

Rewards, perils and pitfalls of untangling spinal pain circuits

Brett A Graham^{1,2} and David I Hughes³

Pain is a complex perception that is fundamental to our daily survival. Under normal circumstances, it serves an important protective function to guard against tissue damage or alert the body to dangerous environments. Under pathological states, however, the perception of pain can become chronic, maladaptive, resistant to treatment, and presents a serious clinical and societal problem. A wealth of literature suggests that disruption of sensory processing within the spinal cord contributes to chronic pain, but our limited understanding of spinal circuitry in health and disease remains a barrier to the development of new therapeutic strategies. The aim of this brief review is to outline current thinking about how individual components of functionally distinct spinal microcircuits can be identified and manipulated to determine their role in influencing our perception of pain in acute and chronic states.

Addresses

¹ School of Biomedical Sciences & Pharmacy, Faculty of Health, University of Newcastle, Callaghan, Australia

² Hunter Medical Research Institute (HMRI), New Lambton Heights, NSW, Australia

³ Institute of Neuroscience Psychology, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, UK

Corresponding author: Hughes, David I (David.I.Hughes@glasgow.ac.uk)

Current Opinion in Physiology 2019, 11:xx–yy

This review comes from a themed issue on **Physiology of Pain**

Edited by **Cheryl Stucky** and **Lucy Donaldson**

<https://doi.org/10.1016/j.cophys.2019.04.015>

2468-8673/© 2019 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Background

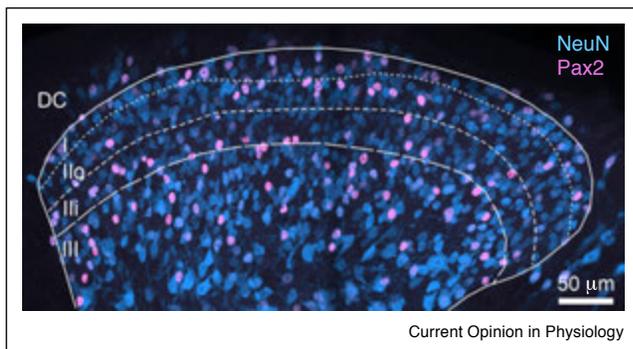
The somatosensory system plays a fundamental role in our ability to perceive, and ultimately react to, our environment. While there is debate about how this sensory information from the periphery is encoded and conveyed to the brain for ultimate perception [1,2], it is well established that the spinal dorsal horn plays a key role in the gating, modulation and relay of nociceptive sensory input to brain higher centres [3,4^{*},5^{**}]. The perception of acute ‘day-to-day’ pain is fundamental to our wellbeing, serving a protective role by raising our awareness to threatening environments and to actual tissue damage. Chronic (persistent) pain, which affects approximately 20% of the global population, results from a pathological

change in either the peripheral and/or central nervous system and is defined as a pain state that has persisted for over three months. Since this form of pain can arise either spontaneously or persist beyond injury, it “lacks the acute warning function of physical nociception” [6], representing a maladaptive state that can be debilitating, and is often resistant to treatment. There is a broad agreement in the field that this burden can only be better addressed by an improved understanding of how the body perceives pain under normal circumstances, and how this compares to chronic pain states, requiring detailed analysis of pain signalling mechanisms from the periphery to the brain. Given the importance of the spinal cord to the transmission of pain this view has driven efforts to define the spinal circuits that process nociceptive information, identify individual neuronal components of these circuits, and determine the functional significance these cells (Figure 1). Here, we aim to provide a brief overview of our current understanding on spinal circuits in the superficial dorsal horn, laminae I to III. The review will then focus on technological advances that help us identify individual elements of pain circuits, and on experimental approaches that can be used to manipulate the activity of these cells to determine their functional significance in pain perception in health and disease.

Defining spinal interneuron populations

The spinal dorsal horn is the principle termination site of primary afferent fibres serving a range of sensory modalities, and is the first site where this information may be modulated by discrete spinal circuits before it is relayed to higher brain centres for conscious perception. The predominant neuronal populations in this region are interneurons, whose role is to process and modulate afferent input before it is relayed to supraspinal sites via projection neurons. Spinal interneurons account for the majority of neurons in lamina I, and virtually all in lamina II [4^{*}]. These cells may be subdivided broadly into two functional populations based on their neurotransmitter content: inhibitory interneurons, which use GABA and/or glycine; and excitatory interneurons, which use glutamate. Other properties of dorsal horn neurons have also been studied widely using anatomical, electrophysiological and pharmacological approaches in animal models [7–12], and these data have provided important insight into the extraordinary complexity of this region, suggesting much heterogeneity still exists within the inhibitory and excitatory interneuron populations. For example, studies combining both *in vitro* electrophysiological experiments in spinal cord slices with subsequent anatomical approaches helped characterise the biophysical properties of both lamina I projection neurons [13,14] and

Figure 1



Neuroanatomical organisation of the mouse spinal dorsal horn. The neuronal architecture of the spinal dorsal horn is highly complex. It is made up of three principal types of neurons (blue): projection neurons, excitatory interneurons, and inhibitory interneurons (magenta). Interneurons modulate and gate sensory information entering the dorsal horn, whereas projection neurons relay this information on to higher supraspinal centres.

interneurons in lamina II [15–19], but rarely addressed questions of connectivity, apart from a few notable exceptions [20–25]. Nonetheless, these studies provide a wealth of information on the heterogeneity of neuronal populations in this region, using untargeted sampling method as all recordings were carried out ‘blind’ to cell identity. While this is an ideal approach for sampling across the population, it is not an efficient approach to resolve discrete synaptic circuits.

