1

Emerging Techniques in Basic Science and Translation

Michael Iadarola, PhD,[a] Stephen B. McMahon, PhD,[b] Sarah E. Ross, PhD,[c,d] Tayler D. Sheahan, PhD,[c,d] and Joost Verhaagen, PhD[e]

[a]Department of Perioperative Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA; [b]King's College London, Wolfson Centre for Age-Related Diseases, Guy's Campus, London, United Kingdom; [c]Department of Neurobiology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [d]Pittsburgh Center for Pain Research, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [e]Laboratory for Regeneration of Sensorimotor Systems, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Educational Objectives

1) Discuss the impact of RNA sequencing on pain and pain transcriptomics.
2) Describe molecular genetic approaches for investigating the neural circuits of pain.
3) Explain the principles of viral vector-mediated gene delivery.
4) Discuss routes of delivery to dorsal root ganglion (DRG) neurons and key applications of adeno-associated viral vectors (AAVs).
5) Highlight anticipated future developments, including the use of AAVs that can deliver genes to DRG neurons via noninvasive routes of delivery.
6) Review methods for in vivo imaging of neuronal activity and provide examples of their use in pain research.

Introduction

Pain research, along with other studies of the nervous system, has benefitted enormously from technical developments that permit studies that even a few decades ago would have been unimaginable. These developments affect almost all types of research. This chapter reviews some of the technical advances in vector-mediated gene delivery that are most relevant to pain research.

Impact of Next-Generation Sequencing on Pain and Pain Transcriptomics

Reaching down to the most basic of biological levels for the investigation of pain has been a challenging task. In biology, the sequence of DNA is frequently the most fundamental level of knowledge and irreducible fact. The objectives of this section are to provide an understanding of RNA sequencing (RNA-Seq, which uses the methods of DNA sequencing), explore the transcriptomic data RNA-Seq generates, and discuss what RNA-Seq analyses of neuronal gene expression tell us about pain processes in both animals and humans and how this knowledge can inform analgesic drug discovery.

The Sequencing Surge

We are in the midst of a totally revolutionary expansion of DNA sequencing technology and, consequently, nucleic acid sequence information emanating from such methodology [41,54,87]. These relatively new methods are just beginning to penetrate into the pain field, and the impact, while gaining strength, has yet to be fully realized. By comparison, massively parallel sequencing has transformed the field of oncology, although comparison to oncology is somewhat unrealistic since we cannot biopsy nervous system tissue from individuals with chronic pain. For the cancer field, high-throughput

Pain 2018: Refresher Courses, 17th World Congress on Pain
Michael S. Gold, Esther M. Pogatzki-Zahn, Mark S. Wallace, editors
IASP Press, Washington, D.C. © 2018

DNA sequencing methods are vastly increasing our detailed understanding of mechanisms of oncogenesis, genetic susceptibility to cancer, diagnostics, identification of biomarkers for precision treatments, the response to chemotherapy, and assessment of tumor eradication [13,60,67,86,102]. It is hoped that the pain field will reach these levels of insight. The use of whole genome sequencing presents challenges to experimental design for pain as well as basic issues of filtering sequence variants and identifying differences relevant to disease causality in an individual out of the 3 billion bases in genomic DNA [67]. In this regard, whole-genome sequencing or whole-exome sequencing (where just the genomic sequences that code for protein are analyzed) has been applied to family pedigrees with migraine or neuropsychiatric disorders, but precise identification of a specific gene or genes responsible for these disorders has remained elusive [36].

A Brief History of Sequencing

Since the introduction of chemical DNA sequencing by Maxam and Gilbert and enzymatic sequencing by Sanger and colleagues in the mid-1970s, sequencing technologies have experienced a steady growth in the amount of DNA that can be sequenced, the speed and accuracy of sequencing, and methods for data analysis, all accompanied by reductions in cost [87]. In the early 1980s, DNA sequencing involved radiolabeling DNA, running a thin slab gel, developing an autoradiogram on film, and reading the base sequence from four lanes of the gel, one for each nucleotide. Obtaining data on ~300 bases of sequence was extremely tedious. Autoradiography was replaced by fluorescent nucleotides and automated reading of the gel to enable analysis of 10,000 bases/day. The slab gel was replaced by capillary electrophoresis devices, eventually having 96 capillaries for highly parallel sequencing that did not require manual loading. Through automation of other preparative steps, a laboratory could generate sequence data 1 to 2 million bases/day. These types of capillary electrophoresis devices were used to sequence the human genome (~3 billion bases), the first draft of which was published in 2001 by the Human Genome Project and Celera Genomics. The present, workhorse, high-throughput sequencing devices do not use electrophoretic separation methods, but rather "sequencing by synthesis" of millions of DNA fragments at once using fluorescent bases (see the Sequencing by Synthesis video on the Illumina web site). These devices can perform 200 million sequencing reactions of ~100 bases for each reaction at once, generating ~20 billion bases of sequence information in a matter of days to hours. One does not have to "do the math" to realize that this is a massive improvement over 300 bases a day. Of equal importance is the parallel evolution of computerized techniques for sequence analysis and the establishment of databases of genomic assemblies and annotations (e.g., the University of California Santa Cruz [UCSC] Genome Browser database, the RefSeq at the National Center for Biotechnology Information [NCBI], and many others), all of which are essential for interpreting and merging genomic and transcriptomic information.

