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Rewards, perils and pitfalls of untangling spinal pain circuits

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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.

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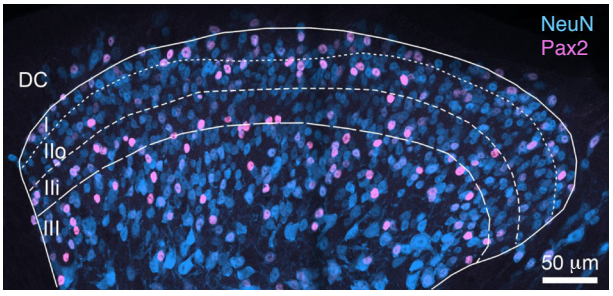
Background

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 projections 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.

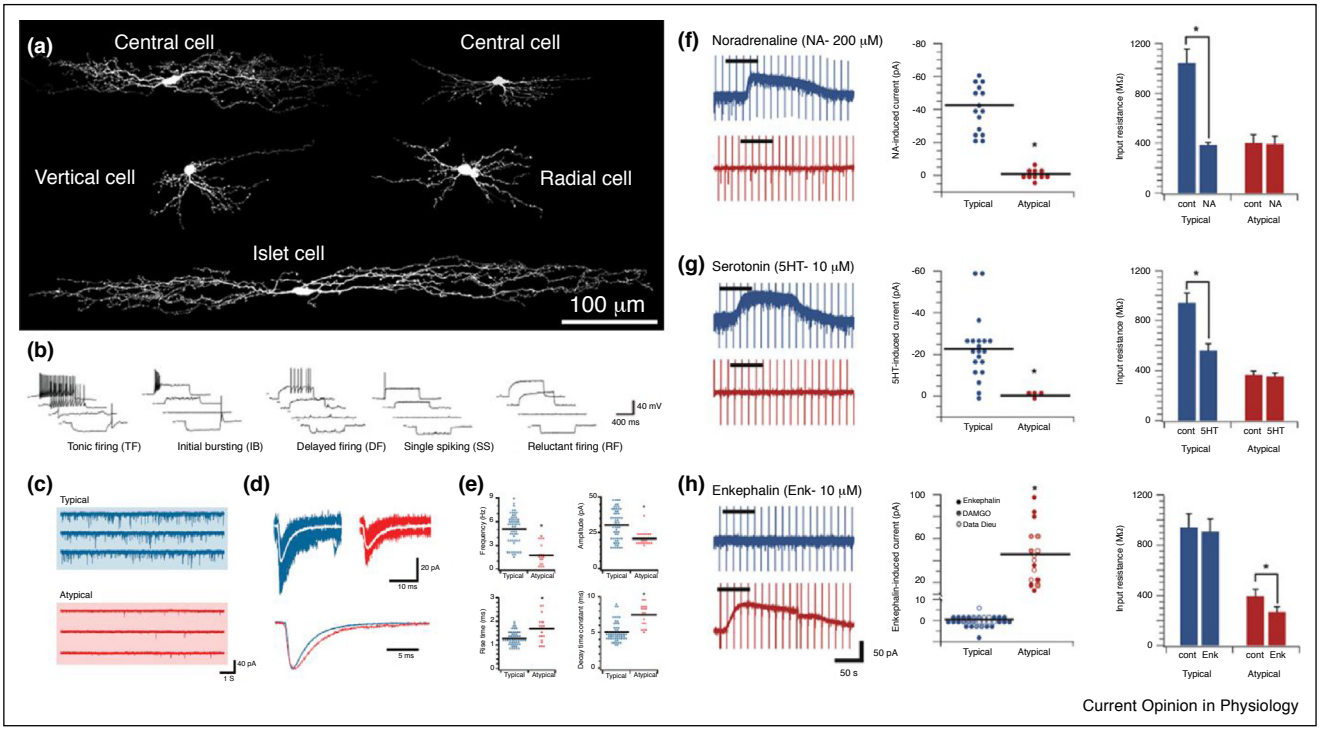
interneurons in lamina II [15–19], but rarely addressed questions of connectivity, apart from a few notable exceptions [20–25]. Nonetheless, these studies provide a wealth of information on the heterogeneity of neuronal populations in this region, using untargeted sampling method as all recordings were 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 promotor 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 promotor [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•].