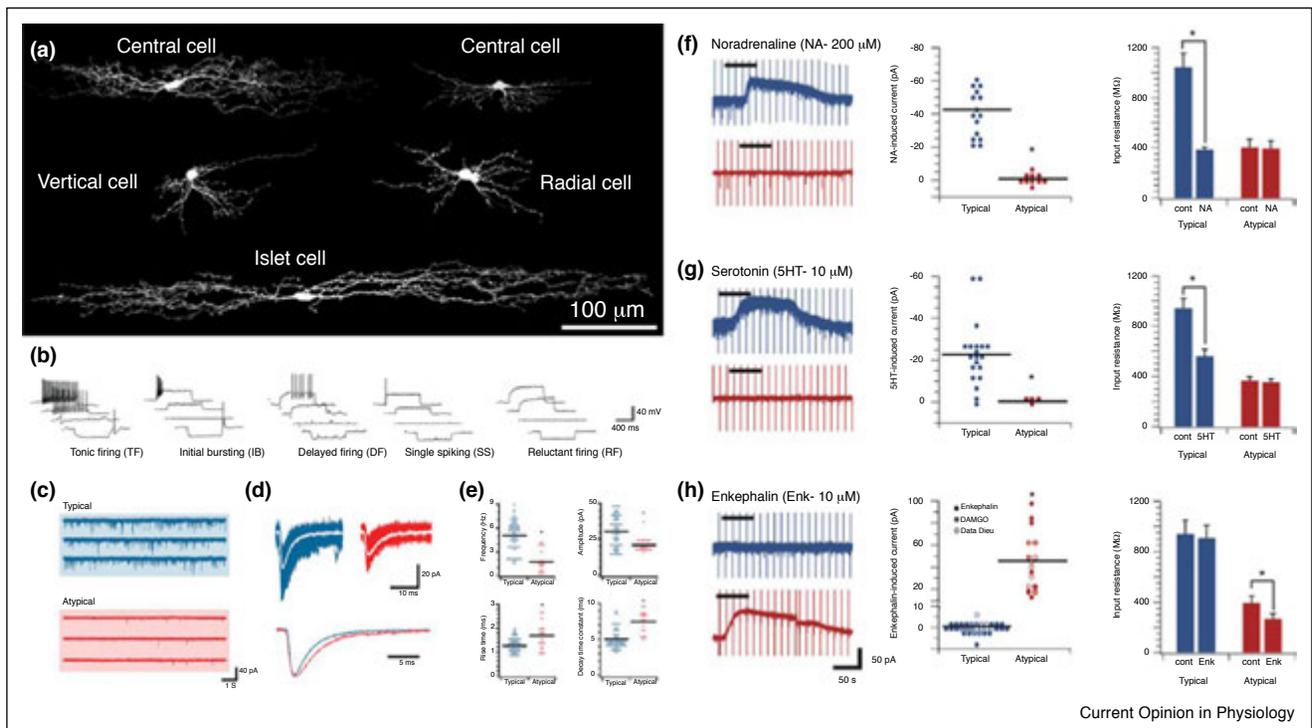
A number of studies have attempted to better resolve dorsal horn heterogeneity by correlating across multiple classification properties. For example, distinct physiological properties have been identified in spinal interneurons based on action potential discharge patterns in response to current injections, subthreshold currents and responsiveness to drug application [10,26], and these appear to correlate with particular classes of interneurons defined by their morphology [16,18]. These studies, carried out in hamsters and mice, identified five classes of morphologically distinct interneuron in lamina II, comprising of islet cells, radial cells, central cells, vertical cells and unclassified cells, and each of these classes typically display consistent physiological properties. Subsequent studies correlated the neurochemical phenotype of spinal interneurons with these anatomical and electrophysiological properties [17,19]. Over time this work has helped to develop generalised schemes ascribing-specific morphological and electrophysiological signatures to different types of inhibitory and excitatory interneuron populations [16,18]. However, caution should be exercised when applying classification schemes developed in one species as they may not necessarily translate to others. For example, criteria used in rat and hamster to morphologically separate islet cells from central cells, with both

exhibiting the same dendritic orientation but the extent of this branching distinguishing them, may not faithfully translate to mouse. Several anatomical and electrophysiological studies have established similar caveats in comparable populations of neurons elsewhere in the central nervous system between rats and mice, and this may also apply to spinal interneurons [27,28]. Nonetheless, these multidisciplinary studies identify a number of anatomical and electrophysiological features that collectively, represent characteristic signatures to help define distinct populations of dorsal horn interneurons.