RNA Sequencing, Databases, and Pain

The use of computer analysis is particularly important to RNA-Seq because each of those 20 to 200 million ~100 base fragments must be aligned to the complementary region of the genome, and the exact gene they correspond to must be determined. Fig. 1 shows an alignment diagram of reads from the mouse μ-opioid receptor [46] extracted from a deep (200 million reads) RNA-Seq study of mouse dorsal root ganglion (DRG) cells [29]. This type of detailed quantitative information can be generated for all of the ~15,000 genes expressed in a DRG. As a result, we now know every gene that is expressed in the DRG of mice, rats, dogs, and humans and in nodose and superior cervical ganglion cells [29,55,75,76,77,82,83]. In fact RNA-Seq transcriptomics now extends to individual neurons in mouse DRG [100] and dorsal spinal cord [37] and in regions of the brain [21,61]. There no longer is any mystery as to what genes are expressed in the various populations of nociceptive neurons. Additionally, databases such as GTEx (https://www.gtexportal.org/home/) and the Human

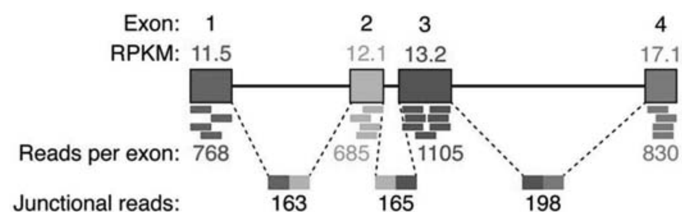

Fig. 1. Diagram of major exons of the mouse μ-opioid receptor. The genome aligned transcript counts were obtained from a deep (200 million reads) RNA-Seq analysis of Trvpv1-lineage DRG neurons [29,30]. Exons 1, 2, 3, and 4 are represented by the boxes. Exonic alignments show approximately equal reads/kilobase of transcript/million bases sequences (RPKM). Detection of "junctional reads" which contain sequences from two adjacent exons delineates splicing events and provides a quantitative measure of mRNA splicing. Junctional reads were detected between each of the major exons, spanning the introns between them (dashed lines). Additional splicing of *Oprm1* was not robustly detected (see [46]).

Protein Atlas (https://www.proteinatlas.org/) contain transcriptome information from multiple human tissues and organs including the spinal cord and 12 brain regions. Unfortunately, no peripheral nervous system ganglia are in these datasets. Nonetheless, by consulting any number of published or online resources, a very good assessment of the quantitative expression of any particular gene can be obtained.

Needless to say, the proliferation of high-throughput sequencing devices has produced a truly vast amount of sequence information that is growing in a seemingly double exponential fashion. Fig. 2 shows the growth of sequences deposited into the public Sequence Read Archive (SRA) of the U.S. Library of Medicine and NCBU. Deposition of information is occurring at a staggering rate: note that the increments on the *y*-axis are in quadrillions of bases! Many different types of experiments are represented in the SRA, and new information is continuously deposited. For example, we were interested in RNA-Seq and wound healing but did not identify relevant research reports in PubMed. However, in early April 2018, results from nearly 2,500 single-cell sequencing reactions had been deposited in SRA that we could download, align with our pipeline, and use for guidance and interpretation of experiments. This resource is a phenomenal savings in time and resources. Our laboratory and others have contributed sequencing runs from mouse, rat, canine, and human DRG and spinal cord, rat sciatic nerve and, in some cases, with and without nociceptive or other manipulations. It is important to point out that the information here is in a raw format and needs to be analyzed for quality and aligned before it can be used. Nonetheless, the sequencing only has to be executed once, and the goal is to establish a database of the animal and human "Nociceptome" as well as evaluations of the impact of pain manipulations on brain regions that comprise the pain matrix [45,103]. Such a database can be consulted to make informed judgements for projects that are being proposed. The most fundamental question to ask is "Is the gene expressed in the target tissue?" The very next question is: "To what degree (how much) is it expressed to make it worthwhile for investigation?" Answering these seemingly simple questions is not always straightforward, and we will discuss various considerations in the next section.

