weighing 240–300 g as previously described\textsuperscript{12} under isoflurane/N\textsubscript{2}O anaesthesia. Physiological homeostasis was maintained and monitored throughout the experiment. Extracellular single-unit activity of spinal WDR neurons in deep laminae IV/V was measured. Natural mechanical stimuli, including brush and von Frey filaments (8 g, 26 g and 60 g) and von Frey
filaments with concurrent ipsilateral noxious ear pinch (to trigger DNIC\(^2\)), were applied in this order to
the receptive field for 10 s per stimulus. DNIC are reflected as the inhibitory effect on WDR neuronal
firing during ear pinch to its immediate respective von Frey filament applied without the conditioning
stimulus (% of inhibition after ear pinch). After collection of predrug baseline control, 100 μg
atipamezole (a α\(_2\)-AR antagonist), or 20 μg prazosin hydrochloride (α\(_1\)-AR antagonist) was administered
topically on the spinal cord. All data plotted represents the time point of peak change (10-30 minutes
post application).

**Optogenetics**

A simultaneous recording and optical stimulation of the transduced LC neurons were made using
microoptrodes as described earlier with minor modifications\(^{13}\) to find optimal stimulus parameters. LC
neurons were identified as described before\(^5\).

The 450 nm laser was used to deliver defined light pulses: 20 ms pulse width at 5 Hz, 30 mW (238
mW/mm\(^2\)) light power density at the tip of the implantable 200 μm fibre cannula\(^5\). Spinal WDR neurons
were characterised by three stable baseline responses followed by three optically modulated responses.
For combined optogenetics and spinal pharmacology, after collecting three stable baseline and three
stable optoactivation responses, a drug was applied topically on the spinal cord surface. At the end of
every experiment, animals were sacrificed by the overdose of isoflurane and transcardially perfused
with saline followed by 4% paraformaldehyde for anatomical evaluation.

**Lidocaine block of LC activity**

Six naïve rats were used for lidocaine (500 nl, 2% in saline) block of neuronal activity in the ipsilateral LC
during electrophysiological WDR neuron recordings. At the end of the experiment the solution in the
pipette was replaced with 0.5% Lucifer Yellow-CH dipotassium salt to mark the injection site.

**Immunohistochemistry**

Cryosected tissue was incubated with primary antibodies against dopamine-β-hydroxylase (DBH, a
marker of noradrenergic neurons: 1:500, Millipore), mCherry (1:500, Abcam) followed by appropriate
fluorophore-conjugated secondary antibodies. DAPI was used as nuclear marker. Samples were imaged
with an LSM 710 laser-scanning confocal microscope (Zeiss) using Zeiss Plan Achromat 10x (0.3 NA) and 20x (0.8 NA) dry objectives and analysed with Fiji Win 64. For quantification, samples were imaged with 20x dry objective on Zeiss Imager Z1 microscope. 6-8 slices were imaged per animal. Cell counting was carried out on the Fiji Win 64 utilising cell counter plugin. On average, 20-30 brainstem sections were imaged for quantification.

Passive Tissue Clearing (PACT)

A passive CLARITY tissue clearing technique (PACT) has been implemented to allow thick (1000-2500 µm) spinal cord fragments imaging. Anti-tyrosine hydroxylase (TH, marker of catecholaminergic neurons; 1:250, Millipore) primary antibody was used followed by Alexa Fluor 647 secondary antibody (1:200, Invitrogen). After achieving equilibrium with refractive index-matching solution (refractive index = 1.47), samples were imaged with a Zeiss LSM 780 confocal upright microscope, equipped with Plan-Neofluar 10x 0.3 NA dry objective and 633 nm laser line. Scans were taken with 2048x2048 pixel resolution, with 4-5 µm optical section typically spanning 400-700 µm of scanned depth (resulting in 100-150 planes) with auto Z-brightness correction. Images were analysed with Zen 2012 Blue Edition software followed by Fiji (ImageJ) equipped with appropriate plugins.