Targeted approaches to studying circuitry

The advent of transgenic mouse lines in which fluorescent proteins, driven by a genetic promoter, label discrete populations of neurons, has been instrumental in furthering our understanding of interneurons and the circuits they form by allowing us to target these cells specifically in electrophysiological experiments [29]. These approaches, initially developed to study circuits in the hippocampus, were soon adopted to target inhibitory interneurons in the spinal dorsal horn. Mice where green fluorescent protein (GFP) was expressed under the control of either the prion promoter or that for the GABA-synthesising enzyme GAD67 allowed the study of GABAergic interneurons [30,31], whereas the glycine transporter 2 gene was used to label glycinergic interneurons [32]. Similar approaches were later employed to study excitatory interneurons in the spinal cord, using the vesicular glutamate transporter type 2 gene as the promoter [33]. Collectively, these studies represent some of the most significant advances in efforts to characterise spinal cord circuitry. Further refinements targeting even more discrete populations of interneurons have followed, driven in part by our understanding of the neurochemical signatures of subpopulations of dorsal horn interneurons [34,35,36]. By mapping the laminar distribution of these cells in the dorsal horn, and defining their responsiveness to various peripheral stimuli [37,38], it has been possible to implicate-specific subpopulations in the processing of nociceptive afferent input. For example, in the spinal dorsal horn, the calcium binding protein calretinin (CR) is expressed primarily in excitatory interneurons and these have been implicated in circuits underlying both acute and chronic pain [39,40]. More recently, CR-expressing cells have been found to comprise a more heterogeneous population than previously appreciated, with approximately 15% being shown to express Pax2, a developmental marker expressed in inhibitory interneurons [38]. Excitatory and inhibitory CR neurons show clear differences in their anatomical, neurochemical, electrophysiological and pharmacological features [38–40,41*,42*], and it is highly likely that these varied properties underpin their respective roles in the spinal circuits they form (Figure 2). More recently, comprehensive classification schemes have been presented where dorsal horn neurons have been grouped into discrete populations based on

Figure 2



Morphological, electrophysiological and pharmacological heterogeneity of calretinin-expressing spinal interneurons.

Calretinin is expressed in approximately 30% of all neurons in laminae I and II, but identifies several subpopulations of interneurons that show clear differences in their anatomy, electrophysiological properties, and pharmacological responses. (a) Several classes of morphologically distinct interneurons express CR, including islet cells, central cells, vertical cells, radial cells and those of unclassified morphology. In these studies, we named excitatory CR interneurons 'Typical' CR interneurons (which include cells with central, vertical, radial and unclassified morphologies), and inhibitory CR interneurons as 'Atypical', all of which displayed islet cell morphology. (b) The physiological properties of Typical (Blue) and Atypical (Red) CR cells are also diverse, showing distinct action potential firing patterns in response to current injection, their excitability following synaptic activation, and their responsiveness to bath application of neuromodulators (f-h). Modified from Smith *et al.* [38] and Smith *et al.* [41].

their molecular and genetic profiles [36,43,44]. These schemes broadly match expression patterns for markers studied using immunohistochemistry [34,35], and are unprecedented as a source of information to differentiate molecular-genetic and neurochemical signatures for distinct interneuron populations.