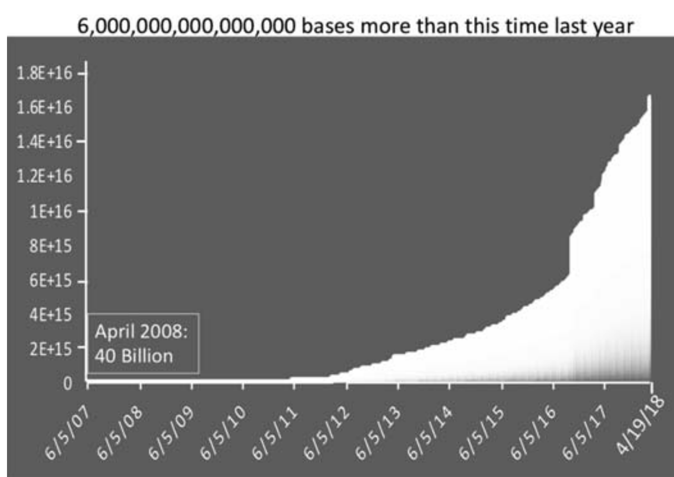

Fig. 2. Bases in the Sequence Read Archive (SRA), April 2018.

Quantitative Considerations, or How Much Is Enough?

From a numerical standpoint, RNA-Seq is basically a counting method. It is suggested that the counts of a particular transcript may provide an informative reference point for predicting the potential success of a drug development project or predicting potential side effects (or at least put a tissue on the "radar screen" for on-target or side effects). The method can provide a good estimate of "zero counts," which can be used as inclusion or exclusion criteria, and observations of DRG gene expression in the literature can be examined in detail across multiple species. For example, in several papers, gastrin-releasing peptide (GRP) was suggested to be the neuropeptide that transmitted itch in mice. Our mouse RNA-Seq datasets [29] unequivocally indicated that GRP was not expressed in mouse DRG or in humans or rats. Further examination revealed that the immunocytochemical results had incorrectly identified GRP immunoreactivity in DRG because of antibody cross-reactivity to the abundant neuropeptides substance P and neuromedin B [30]. Species differences also play a role. Tyrosine hydroxylase (TH) is abundantly expressed in neurons of mouse DRG. Immunocytochemistry and single-cell sequencing show these neurons innervating multiple mouse hair follicles. However, the TH gene exhibits very low expression in rat DRG and no expression in human DRG [30]. Establishing a rough idea of equivalency in expression patterns between model species and humans is tremendously important for understanding pain mechanisms in humans and for translational analgesic drug development efforts. This is a task for which RNA-Seq is particularly well suited.

Other genes originally identified in DRG and subjected to translational efforts present more of an enigma and may require further investigation. A particularly intriguing case is the angiotensin II receptor 2 gene (AGTR2). After this receptor was identified in

DRG neurons using immunohistochemistry, the availability of antagonist drugs fueled a translational effort. However, RNA-Seq shows that this gene is not expressed in mouse, rat, or human DRG, suggesting that the foundational data with antibodies and reverse-transcription/polymerase chain reaction amplification may have been measuring a cross-reacting protein or an inconsequential amount of transcript [33]. What might this mean for drug development? From the present vantage of RNA-Seq, the lack of expression would suggest not starting in the first place! Nevertheless, based on the foundational data, AGTR2 antagonists were developed and tested in pain models. Eventually, the lead compound EMA401 reached a Phase II clinical trial for postherpetic neuropathic pain, where it outperformed placebo [79]. Subsequent studies in animal models attribute the analgesic effects to infiltrating macrophages or T cells [49,88]. The potential lesson here is that basal state expression may not reflect important elements of a dynamic state that occurs with persistent inflammation or nerve-injury-induced neuropathic pain. RNA-Seq of such models needs to be conducted and added to the transcriptomic database of pain.