Quantification and Statistical Analysis

Typically, up to 4 WDR neurons were characterised per preparation (n), and data were collected from at least 6 rats per group (N). Single pharmacological investigation was performed on one neuron per animal. Statistical analysis was performed either on n for populational studies, or N for pharmacological studies. Uncorrected two-way repeated-measures (RM) ANOVA with the Tukey post-hoc was used to assess von Frey and DNIC responses in the baseline conditions. For pharmacological experiments, Geisser-Greenhouse correction was used for RM-ANOVA. Paired student t-test was used to assess brush-evoked responses. GraphPad Prism was used to analyse the data. P < 0.05 was considered significant.
Data availability

Data are available upon request.

Results

A ventral coerulean neuronal population inhibits spinal nociceptive processing via $\alpha_1$-adrenoceptors

Hypothesising that the contrasting impact of spinal atipamezole on spinal neuronal activity following activation of a descending noradrenergic control$^{4,5}$ reflected the activation of discrete rather than identical top-down modulatory circuits, we microinjected CAV spinally (thus retrogradely labelling an LC:SC module) or in the LC itself (thus labelling an LC:LC module) to deliver ChR2 under noradrenergic promoter$^{11}$ (Fig. 1a). After confirming ipsilateral ventral LC labelling following spinal CAV injection (12.4±2.4% ventral vs. 4.0±1.5% dorsal noradrenergic LC neurons expressed mCherry) (Fig. 1b, Table S2) as performed in the previous study$^{4,6}$, we demonstrated that optoactivation of both LC:LC and LC:SC modules inhibited mechanically-evoked spinal WDR neuron activity (Fig. 1c, S1), while stimulus intensity coding (8, 26, 60 g von Frey) was maintained (Fig. 1d-j). LC-mediated inhibition of WDR neuronal activity upon stimulation with mechanical modalities was reversed by $\alpha_1$-AR antagonist prazosin (Fig. 1k-l) but enhanced by local application of atipamezole (Fig. 1m-n). Our results suggest that phasic activation of the LC inhibits spinal WDR neuron activity via an $\alpha_1$-AR-mediated mechanism. Unlike $\alpha_2$-ARs, which directly mediate inhibition by coupling with small Gi proteins, $\alpha_1$-ARs couple with facilitatory G-proteins$^{15}$. Therefore, $\alpha_1$-ARs-mediated inhibition of WDR neurons is likely to be mediated indirectly, for example via noradrenergic activation of inhibitory interneurons therein. In fact, activation of $\alpha_1$-ARs expressed on spinal GABAergic interneurons has been reported previously as a plausible mechanism for descending coerulean inhibitory controls$^{16,17}$.

DNIC expression is inhibited by LC:SC module optoactivation

Hypothesising that the impact of atipamezole on WDR activity in the study by Hirschberg and colleagues$^4$ was a result of the malfunction of another, non-coerulean, inhibitory noradrenergic control,
we investigated the impact of LC:LC or LC:SC module opto-activation on the expression of DNIC where DNIC were previously shown to be abolished by spinal atipamezole\(^3\). Interestingly, LC:SC opto-activation abolished DNIC, while LC:LC opto-activation only marginally decreased its potency (Fig. 2a-c). Meanwhile, while prazosin partially restored the LC:LC or LC:SC optoactivation-evoked decrease in DNIC expression (Fig. 2d-g), atipamezole facilitated it (Fig. 2h-k). We propose that, upon opto-activation of the LC:LC module, it is likely that a proportion of dorsal as well as ventral LC neurons are stimulated, causing a decrease in DNIC potency due to communication between the LC and the DNIC origin nucleus. Thus, it is likely that the dorsal LC has either no effect or facilitates DNIC functionality, while the LC:SC direct pathway inhibits WDR activity via spinal α\(_1\)-ARs with simultaneous direct brainstem located inhibition of the DNIC origin nucleus.