Refinements for selective targeting of discrete neuronal populations

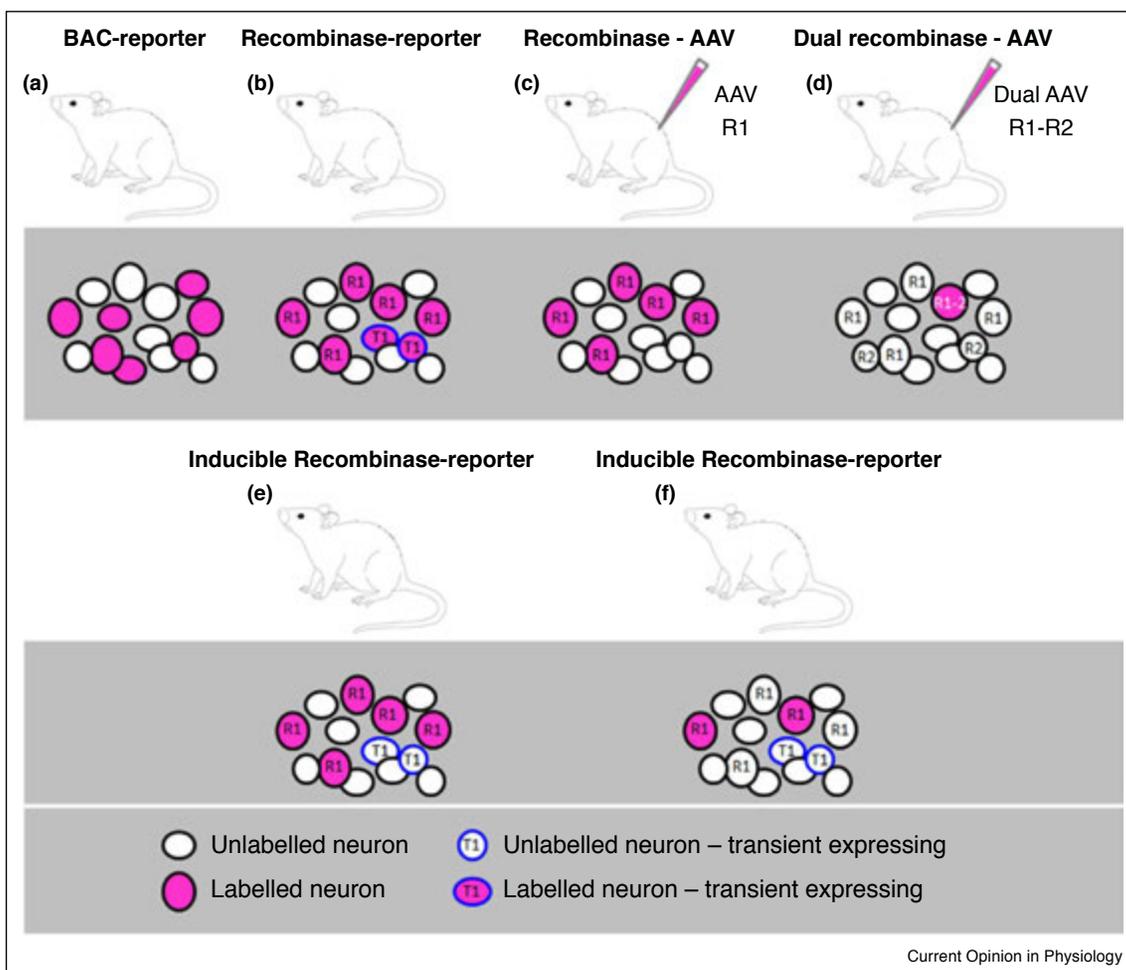
The rapid development of molecular and genetic approaches enabling identification, targeting and manipulation of neurons in both the peripheral and central nervous system has been instrumental in furthering our understanding of the neuronal circuitry underlying somatosensation. Transgenic mice where various site-specific recombinases (SSRs) are expressed in restricted neuronal populations now offer a tool-box that can be used to dissect spinal circuits and study their contribution to the perception of different sensory inputs. Such mice are routinely crossed with recombinase-specific reporter lines, leading to the expression of fluorescent markers (e.g. GFP or tdTomato), light-sensitive ion channels (e.g. channelrhodopsin,

archaerhodopsin or halorhodopsin), toxins (e.g. diphtheria toxin or tetanus toxin), or drug-sensitive receptors (Designer Receptors Exclusively Activated by Designer Drugs; DREADDs for example hM3Dq or hM4Di) in distinct neuronal populations. These approaches can be used to map populations of neurons derived from a cell population defined through their expression of a particular gene. Various genes are switched on (and off) during development, and this transient gene expression within a cell lineage will result in the permanent, and continued expression of the reporter protein within that population. While this can be advantageous in many cases, it can also introduce a degree of ambiguity in reporter molecule expression patterns, especially in instances where gene expression is transient during stages of development and differ from the more stable expression patterns in adulthood [45,46]. Of particular relevance to spinal cord circuitry, the genes controlling the expression of neurochemical markers define several populations of dorsal horn interneurons implicated in mechanical pain hypersensitivity, including calretinin (CR), vesicular glutamate transporter type 3, somatostatin, and dynorphin, exhibit

different developmental and adult expression patterns [39,40]. When using reporter lines to express light-sensitive ion channels or drug-sensitive receptors in discrete neuronal populations for subsequent *in vivo* or *in vitro* experiments, the caveat of transiently expressing populations can introduce discrepancies between studies and the results generated. This emphasises the importance of verifying the expression patterns of reporter molecules are appropriately restricted to the intended neurochemically defined populations, for example using correlated immunohistochemical approaches. More recently, experimental refinements have been devised to avoid the problem of transient gene

activation during development, including the administration of transgene inducing agents in mice controlling inducible recombinase activation [45,47], or the delivery of recombinase-dependent adeno associated viruses (AAVs) either directly into the spinal cord [48–50] or into primary afferent fibres [51] at time-points that avoid developmental expression. An additional advantage of employing a viral vector-based approach for labelling recombinase-expressing cells with light-sensitive ion channels, toxins or DREADDs over crossing with a reporter line, is that continued replication of the AAV in the transfected animal increases reporter molecule expression over time,

Figure 3



Rewards, perils and pitfalls of using transgenic mouse lines.