Quantitative considerations also inform targets such as ion channels (e.g., TRPV1, sodium channels) which generally require high expression to produce depolarization of a nerve ending or action potential propagation. By comparison, the expression of G-protein receptors varies greatly, and the rules governing the interplay between expression, translation to protein, protein localization in the cell (e.g., pre- or postsynaptic), and turnover rate are still being delineated. The ability of a metabotropic receptor to occupy a critical subcellular niche particularly suited to controlling key processes of intercellular communication such as neurotransmitter release is likely to be an important determinant of how much expression is needed to influence function.

Future Directions

This short exposition has not touched on different types of RNAs such as long noncoding RNAs, the variety of sequencing devices, or the potential impact of whole-genome sequencing. Combinations of these methods will be useful for identifying mutations underlying undiagnosed inherited neuropathies [82] and genes underlying gain or loss of function mutations that affect pain sensitivity, such as in the pain channelopathies [34]. Progress in the use of whole genomic information will be driven by many factors, not the least of which is the increased accessibility of direct-to-consumer genetic testing [105] and sequencing. Penetration of sequencing to the public at large is likely to accelerate demands for physicians to evaluate an individual patient's self-obtained genomic information as part of a pretreatment evaluation. Benefits for pain management accrue through an understanding a particular patient's pharmacogenetics for drug metabolism [15], risk for anesthesia-related side effects, and other factors that have a clear genetic profile. In summary, it is evident that the pain field is just beginning to reap the rewards of the new sequencing technologies, that it will bring clarity to many areas, and that the field as a whole will advance.

Molecular Genetic Approaches for Investigating the Neural Circuits of Pain

The ability to target distinct cell types using molecular genetic tools has revolutionized the way we study the neural circuits that underlie pain. Here we explain how these tools work and how they can be used to glean new insight into nociceptive processing.

Genetically Modified Mice

Genetically modified mice have been widely used to study the function of gene products in normal and pathological pain conditions. The null mutant, or knockout, was the first and continues to be the most common approach employed, despite the growing body of evidence indicating that one should be extremely cautious in drawing conclusions about the function of a protein based on the phenotype of the mouse that developed in the absence of the protein in question. However, as individual cells are the functional units of neural circuits, there is growing interest in the properties and connections of specific neuronal cell types within circuits and their roles in nociceptive and pain processing.

A major challenge to targeting specific cell types has been understanding what defines a distinct neuron population. In some cases, cell types have been defined by the expression of a gene thought to be important for the manifestation of pain. For example, *Scn10a*, the gene encoding the voltage-gated sodium channel $Na_V1.8$, has been used to broadly define nociceptive sensory neurons [4,19], whereas smaller subpopulations of putative nociceptive afferents have been

defined by the expression of TRPV1, TRPM8, and MrgprD. Identification of these genes has enabled the generation of mice in which it is not only possible to manipulate expression of the gene of interest, but through site-specific recombinase technology, it is now possible to employ a growing array of genetic tools that give us the ability to visualize, manipulate the function of, and record the activity of specific neuronal subtypes. As noted above, the advent of single-cell RNA-sequencing technology has led to the identification of additional cell-type-specific markers for physiologically and/or morphologically distinct neuron populations. For instance, in a recent wave of single-cell transcriptome analyses, it has been suggested that there are at least 11 sensory neuron and 30 spinal cord dorsal horn neuron types that can be defined based on their gene expression patterns [37,69,84,100]. This type of analysis is important because the identification of gene expression patterns will allow us to target neuron populations with even more specificity.

As just noted, a widespread approach for gaining genetic access to a given neuron population of interest is site-specific DNA recombination [7,58,59]. Two commonly used site-specific recombination systems include the bacteriophage recombinase Cre/locus of cross over (Cre-LoxP) and the yeast flipase/FLP recognition site (Flp-FRT) systems. These systems require two transgenes: (1) a cell-type-specific driver transgene that encodes a recombinase to promote genetic recombination and (2) a target transgene that encodes an effector protein.

Cre or Flp driver mouse lines can be generated using multiple strategies. For instance, a modified bacterial artificial chromosome (BAC) can be used to randomly integrate a driver transgene that is under the control of the promoter sequence of an endogenous gene of interest into the mouse genome [51]. While BACs mediate transgenesis in fertilized eggs with high efficiency, the regulatory elements for the endogenous pain-related gene of interest driving the expression of the transgene may be absent owing to random integration. Thus, expression of the BAC transgene may not faithfully recapitulate endogenous expression levels. Alternatively, homologous recombination in embryonic stem cells can be used to insert a driver transgene's coding sequence into the locus of the endogenous pain-related gene of interest. This so-called “knockin” approach can disrupt the DNA sequence that encodes the endogenous gene with the driver transgene. In some cases, loss of one endogenous gene copy may produce unintended phenotypic changes. To maintain endogenous gene expression, the internal ribosomal entry site (IRES) or self-cleaving 2A peptide can be included in the targeting vector, and both the endogenous gene and the transgene will be expressed [66,94]. Examples of pain-related driver lines and the neuron populations they target are described in Table I.