Ablation of coerulean noradrenergic fibres does not affect basal spinal convergent neuron activity nor DNIC expression.

To investigate this separation of LC:SC and DNIC pathway functionality further we systemically injected the neurotoxin DSP4 to deplete noradrenergic projections from the LC\(^9,10\) (Fig. 3a). Eighteen to twenty days following treatment, no impact on WDR activity was observed (Fig. 3b-d), and DNIC were expressed (Fig. 3e). Elsewhere, we microinjected lidocaine (sodium channel blocker) to the LC ipsilateral to the recorded WDR neuron (Fig. 3f). This had no effect on WDR evoked activity (Fig. 3g-h), nor on DNIC expression (Fig. 3i-j). These results suggest that tonic activity in the LC a) is not required to maintain DNIC expression and b) does not modulate basal stimulus-evoked firing of spinal WDR neurons in health.

Discussion

Herein we evidence that in health, phasic activity of the LC (upon LC:LC or LC:SC optoactivation) inhibits spinal WDR neurons via an α\(_1\)-AR-mediated mechanism, while discrete LC:SC optoactivation abolishes the expression of diffuse noxious inhibitory controls (DNIC), a brainstem to spinal cord pathway that inhibits spinal WDR neurons via an α\(_2\)-AR-mediated mechanism. Tonic LC activity has no effect on DNIC expression nor on the tonic modulation of WDR neuronal firing rate.

Our study points towards an interaction between, but a functional distinction of, DNIC and LC-spinal cord pathways. Both are encompassed by the descending pain modulatory system (DPMS) whose
output likely represents a conglomerate of operationally unique systems engaged by discrete circuits that are each influenced differentially by sensory drivers. If true, this would have consequences for the way in which targeted pain management is prescribed in chronicity, where we know that DPMS-restorative pharmacotherapies do not alleviate pain in all patients. This ‘one size doesn’t fit all’ phenomenon is unsurprising given the complexity of the circuits therein. For example, the coerulean neuronal population is developmentally diverse, and distinct anatomical projections from within mediate discrete aspects of the sensory and affective experience. The LC’s modular functional organisation thus lends itself to facilitatory as well as inhibitory influences on spinal nociceptive activity, where the underlying mechanism(s) involved will include neuro-immune interactions since superficial dorsal horn astrocytes expressing α1-ARs were shown to be critical analgesic regulators in monoaminergic transmission terms.

In the present study our demonstration that activating the LC:SC pathway in health abolishes DNIC while LC neuronal ablation does not point towards the likelihood of maladaptive communication between LC and DNIC circuits being an underlying mechanism of certain chronic pain phenotypes. DNIC are not expressed in varied animal models of chronic pain and we have previously demonstrated that DNIC is expressed in a disease-stage specific manner in rodent models of osteoarthritis and cancer induced bone pain. Disease-related changes to descending modulatory controls likely impact endogenous inhibitory modulation in the long term. If it were evidenced that a noradrenergic drive from the LC exacerbates pain in early stages of disease, one could envisage that the therapeutic application of pharmacological manipulators of specific spinal adrenoceptors would benefit patients at certain stages of specific diseases (and we include a review of the spinal anatomical distribution of adrenoceptors including consideration of single cell RNAseq data tied to a prediction of the potential mechanisms involved in supplementary figure 2). Indeed, previous research has demonstrated abolished DNIC expression in the late stage of a model of chronic joint inflammatory pain and impaired descending noradrenergic modulation with relation to the LC. This insight, specifically linking stage specific DNIC attenuation to impaired LC functionality, lends weight to the argument that communication between LC and DNIC origin nuclei governs the final output of descending modulatory controls that are subserved by noradrenaline. However, the nature of the influence is unknown, and a future research goal includes employing genetic strategies to determine the nature of the neuronal populations that mediate crosstalk between the LC and DNIC origin nuclei.
Summarising, defining the functional relationship between the LC and DNIC-origin nuclei will allow identification of the underlying circuitry responsible for descending inhibitory controls in health and their perturbation in chronic pain. Do chronic pain inducing diseases lead to altered brainstem nucleus crosstalk and/or spinal pharmacological functionality that is specific in terms of disease type and stage? Revealing novel mechanisms that underlie abnormal nociceptive processing is the key to uncovering analgesic targets. Relevant, the work presented herein has uncovered a mechanism by which the body inhibits pain in an endogenous manner in health. Future studies will endeavour to uncover aspects of the postulated mechanism, whereby inferences regarding adrenoceptor subtype involvement will require full elucidation of their anatomical distribution. The potential clinical relevance is apparent when considering that dysregulation of DNIC in rodent pain models translates to the clinic. Conditioned pain modulation (CPM), the proposed human counterpart of DNIC, is dysfunctional in chronic pain patients\textsuperscript{25,26} and its maladaptive expression is associated with chronicity\textsuperscript{27}; translatable mechanisms between DNIC and CPM have been evidenced\textsuperscript{28}.