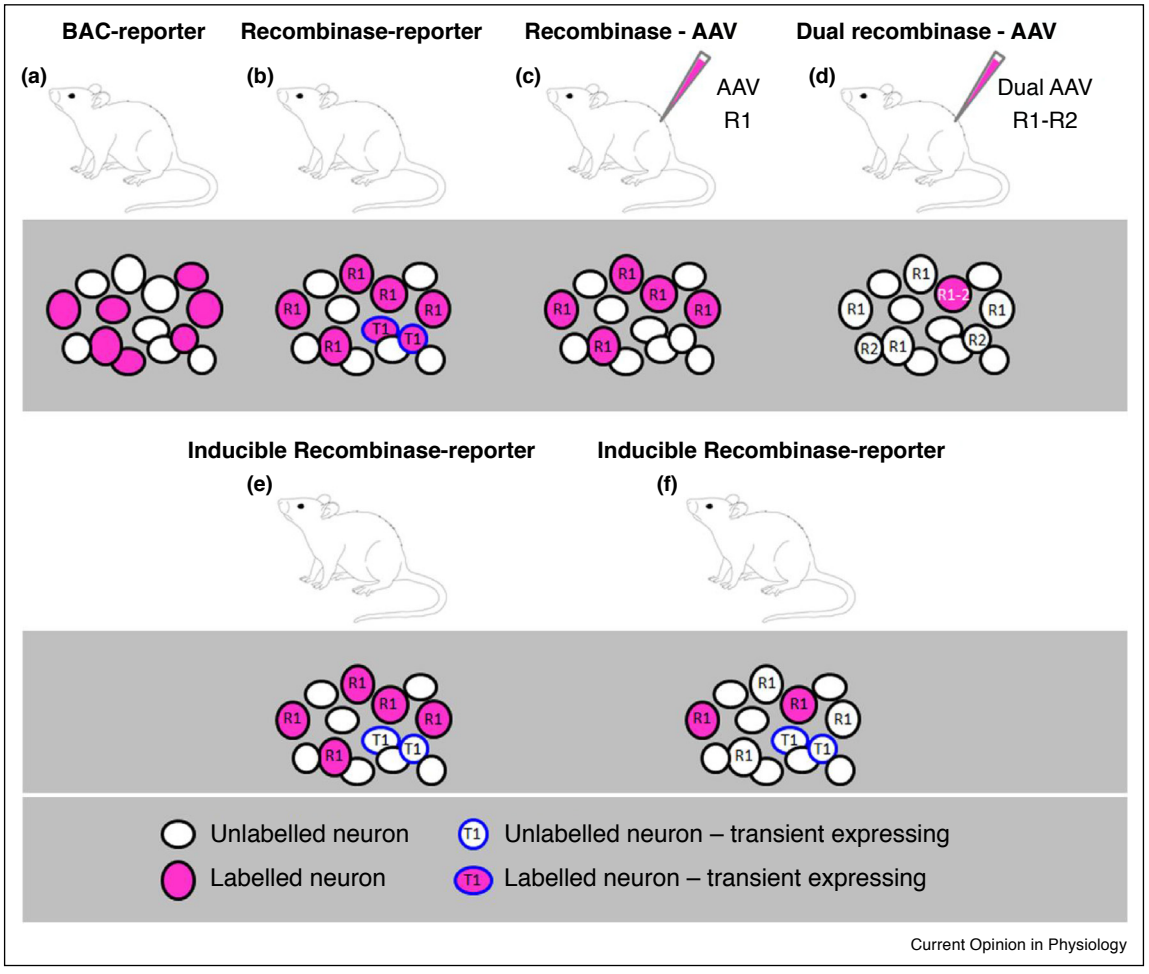
their molecular and genetic profiles [36•,43••,44••]. These schemes broadly match expression patterns for markers studied using immunohistochemistry [34,35], and are unprecedented as a source of information to differentiate molecular-genetic and neurochemical signatures for distinct interneuron populations.

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 promotor 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 resources 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.

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