When both a driver and target transgene are expressed in an animal, the site-specific recombinase (Cre or FLP) acts as a molecular scissors and irreversibly removes transcriptional stops (flanked by LoxP/FRT sites), initiating expression of the effector protein in a genetically distinct cell type (Fig. 1A). Thus, target transgenes are typically placed into the locus of a gene that provides close to ubiquitous expression, such as the Rosa26 locus [111], but the expression of the effector only occurs in the cell types that express the recombinase, thereby conferring cell type specificity (Fig. 1A).

Optogenetic and Chemogenetic Tools

We now have a wide range of effector tools that allow for the visualization of genetically distinct neuron populations, as well as manipulation of their activity (Fig. 1A). While genetically encoded voltage sensors that enable monitoring of neuronal activity have been developed, limitations in both the genetic tools and the actual hardware for data acquisition have necessitated the use of genetically encoded calcium sensors as an indirect measure of neuronal activity. Using these tools, the field has already made great strides in understanding pain neural circuitry. For instance, fluorescent reporters have been widely used in targeted electrophysiology recordings to characterize cell types and have demonstrated that increased excitability in neuron populations, including GABAergic medium spiny neurons of the indirect pathway within the nucleus accumbens, contribute to nerve-injury-induced pain behavior [78].

Fluorescent reporters are often tagged to optogenetic and chemogenetic tools for manipulating neuronal activity. Light-sensitive proteins that rapidly excite or inhibit cells have provided unprecedented power for probing the contribution of neuron populations within pain circuits [8,14]. For example, illumination of peripheral nerve terminals in the skin expressing the blue-light-activated ion channel, channelrhodopsin, demonstrated that activation of $Na_V1.8$ primary afferents elicits pain behavior and causes long-term sensory neuron sensitization [17]. Chemogenetic strategies such

Table I
Examples of pain-related recombinase driver mouse lines

Driver Line	Neuron Population	Genetic Targeting	Reference
Sensory Neuron Driver Lines			
Advillin-Cre	Sensory neurons	BAC transgenic	[114]
Advillin-CreERT2	Sensory neurons	BAC transgenic	[56]
Pirt-IRES-Cre	Sensory neurons	Knockin	[52]
Brn3a-CreERT2	Sensory neurons*	BAC transgenic	[70]
$Na_V1.8$-Cre	Nociceptors	Knockin	[92]
$Na_V1.8$-Cre (SNS-Cre)	Nociceptors	BAC transgenic	[3]
$Na_V1.8$-CreERT2	Nociceptors	Knockin	[112]
TRPV1-Cre	TRPV1-lineage sensory neurons*	BAC transgenic	[65]
TRPV1-IRES-Cre	TRPV1-lineage sensory neurons*	Knockin	[9]
TRPM8-Cre	Cold-sensitive sensory neurons	BAC transgenic	[109]
Mrgprd-Cre	Nonpeptidergic polymodal nociceptors	BAC transgenic	[104]
Mrgprd-CreERT	Nonpeptidergic polymodal nociceptors	Knockin	[71]
Tac1-IRES2-Cre	Substance P-expressing sensory neurons*	Knockin	[38]
Mrgpra3-Cre	Itch-sensitive sensory neurons	BAC transgenic	[35]
Sst-IRES-Flp	Itch-sensitive sensory neurons*	Knockin	[40]
Sst-IRES-Cre	Itch-sensitive sensory neurons*	Knockin	[96]
Spinal Cord Dorsal Horn Driver Lines			
GRP-Cre	Excitatory interneurons**	BAC transgenic	[110]
PKCγ-Cre	Excitatory interneurons**	Knockin	[18]
PKCγ-CreER	Excitatory interneurons**	Knockin	[1]
CR-IRES-Cre	Excitatory interneurons**	Knockin	[96]
CR-CreER	Excitatory interneurons**	Knockin	[96]
VGluT3-Cre	Excitatory interneurons**	BAC transgenic	[32]
RORα-IRES-Cre	Excitatory interneurons**	Knockin	[12]
GRPR-Cre	Excitatory itch-responsive interneurons**	BAC transgenic	[5]
CCK-CreER	Lineage excitatory interneurons**	Knockin	[96]
CCK-IRES-Cre	Lineage excitatory interneurons**	Knockin	[96]
NPY-Cre	Inhibitory interneurons**	Knockin	[64]
PV-Cre	Inhibitory interneurons**	Knockin	[28]
Cdh3-CreER	Inhibitory interneurons**	BAC transgenic	[1]
nNos-CreER	Inhibitory interneurons**	Knockin	[96]
Pdyn-IRES-Cre	Lineage inhibitory interneurons**	Knockin	[53]
Lbx1-Cre	Lbx1-lineage interneurons**	Knockin	[90]
Lbx1-Flp	Lbx1-lineage interneurons**	Knockin	[20]
Tlx3-Cre	Tlx3-lineage interneurons**	Knockin	[107]
Bhlhb5-Cre	Excitatory and inhibitory interneurons**	Knockin	[80]
NK1R-CreER	Spinal projection neurons**	Knockin	[44]