Ultimately, the results of our present study could help elucidate the origins of chronic pain where the LC, a complex multi-functional nucleus with a yet-to-be-fully-defined role in pain (especially chronicity), demands further investigation.

Acknowledgements

The authors would like to thank Professor Anthony Pickering for supplying the CAV virus used in this study, and Professor Stephen McMahon for start-up equipment funding.

Funding

This work was funded courtesy of an Academy of Medical Sciences Springboard Grant awarded to KB (RE15263). FDD is funded by a National Centre for the Replacement, Refinement and Reduction of Animals in Research studentship (NC/T002115/1).

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.
References

1. Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci*. 1991;14(5):219-245. doi:10.1146/annurev.ne.14.030191.001251

2. Le Bars D, Dickenson AH, Besson JM. Diffuse noxious inhibitory controls (DNIC). I. Effects on dorsal horn convergent neurones in the rat. *Pain*. 1979;6(3):283-304. doi:10.1016/0304-3959(79)90049-6

3. Bannister K, Patel R, Goncalves L, Townson L, Dickenson AH. Diffuse noxious inhibitory controls and nerve injury: restoring an imbalance between descending monoamine inhibitions and facilitations. *Pain*. 2015;156(9):1803-1811. doi:10.1097/j.pain.0000000000000240

4. Hirschberg S, Li Y, Randall A, Kremer EJ, Pickering AE. Functional dichotomy in spinal-vsprefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats. *Elife*. 2017;6(Lc):1-26. doi:10.7554/eLife.29808.001

5. Hickey L, Li Y, Fyson SJ, et al. Optoactivation of Locus Ceruleus Neurons Evokes Bidirectional Changes in Thermal Nociception in Rats. *J Neurosci*. 2014;34(12):4148-4160. doi:10.1523/JNEUROSCI.4835-13.2014

6. Li Y, Hickey L, Perrins R, et al. Retrograde optogenetic characterization of the pontospinal module of the locus coeruleus with a canine adenoviral vector. *Brain Res*. 2016;1641:274-290. doi:10.1016/j.brainres.2016.02.023

7. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*. 1983;16(2):109-110. doi:10.1016/0304-3959(83)90201-4

8. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8(6):e1000412. doi:10.1371/journal.pbio.1000412

9. Szot P, Miguelez C, White SS, et al. A comprehensive analysis of the effect of DSP4 on the locus coeruleus noradrenergic system in the rat. *Neuroscience*. 2010;166(1):279-291. doi:10.1016/j.neuroscience.2009.12.027

10. Ross SB, Stenfors C. DSP4, a Selective Neurotoxin for the Locus Coeruleus Noradrenergic System. A Review of Its Mode of Action. *Neurotox Res*. 2015;27(1):15-30. doi:10.1007/s12640-014-9482-z