Selection of optimal animal model is central to the success of an experiment. **(a)** BAC-reporter lines provide an efficient means of labelling cells (magenta), but run the risk of incomplete expression and expression in aberrant populations. **(b)** Recombinase lines crossed with a reporter line give offspring where labelling in the desired population is more complete (R1, magenta), but will also capture cells where the promoter gene is active transiently during development (T1, magenta with blue outline). **(c)** Intraspinal injection of recombinase-specific AAVs that encode for a reporter molecule (AAV R1) label cells where the reporter is expressed at that developmental time point (R1, magenta). **(d)** Generation of dual-recombinase expressing mice, followed by intraspinal injections of dual recombinase-specific AAV (AAV R1-R2) will label discrete cell populations only (R1-2, magenta). **(e)** Another means of avoiding labelling in cells where promoter genes are only active transiently is to administer transgene-inducing agents at appropriate developmental time points in mice where reporter expression is under the control of an inducible recombinase. **(f)** Sparse labelling of reporter-expressing cells (R1) can be achieved in these mice by altering the dose or the timing of delivery of the transgene-inducing agents.

enhancing the sensitivity of cells to subsequent experimental manipulation. Although the principal objective of most experiments that adopt these approaches is to label as many of the selected population as possible, in instances where the aim is to recover the morphology of individual neurons, labelling a dense plexus of cells can be problematic. Another advantage of viral vector-based and inducible approaches addresses this challenge by producing sparse labelling patterns by controlling the timing of the transgene inducing agent administration, varying the dose administered, or lowering the viral titres injected [52,53,54*].

Finally, building on the traditional neurochemical approach to differentiate subpopulations of dorsal horn neurons, molecular and genetic profiles are increasingly being used to develop classification schemes that provide an invaluable resource to help target neuronal populations more precisely than previously possible [36*,43**,44**]. Identifying unique molecular signatures for discrete neuronal populations allows us to devise intersectional genetic strategies where distinct recombinases (e.g. Cre and Flp, Cre and Dre, or Dre and Flp) are expressed in largely non-overlapping neuronal populations, but where co-expression is restricted to the target population. By adopting such approaches, injecting dual recombinase-specific AAVs into double-transgenic mice provides a means of targeting and manipulating neuronal populations with far greater precision than previously possible [55,56]. As studies implement these latest experimental approaches, the prospect of building a detailed understanding of individual components within functionally distinct spinal cord circuits has never been more attainable. Furthermore, as this field advances, so does the hope that this information better instructs future targeted analgesic development.

New insights into old problems: defining spinal circuits in chronic pain states

The literature contains several models attempting to explain how neuronal circuits in the spinal dorsal horn change in pathological conditions, leading to the development of chronic pain states. For example, structural reorganisation and altered peptide expression in of low threshold mechanoreceptors (LTMRs) have been proposed to underlie the development of tactile allodynia after peripheral nerve injury [57–59], whereas others have proposed that the disinhibition of spinal horn circuits through either the selective loss of inhibitory interneurons [60,61], or the disruption of anion gradients in pain projecting neurons [62], contribute significantly to these altered sensory perceptions. While most of these hypotheses have been challenged, a significant body of evidence is emerging that alterations in the inhibition of spinal circuits plays an important role in the development of mechanical hypersensitivity [39,40,50,63,64*,65**,66**]. These detailed studies of neuronal activity in functionally distinct circuits have only been possible through the

technical advances described above, allowing manipulation of selective interneuron populations using molecular genetic approaches (Figure 3). With further refinements in experimental approaches, and more selective means of targeting discrete neuronal populations, there is great optimism that our understanding of spinal pain circuits underlying acute and chronic pain states will improve drastically. In doing so, the detailed understanding that will follow is likely form the basis for new spinally-based analgesic approaches and help develop better pain management treatments.

Conflict of interest statement

Nothing declared.

Acknowledgements

Support from the BBSRC (grants BB/J000620/1 and BB/P007996/1 to DIH) and the National Health and Medical Research Council (NHMRC) of Australia (grants 631000 and 1043933 to BAG) is gratefully acknowledged.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Doetsh GS: **Patterns in the brain: neuronal population coding in the somatosensory system.** *Physiol Behav* 2000, **69**:187-201.
 2. Prescott SA, Ma Q, De Koninck Y: **Normal and abnormal coding of somatosensory stimuli causing pain.** *Nature Neurosci* 2014, **17**:183-191.
 3. Peirs C, Seal RP: **Neural circuits for pain: recent advances and current views.** *Science* 2016, **354**:578-584.
 4. Todd AJ: **Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn.** *Mol Pain* 2017, **13**:1-19.
- This article provides a detailed description of neurochemically distinct subpopulations of spinal interneurons and their contribution to dorsal horn circuits that influence the perception of pain and itch.
5. Moehring M, Halder P, Seal RP, Stucky CL: **Uncovering the cells and circuits of touch in normal and pathological settings.** *Neuron* 2018, **100**:349-360.
- A comprehensive review of the spinal circuits, sensory afferents and peripheral (cutaneous) endings involved in the perception of both innocuous and noxious mechanosensation, but also highlighting the role of non-neuronal cells in activating touch and pain pathways.
6. Treede R-D, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, Cohen M, Evers S, Finnerup NB, First MB *et al.*: **A classification of chronic pain for ICD-11.** *Pain* 2015, **156**:1003-1007.
 7. Todd AJ: **Electron microscope study of Golgi-stained cells in lamina II of the rat spinal dorsal horn.** *J Comp Neurol* 1988, **275**:145-157.
 8. Todd AJ, McKenzie J: **GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord.** *Neuroscience* 1989, **31**:799-806.
 9. Woolf CJ, Fitzgerald M: **The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord.** *J Comp Neurol* 1983, **221**:313-328.
 10. Yoshimura M, Jessell TM: **Membrane properties of rat substantia gelatinosa neurons *in vitro*.** *J Neurophysiol* 1989, **62**:109-118.
 11. Ruscheweyh R, Sandkühler J: **Lamina-specific membrane and discharge properties of rat spinal dorsal horn neurones *in vitro*.** *J Physiol* 2002, **541**:231-244.