*Not specific to primary afferents. **Not specific to spinal cord. *Abbreviations:* BAC, bacterial artificial chromosome; Bhlhb5, base helix loop helix protein 5; Brn3a (Pou4F1), Pou class 4 homeobox 1; CCK, cholecystokinin; Cdh3, cadherin 3; CR, calretinin; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; Lbx1, ladybird homeobox 1; Mrgpra3, Mas-related GPCR member 3; Mrgprd, Mas-related GPCR member D; NK1R, neurokinin-1 receptor; nNos, neuronal nitric oxide synthase; NPY, neuropeptide Y; Pdyn, preprodynorphin; PKCγ, protein kinase C gamma; PV, parvalbumin; RORα, RAR-related orphan receptor alpha; Sst, somatostatin; Tac1, tachykinin 1; Tlx3, T-cell leukemia, homeobox 3; TRPM8, transient receptor potential channel melastatin 8; TRPV1, transient receptor potential channel vanilloid 1; VGluT3, vesicular glutamate transporter type 3.

as designer receptors exclusively activated by designer drugs (DREADDs) operate on a longer timescale, and they have similarly been used to show that inhibition of TRPV1-lineage sensory neurons produces antinociception and decreases neuronal excitability [81]. Lastly, calcium and voltage sensors can also be used to perform cell-type-specific activity monitoring. For instance, using the genetically encoded calcium indicator GCaMP to monitor activity of spinal cord dorsal horn interneurons has revealed that heat-responsive neurons encode absolute skin temperature, whereas cold-responsive neurons encode temperature change [74].

These molecular genetic approaches to study neural circuit function have already begun to provide new insights into the processing of noxious input. However, there are important limitations that should be considered when interpreting findings obtained from these approaches. Foremost, a single gene does not define a specific cell type. Rather, cell types are distinguished by complex gene expression profiles. To target a more refined genetic cell type, intersectional genetics strategies that combine the Cre/LoxP and Flp/FRT systems can be used to target a neuron population that actively expresses two genes of interest (Fig. 1B) [57,72]. This intersectional genetic strategy has been used to target somatostatin and dynorphin lineage spinal interneurons, which were found to comprise a microcircuit that gates and transmits mechanical pain [20].

Another important consideration is that Cre and Flp driver lines will lead to recombination in every cell with an active promoter for the endogenous gene of interest throughout development, which often overrepresents the number of neurons that functionally express the gene in adulthood. Strategies developed to gain temporal control over recombinase activity include the tetracycline (tet)-responsive system [28] and the more widely used ligand-regulated form of Cre, called CreER [26,63]. CreER is a fusion protein with Cre and a mutated form of the estrogen-receptor-binding domain. Translocation of CreER to the nucleus is induced by administration of the estrogen receptor antagonist tamoxifen, and thus the timing of transgene expression can be controlled.

To summarize, these molecular genetic tools have opened the door to cell-type-specific functional analyses of the neurons comprising nociceptive and pain circuits. To date, we have made many foundational insights into the neuron cell types that integrate nociceptive input in both the peripheral and central nervous system. As we enhance our understanding of the genetic identity of these neurons, we will be poised to further dissect the intricacies of circuit organization.