11. Hwang DY, Carlezon WA, Isacson O, Kim KS. A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther*. 2001;12(14):1731-1740. doi:10.1089/104303401750476230
12. Urch CE, Dickenson a. H. In vivo single unit extracellular recordings from spinal cord neurones of rats. *Brain Res Protoc.* 2003;12:26-34. doi:10.1016/S1385-299X(03)00068-0

13. LeChasseur Y, Dufour S, Lavertu G, et al. A microprobe for parallel optical and electrical recordings from single neurons in vivo SUPPLEMENT. *Nat Methods.* 2011;8(4):319-325. doi:10.1038/nmeth.1572

14. Treweek JB, Chan KY, Flytzanis NC, et al. Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping. *Nat Protoc.* 2015;10(11):1860-1896. doi:10.1038/nprot.2015.122

15. Millan MJ. Descending control of pain. *Prog Neurobiol.* 2002;66(6):355-474. doi:10.1016/S0301-90082(02)00009-6

16. Gassner M, Ruscheweyh R, Sandkühler J. Direct excitation of spinal GABAergic interneurons by noradrenaline. *Pain.* 2009;145(1-2):204-210. doi:10.1016/j.pain.2009.06.021

17. Sonohata M, Furue H, Katafuchi T, et al. Actions of noradrenaline on substantia gelatinosa neurones in the rat spinal cord revealed by in vivo patch recording. *J Physiol.* 2004;555(Pt 2):515-526. doi:10.1113/jphysiol.2003.054932

18. Robertson SD, Plummer NW, de Marchena J, Jensen P. Developmental origins of central norepinephrine neuron diversity. *Nat Neurosci.* 2013;16(8):1016-1023. doi:10.1038/nn.3458

19. Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E. Noradrenergic Locus Coeruleus pathways in pain modulation. *Neuroscience.* 2016;338:93-113. doi:10.1016/j.neuroscience.2016.05.057

20. Llorca-Torralba M, Camarena-Delgado C, Suárez-Pereira I, et al. Pain and depression comorbidity causes asymmetric plasticity in the locus coeruleus neurons. *Brain.* 2021;(2021):1-33. doi:10.1093/brain/awab239

21. Kohro Y, Matsuda T, Yoshihara K, et al. Spinal astrocytes in superficial laminae gate brainstem descending control of mechanosensory hypersensitivity. *Nat Neurosci.* 2020;23(11):1376-1387. doi:10.1038/s41593-020-00713-4

22. Lockwood SM, Bannister K, Dickenson AH. An investigation into the noradrenergic and serotonergic contributions of diffuse noxious inhibitory controls in a monoiodoacetate model of osteoarthritis. *J Neurophysiol.* 2019;121(1):96-104. doi:10.1152/jn.00613.2018

23. Kucharczyk MW, Derrien D, Dickenson AH, Bannister K. The Stage-Specific Plasticity of Descending Modulatory Controls in a Rodent Model of Cancer-Induced Bone Pain. *Cancers (Basel).* 2020;12(11):1-17. doi:10.3390/cancers12113286
24. Pereira-Silva R, Costa-Pereira JT, Alonso R, Serrão P, Martins I, Neto FL. Attenuation of the Diffuse Noxious Inhibitory Controls in Chronic Joint Inflammatory Pain Is Accompanied by Anxiodepressive-Like Behaviors and Impairment of the Descending Noradrenergic Modulation. *Int J Mol Sci.* 2020;21(8):1-28. doi:10.3390/ijms21082973

25. Yarnitsky D, Granot M, Nahman-Averbuch H, Khamaisi M, Granovsky Y. Conditioned pain modulation predicts duloxetine efficacy in painful diabetic neuropathy. *Pain.* 2012;153(6):1193-1198. doi:10.1016/j.pain.2012.02.021

26. Graven-Nielsen T, Wodehouse T, Langford RM, Arendt-Nielsen L, Kidd BL. Normalization of widespread hyperesthesia and facilitated spatial summation of deep-tissue pain in knee osteoarthritis patients after knee replacement. *Arthritis Rheum.* 2012;64(9):2907-2916. doi:10.1002/art.34466