12. Yaksh TL: **Behavioral and anatomic correlates of the tactile-evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists.** *Pain* 1989, **37**:111-123.
13. Prescott SA, De Koninck Y: **Four cell types with distinctive membrane properties and morphologies in lamina I of the spinal dorsal horn of the adult rat.** *J Physiol* 2002, **539**:817-836.
14. Ruscheweyh R, Ikeda H, Heinke B, Sandkühler J: **Distinctive membrane and discharge properties of rat spinal lamina I projection neurones *in vitro*.** *J Physiol* 2004, **555**:527-543.
15. Light AR, Trevino DL, Perl ER: **Morphological features of functionally defined neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn.** *J Comp Neurol* 1979, **186**:151-171.
16. Grudt TJ, Perl ER: **Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn.** *J Physiol* 2002, **540**:189-207.
17. Maxwell DJ, Belle MO, Cheunsuang O, Stewart A, Morris R: **Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn.** *J Physiol* 2007, **584**:521-533.
18. Yasaka T, Kato G, Furue H, Rashid MH, Sonohata M, Tamae A, Murata Y, Masuko S, Yoshimura M: **Cell-type-specific excitatory and inhibitory circuits involving primary afferents in the substantia gelatinosa of the rat spinal dorsal horn *in vitro*.** *J Physiol* 2007, **581**:603-618.
19. Yasaka T, Tiong SY, Hughes DI, Riddell JS, Todd AJ: **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* 2010, **151**:475-488.
20. Lu Y, Perl ER: **A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input.** *J Neurosci* 2003, **23**:8752-8758.
21. Lu Y, Perl ER: **Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II).** *J Neurosci* 2005, **25**:3900-3907.
22. Lu Y, Dong H, Gao Y, Gong Y, Ren Y, Gu N, Zhou S, Xia N, Sun Y-Y, Ji R-R, Xiong L: **A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia.** *J Clin Invest* 2013, **123**:4050-4062.
23. Kato G, Kawasaki Y, Ji RR, Strassman AM: **Differential wiring of local excitatory and inhibitory synaptic inputs to islet cells in rat spinal lamina II demonstrated by laser scanning photostimulation.** *J Physiol* 2007, **580**:815-833.
24. Kato G, Kawasaki Y, Koga K, Uta D, Kosugi M, Yasaka T, Yoshimura M, Ji R-R, Strassman AM: **Organization of intralaminar and translaminar neuronal connectivity in the superficial spinal dorsal horn.** *J Neurosci* 2009, **29**:5088-5099.
25. Kosugi M, Kato G, Lukashov S, Pendse G, Puskar Z, Kozsuresk M, Strassman AM: **Subpopulation-specific patterns of intrinsic connectivity in mouse superficial dorsal horn as revealed by laser scanning photostimulation.** *J Physiol* 2013, **591**:1935-1949.
26. Graham BA, Brichta AM, Callister RJ: **Moving from an averaged to specific view of spinal cord pain processing circuits.** *J Neurophysiol* 2007, **98**:1057-1063.
27. Routh BN, Johnston D, Harris K, Chitwood RA: **Anatomical and electrophysiological comparison of CA1 pyramidal neurons of the rat and mouse.** *J Neurophysiol* 2009, **102**:2288-2302.
28. Ellenbroek B, Youn J: **Rodent models in neuroscience research: is it a rat race?** *Dis Models Mech* 2016, **9**:1079-1087.
29. Oliva AA, Jiang M, Lam T, Smith KL, Swann JW: **Novel hippocampal interneuronal subtypes identified using transgenic mice that express green fluorescent protein in GABAergic interneurons.** *J Neurosci* 2000, **20**:3354-3368.
30. Hantman AW, van den Pol AN, Perl ER: **Morphological and physiological features of a set of spinal substantia gelatinosa neurons defined by green fluorescent protein expression.** *J Neurosci* 2004, **24**:836-842.
31. Heinke B, Ruscheweyh R, Forsthuber L, Wunderbaldinger G, Sandkühler J: **Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurons identified by expression of green fluorescent protein in mice.** *J Physiol* 2004, **560**:249-266.