Genetic Modification of Primary Sensory Neurons with Adeno-Associated Viral Vectors

The primary sensory neurons of the peripheral nervous system have an important role in major neurological diseases, including peripheral neuropathies and pain. These neurons are therefore an attractive target for gene delivery. Gene delivery to neurons of the peripheral nervous system has a history that dates back to the end of the 1980s [27]. Initially, vectors based on herpes simplex virus and adenovirus were adapted for gene transfer to neurons. These recombinant viral vectors expressed endogenous viral genes, in addition to the transgene, resulting in an adverse tissue response and short-lived transgene expression. Over the years adeno-associated viral vectors (AAVs) have become popular and powerful gene delivery agents for sensory neurons. AAVs lack endogenous viral genes, have a low risk of insertional mutagenesis and immunogenicity, and can be produced relatively easily at high titer and at clinical grade [39].

With the use of AAV vectors to manipulate gene expression it is possible to study the functions of specific proteins in primary sensory neurons in, for example, axon regeneration, peripheral neuropathies, and neuropathic pain. In addition to the study of gene function, AAV vectors have been used to deliver light-sensitive ion channels or DREADDS to manipulate neuronal activity [47] or genetically encoded calcium channels for optical in vivo assessment of the functional characteristics of primary sensory neurons [11]. A recent and exciting application of AAV vectors is their use for in vivo gene editing [73]. Thus, AAV vectors are powerful tools for experimental genetic manipulation of primary sensory neurons and are gaining increasing acceptance as a clinical gene delivery platform.

Production of AAV Vectors

Many laboratories obtain their AAV vectors conveniently from local or commercial vector cores. However, small-scale production of AAV vectors is now feasible in any well-equipped molecular biology laboratory. All commonly used AAV serotypes are produced by co-transfection in human embryonic kidney (HEK) cells of a vector plasmid, which harbors the transgene of interest under an appropriate promoter flanked by the AAV-ITRs (inverted terminal repeats), and helper plasmids encoding the capsid genes of the desired serotype. Following co-transfection the cells start to produce AAV vector particles, which can be purified by iodixanol gradient ultracentrifugation, resulting in research stocks with titers ranging from 5 × 1012 to 2 × 1013 genomic copies/mL [101]. Large-scale production protocols in either insect cells [91] or mammalian cells [31] have also been developed. These production systems are mainly used to generate large

amounts of AAV for preclinical studies in large animals or for clinical studies.

AAV Serotypes and Transduction Efficiency of Sensory Neurons

Following the demonstration that human AAV2 efficiently transduced central nervous system (CNS) neurons in rodents [50], several other AAV serotypes (AAV1 and AAV3 to AAV9) have been identified with natural variations in amino-acid capsid identity [39]. Variation in capsid structure profoundly affects cellular tropism and expression kinetics. We investigated the transduction efficiency of AAV1 to AAV6 and AAV8 in adult rat primary sensory neurons [62]. Titer-matched AAV serotype vectors were directly infused in two lumbar DRG (L4, L5). All serotypes preferentially transduced neurons (albeit at different transduction efficiency), except AAV6, which transduced neurons and satellite cells. Our best-performing serotype, AAV5, transduced 48% of primary sensory neurons after 2 weeks, followed by AAV1, which transduced 37% of DRG neurons. The number of cells that expressed green fluorescent protein (GFP) increased over time, reaching 90% transduction efficiency in AAV5-injected DRG 12 weeks after injection. To our knowledge no systematic serotype comparison has been performed for mouse DRG, but AAV1 [97] and AAV9 [11,106] exhibit high transduction efficiencies in mouse sensory neurons.

Routes of Delivery of AAV Vectors to Primary Sensory Neurons

While we have established effective gene transfer to the DRG by direct injection of AAV, alternative delivery routes for the transduction of DRG neurons have also been explored. Intrathecal delivery of self-complementary AAV1, AAV8 [93], and AAV5 [23,24] produced significant transgene expression in the DRG of adult rats comparable to that seen after direct injection. AAV6 and AAV9 transduced neurons of the DRG efficiently in adult mice following intrathecal injection or injection via the sciatic nerve [11,99]. Since transgene expression was studied at 3 weeks after intrathecal delivery of AAV6, it is not known if transgene expression declines at longer post-injection time points as we observed after direct injection of AAV6 in the DRG of rats [62]. When we compare direct and intrathecal injection, the advantages of direct injection are that transduction remains local in the injected ganglia, with minimal leakage to adjacent tissues, and that low quantities of viral vector are needed. Intrathecal injection is a fast and simple procedure, but high quantities of viral vector are required, and leakage of vector to the cord and periphery occurs.