27. Yarnitsky D. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): Its relevance for acute and chronic pain states. *Curr Opin Anaesthesiol.* 2010;23(5):611-615. doi:10.1097/ACO.0b013e32833c348b

28. Cummins TM, Kucharczyk M, Graven-Nielsen T, Bannister K. Activation of the descending pain modulatory system using cuff pressure algometry: Back translation from man to rat. *Eur J Pain.* Published online April 29, 2020. doi:10.1002/ejp.1580
Figure legends

Figure 1. A ventral coerulean neuronal population inhibits spinal nociceptive processing via α₁-adrenoceptors. a) Immunohistochemical analysis of locus coeruleus (LC) dopamine-β-hydroxylase (DBH)-expressing noradrenergic neurons transduced by canine adenovirus (CAV) delivering channelrhodopsin-2-mCherry construct under catecholamine specific promoter (PRS) injected locally (LC:LC module) or in the ipsilateral lumbar dorsal horns (LC:SC module). b) Percentage of mCherry-expressing DBH neurons in the ipsi- and contralateral LC following LC:SC module labelling. Mean±SEM of N=3 animals per group, n=6-8 slices per animal, unpaired One-Way ANOVA performed on N, [structure] P=0.0018, F(5, 12)=7.747. c) Schematic of the in vivo electrophysiological experiments. d) WDR neuron units code upon stimulation with natural stimuli. e) WDR neuron inhibition following LC:LC module ChR2-mediated activation (450 nm laser pulses: 5 Hz, 20 ms, 238 mW/mm²) f) The equivalent LC:SC module activation is shown. Quantification of g) brush and h) von Frey evoked activity before/after LC:LC module activation. Brush and von Frey: mean±SEM of N=13 animals per group, n=13 cells per group; Paired t-test performed on n: P>0.05 (brush) and Two-Way ANOVA (von Frey) performed on n, [von Frey] P<0.0001, F(2, 36)=24.37, [450 nm] P<0.0001, F(1, 36)=47.29. Quantification of i) brush and j) von Frey evoked activity before/after LC:SC module activation. Brush and von Frey: mean±SEM of N=14 animals per group, n=14 cells per group; Paired t-test performed on n: P<0.05 (brush); Two-Way ANOVA (von Frey) performed on n, [von Frey] P<0.0001, F(2, 39)=23.75, [450 nm] P<0.0001, F(1, 39)=89.83. Prazosin (α₁-adrenoreceptors antagonist) reversed the inhibitory effect of k) LC:LC and l) LC:SC module activation. LC:LC or LC:SC prazosin: mean±SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [LC:LC-group] P<0.05, F(1.03, 5.17)=3.306 and [LC:SC-group] P<0.01, F(1.23, 6.17)=14.24, respectively. m) LC:LC- and n) LC:SC-mediated inhibition of WDR neurons was further potentiated after spinal application of 100 µg atipamezole (α₂-adrenoreceptors antagonist). LC:LC or LC:SC atipamezole: mean±SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [LC:LC-group] P<0.05, F(1.39, 6.94)=5.635, and [LC:SC-group] P<0.001, F(1.82, 9.09)=26.58. Tuckey post-hoc used for all ANOVA: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. See supplementary material: Supplementary Figure 1, and Supplementary Table 1 (statistics).
Figure 2. Diffuse noxious inhibitory controls (DNIC) expression is inhibited by LC:SC module optoactivation. a) DNIC expression, quantified as the inhibitory effect of a conditioning stimulus (ear pinch), decreased following LC:LC module optoactivation (450 nm laser pulses), and was abolished following identical LC:SC module activation. b) Percentage of inhibition after DNIC activation before/after LC:LC module activation. Mean±SEM of N=13 animals per group, n=13 cells per group; Two-Way ANOVA performed on n, [450 nm] P<0.01, F(1, 36)=10.75. c) Identical experiments before/after the LC:SC module activation. Mean±SEM of N=14 animals per group, n=14 cells per group; Two-Way ANOVA performed on n, [450 nm] P<0.001, F(1, 39)=46.01. Prazosin partially reversed the impact of d) LC:LC or e) LC:SC module activation on DNIC expression: f) LC:LC prazosin: mean±SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [group] P<0.05, F(1.38, 6.91)=8.056. g) LC:SC prazosin: mean±SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [group] P<0.05, F(1.17, 5.87)=8.215. The inhibitory effect of h) LC:LC and i) LC:SC module activation on DNIC expression was facilitated by spinal atipamezole: j) LC:LC atipamezole: mean ± SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [group] P>0.05, F(1.06, 5.31)=4.950. k) LC:SC atipamezole: mean ± SEM shown as percentage of baseline for N=6 animals per group, one cell per animal; Two-Way ANOVA with Geisser-Greenhouse correction [group] P<0.001, F(1.82, 9.01)=26.58. Tuckey post-hoc used for all ANOVA: *P<0.05, **P<0.01, ****P<0.0001. See supplementary material: Supplementary Table 1 (statistics).