32. Zeilhofer HU, Studler B, Arabadzisz D, Schweizer C, Ahmadi S, Layh B, Bösl MR, Fritschy JM: **Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice.** *J Comp Neurol* 2005, **482**:123-141.
33. Punnakkal P, von Schoultz C, Haenraets K, Wildner H, Zeilhofer HU: **Morphological, biophysical and synaptic properties of glutamatergic neurons of the mouse spinal dorsal horn.** *J Physiol* 2014, **592**:759-776.
34. Gutierrez-Mecinas M, Furuta T, Watanabe M, Todd AJ: **A quantitative study of neurochemically defined excitatory interneuron populations in laminae I-III of the mouse spinal cord.** *Mol Pain* 2016, **12**:1-18.
35. Polgár E, Durrieux C, Hughes DI, Todd AJ: **A quantitative study of inhibitory interneurons in laminae I-III of the mouse spinal dorsal horn.** *PLoS One* 2013, **8**:e78309.
36. Abraira VE, Kuehn ED, Chirila AM, Springel MW, Toliver AA, Zimmerman AL, Orefice LL, Boyle KA, Bai L, Song BJ *et al.*: **The cellular and synaptic architecture of the mechanosensory dorsal horn.** *Cell* 2017, **168**:295-310.
- This paper provides a comprehensive description of the molecular, functional and organisational properties of spinal interneurons in the low-threshold mechanoreceptor recipient zone of the dorsal horn.
37. Polgár E, Campbell AD, MacIntyre LM, Watanabe M, Todd AJ: **Phosphorylation of ERK in neurokinin 1 receptor-expressing neurons in laminae III and IV of the rat spinal dorsal horn following noxious stimulation.** *Mol Pain* 2007, **3**:4.
38. Smith KM, Boyle KA, Madden JF, Dickinson SA, Jobling P, Callister RJ, Hughes DI, Graham BA: **Functional heterogeneity of calcitonin-expressing neurons in the mouse superficial dorsal horn: implications for spinal pain processing.** *J Physiol* 2015, **593**:4319-4339.
39. Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X *et al.*: **Identification of spinal circuits transmitting and gating mechanical pain.** *Cell* 2014, **159**:1417-1432.
40. Peirs C, Williams S-PG, Zhao X, Walsh CE, Gedeon JY, Cagle NE, Goldring AC, Hioki H, Liu Z, Marell PS, Seal RP: **Dorsal horn circuits for persistent mechanical pain.** *Neuron* 2015, **87**:797-812.
41. Smith KM, Boyle KA, Mustapa M, Jobling P, Callister RJ, Hughes DI, Graham BA: **Distinct forms of synaptic inhibition and neuromodulation regulate calcitonin-positive neuron excitability in the spinal cord dorsal horn.** *Neuroscience* 2016, **326**:10-21.
- This study describes morphological, electrophysiological and pharmacological heterogeneity in a neurochemically distinct population of lamina II interneurons.
42. Gutierrez-Mecinas M, Davis O, Polgár E, Shahzad M, Navarro-Batista K, Furuta T, Watanabe M, Hughes DI, Todd AJ: **Expression of calcitonin among different neurochemical classes of interneuron in the superficial dorsal horn of the mouse spinal cord.** *Neuroscience* 2018, **398**:171-181.
- The neurochemical heterogeneity of calcitonin-expressing spinal interneurons is examined further using a combination of immunohistochemical approaches and viral labelling approaches in recombinase-expressing transgenic mouse lines.
43. Sathyamurthy A, Johnson KR, Matson KJE, Dobrott CI, Li L, Ryba AR, Bergman TB, Kelly MC, Kelley MW, Levine AJ: **Massively parallel single nucleus transcriptional profiling defines spinal cord neurons and their activity during behaviour.** *Cell Rep* 2018, **22**:2216-2225.
- One of two pioneering studies using transcriptomics to characterise neuronal populations in the mouse spinal cord, providing a catalogue of molecular markers that researchers can use to define subpopulations of spinal interneurons with greater precision.

44. Häring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lönnerberg P, Manno GL, Sharma N, Borgius L, Kiehn O *et al.*: **Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types.** *Nat Neurosci* 2018, **21**:869-880.
- The second comprehensive study where a single-cell transcriptomic approach has been used to characterise dorsal horn interneurons specifically, based on their expression of molecular markers and laminar distribution. The paper provides a links to an on-line database where researchers can map the expression patterns of various genes across the defined neuronal populations.
45. Branda C, Dymecki SM: **Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice.** *Dev Cell* 2004, **6**:7-28.
46. Joyner A, Zervas M: **Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system.** *Dev Dyn* 2006, **235**:2376-238550.
47. Dymecki SM, Kim JC: **Molecular neuroanatomy's "Three Gs": a primer.** *Neuron* 2007, **54**:17-34.
48. Foster E, Wildner H, Tudeau L, Haueter S, Ralvenius WT, Jegen M, Johannssen H, Hösl L, Haenraets K, Ghanem A *et al.*: **Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch.** *Neuron* 2015, **85**:1289-1304.
49. Zhang Y, Zhao S, Rodriguez E, Takatoh J, Han B-X, Zhou X, Wang F: **Identifying local and descending inputs for primary sensory neurons.** *J Clin Invest* 2015, **125**:3782-3794.
50. Huang J, Polgár E, Solinski HJ, Mishra SK, Tseng P-Y, Iwagaki N, Boyle KA, Dickie AC, Kriegbaum MC, Wildner H *et al.*: **Circuit dissection of the role of somatostatin in itch and pain.** *Nat Neurosci* 2018, **21**:707-716.
51. Towne C, Pertin M, Beggah AT, Aebischer P, Decosterd I: **Recombinant adeno-associated virus serotype 6 (rAAV2/6)-mediated gene transfer to nociceptive neurons through different routes of delivery.** *Mol Pain* 2009, **5**:52.
52. Badea TC, Hua ZL, Smallwood PM, Williams J, Rotolo T, Ye X, Nathans J: **New mouse lines for the analysis of neuronal morphology using CreER(T)/loxP-directed sparse labeling.** *PLoS One* 2009, **4**:e7859.
53. Li L, Rutlin M, Abaira VE, Cassidy C, Kus L, Gong S, Jankowski MP, Luo W, Heintz N, Koerber HR *et al.*: **The functional organization of cutaneous low-threshold mechanosensory neurons.** *Cell* 2011, **147**:1615-1627.
54. Olson W, Abdus-Saboor I, Cui L, Burdge J, Raabe T, Ma M, Luo W: **Sparse genetic tracing reveals regionally specific functional organization of mammalian nociceptors.** *eLife* 2017, **6**:e29507.
- In this study, the cutaneous patterns of innervation of unmyelinated mechanonociceptors in hairy and glabrous skin is assessed in a transgenic mouse where Cre recombinase expression within these sensory afferents can be activated by administration of a transgene inducing agent. The study demonstrates how the timing of administration of the transgene inducing agent determines the extent of recombinase expression, thus providing a useful means of manipulating the level of reporter expression in these afferents.
55. Luo L, Callaway EM, Svoboda K: **Genetic dissection of neural circuits.** *Neuron* 2008, **57**:634-660.
56. Fenno LF, Mattis J, Ramakrishnan C, Hyun M, Lee SY, He M, Tucciarone J, Selimbeyoglu A, Berndt A, Grosenick L *et al.*: **Targeting cells with single vectors using multiple-feature Boolean logic.** *Nat Methods* 2014, **11**:763-772.
57. Woolf CJ, Shortland P, Coggeshall RE: **Peripheral nerve injury triggers central sprouting of myelinated afferents.** *Nature* 1992, **355**:75-78.
58. Miki K, Fukuoka T, Tokunaga A, Noguchi K: **Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: up-regulation in a subpopulation of primary afferent sensory neurons.** *Neuroscience* 1998, **82**:1243-1252.
59. Ma W, Ramer MS, Bisby MA: **Increased calcitonin gene-related peptide immunoreactivity in gracile nucleus after partial sciatic nerve injury: age-dependent and originating from spared sensory neurons.** *Exp Neurol* 1999, **159**:459-473.
60. Ibuki T, Hama AT, Wang X-T, Pappas GD, Sagen J: **Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts.** *Neuroscience* 1997, **76**:845-858.
61. Eaton MJ, Plunkett JA, Karmally S, Martinez MA, Montanez K: **Changes in GAD- and GABA-immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplants of immortalized serotonergic precursors.** *J Chem Neuroanat* 1998, **16**:57-72.
62. Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sök A, de Koninck P, de Koninck Y: **Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain.** *Nature* 2003, **424**:938-942.
63. Petitjean H, Pawlowski SA, Fraine SL, Sharif B, Hamad D, Fatima T, Berg J, Brown CM, Jan LY, Ribeiro-da-Silva A *et al.*: **Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury.** *Cell Rep* 2015, **13**:1246-1257.
64. François A, Low SA, Sypek EI, Christensen AJ, Sotoudeh C, Beier KT, Ramakrishnan C, Ritola KD, Sharif-Naeini R, Deisseroth K *et al.*: **A brainstem-spinal cord inhibitory circuit for mechanical pain modulation by GABA and enkephalins.** *Neuron* 2017, **93**:1-18.
- A comprehensive multidisciplinary study that uses optogenetic and chemogenetic manipulation (both *in vivo* and *in vitro*) to determine the influence of descending inhibitory projections from the RVM on spinal circuits that play a key role in mechanical pain.
65. Haenraets K, Foster E, Johannssen H, Kandra V, Frezel N, Steffen T, Jaramillo V, Paterna J-C, Zeilhofer HU, Wildner H: **Spinal nociceptive circuit analysis with recombinant adeno-associated viruses: the impact of serotypes and promoters.** *J Neurochem* 2017, **142**:721-733.
- This study addresses the efficiency of AAV vector-mediated gene transfer into genetically defined neuron subtypes in both the central and peripheral nervous system, and serves to illustrate that the extent of transfection into certain cell populations may be dependent on AAV serotype.
66. Haenraets K, Albisetti GW, Foster E, Wildner H: **Adeno-associated virus-mediated transgene expression in genetically defined neurons of the spinal cord.** *J Vis Exp* 2018, **135**:e57382 <http://dx.doi.org/10.3791/57382>.
- A step-by-step guide to performing intraspinal injections for delivery of viral vectors into the spinal dorsal horn, with accompanying video to demonstrate surgical procedures, preparation of glass pipettes, and injection protocol.