Figure 3. Ablation of coerulean noradrenergic fibres does not affect basal spinal convergent neuron activity nor DNIC expression.

a) A PACT-cleared 500 µm thick lumbar spinal cord section (saline versus DSP4-treated rats) evidences a decrease in tyrosine hydroxylase (TH) immunolabelled fibres in the superficial but not deep dorsal horn (SDH/DDH). b) DSP4 treatment did not impact WDR neuron sensory coding nor DNIC expression. Quantification of c) brush and d) von Frey evoked action potentials in saline and DSP4 treated rats. Brush and von Frey: mean±SEM of N=6 animals per group, n=15 cells per group; unpaired t-test performed on n: P>0.05 (brush); Two-Way ANOVA (von Frey) performed on n, [von Frey] P<0.0001, F(2, 30)=128.7, [DSP4] P>0.05, F(1, 15)=1.851. e) Percentage of inhibition after DNIC activation as shown in b). DNIC: mean±SEM of N=6 animals per group, n=15 cells per group; Two-Way ANOVA performed on n, [DSP4] P>0.05, F(1, 15)=0.2105. f) Analogously to DSP4 treatment, ipsilateral LC microinjection of 2% lidocaine (marked by Lucifer Yellow) does not affect WDR neuronal activity nor DNIC expression, as...
quantified in g) for brush and h) for von Frey. Brush: mean±SEM of N=5 animals per group, n=5 cells per group; paired t-test performed on n: P>0.05. von Frey: mean±SEM of N=6 animals per group, n=6 cells per group; Two-Way ANOVA performed on n, [von Frey] P<0.001, F(2, 10)=23.97, [DSP4] P>0.05, F(1, 10)=0.78. i) Percentage of inhibition after DNIC activation as shown in j). DNIC: mean±SEM of N=6 animals per group, n=6 cells per group; Two-Way ANOVA performed on n, [DSP4] P>0.05, F(1, 5)= 0.063. Tuckey post-hoc used for all ANOVA: *P<0.05, **P<0.01, ****P<0.0001. See supplementary material: Supplementary Table 1 (statistics).
Figure 1

190x254 mm (4.9 x DPI)
Figure 2
190x254 mm (4.9 x DPI)
Figure 3
190x254 mm (4.9 x DPI)
Figure 4

ChR2-evoked phasic activation

|          | LC:LC | LC:SC | LC:SC + spinal adrenoceptor | LC:SC α₁ block | LC:SC α₂ block |
|----------|-------|-------|-----------------------------|----------------|----------------|
| Spinal WDR neuron firing | ▼   | ➖   | ➖                          |                |                |
| DNIC expression      | ✓   | ➖   | ✓                          |                |                |

Dorsal horn

Locus Coeruleus: Locus Coeruleus (LC:LC)

Locus Coeruleus: Superior Colliculus (LC:SC)