Recently, AAV9 capsids were engineered to yield capsids that enabled efficient noninvasive gene delivery to the brain (PHP.eB) and primary sensory neurons (PHP.S) in mice [10]. Intravenous administration of AAV-PHP.S transduced 82% of DRG neurons as well as cardiac and enteric neurons. The discovery of these newly engineered variants opens up many new possibilities for research and clinical application. First, intravenous AAV delivery will most likely become the approach of choice for genetic targeting of specific organs and cell types in mice, including the DRG. By combining the PHP.S capsid with specific promoter/enhancer elements, one would be able to target subtypes of sensory neurons in the DRG and to prevent transgene expression in other peripheral tissues. Second, it is conceivable that AAV capsids can be developed that have further specificity for distinct populations of sensory neurons, thus allowing noninvasive targeting of sensory neurons with distinct properties. This advance would further enhance the transduction specificity and safety of intravenous AAV delivery.

In Vivo Imaging of Neuronal Activity

The human brain consists of about 10^{10} individual neurons (the functional unit of the nervous system), and each of these neurons typically makes thousands or tens of thousands of synaptic connections with other neurons, producing a truly staggering level of complexity unmatched by any other system in the body. Understanding how this system functions remains a considerable challenge, but one that is benefiting from recent technological developments. The traditional method that has been pursued for more than half a century is to record either single neuron activity or composite ensemble activity either directly (e.g., EEG) or indirectly (e.g., fMRI). Other than in rare circumstances (e.g., intraoperatively), the single-unit method has only been possible in animal studies. Using these techniques, we have learned a great deal about the brain, including the processing of pain-related information. However, novel techniques are emerging that are having a dramatic effect on our understanding of neuronal circuits, and one that we review here is the use of optical methods

to indirectly observe the activity of hundreds of neurons simultaneously.

Calcium Indicators

Calcium-sensitive fluorophores exploit the fact that each action potential that invades the soma of a neuron is normally associated with a significant transient rise in intracellular calcium. If such sensors are introduced into neurons and then appropriately illuminated, their fluorescence increases with each action potential. This approach has been much used in vitro where tissue slices or dissociated neurons are "loaded" with dyes such as Indo-1, Fura-2, and Oregon Green. After washing, the fluorescence of cells containing the fluorophores reflects intracellular calcium, which is used as a proxy for neuronal activity. These dyes can in some cases be used in vivo, but they suffer from a major drawback—they cannot be targeted to chosen cells. This limitation has been overcome with the introduction and refinement of genetically encodable calcium indicators. The most used of these are GCaMPs, a series of sensors that consist of a calcium sensor, calmodulin, fused with a fluorophore (a variant of GFP), and a third protein, M13, which is a domain of myosin light chain that can bind calmodulin. The GFP only weakly fluoresces in the absence of calcium, but when calcium binds to calmodulin, a conformational change in the complex results in strong fluorescence [2]. The first of these sensors [68] was thermally unstable, but later versions were introduced that could be used at mammalian body temperatures [95]. Further modified versions have been introduced that have greater sensitivity, signal-to-noise ratio, and dynamic responsiveness. The most extensively used sensors currently are CGaMP3 and GCaMP6 (which come in three versions, "slow," "medium," and "fast"), but a version, GCaMP8, with an even wider dynamic range is now available. Fig. 2 shows the excitation and emission spectra for one GCaMP6 variant with low (Fig. 2A) and high (Fig. 2B) calcium. Accordingly, 488nm light is typically used as the optimal excitation wavelength. Since GFP has many uses, a "red" version of the calcium sensor (RCaMP) is also available with red-shifted spectral characteristics [16,113].

Targeting Calcium Sensors

GCaMPs can be expressed in neurons in a variety of ways, a great number using commercially available resources. One method is to use viral vectors. This approach and its limitations is discussed more fully above, but in brief, GCaMPs can be expressed by a variety of AAVs and other viral vectors. Constructs under various promoters and utilizing different serotypes are commercially available from a variety of suppliers including Addgene (https://www.addgene.org/viral-service/), UPenn Vector Core (http://www.med.upenn.edu/gtp/vectorcore/), and the University of Zurich vector facility (http://www.vvf.uzh.ch/en/viralvectorproduction.html). These vectors have been successfully used to transduce mature peripheral and central mammalian neurons, including primary afferent nociceptors, spinal cord projection neurons, and somatosensory cortical neurons [11,,22,48,52,85,98]. A second approach, as described above, is to use GCaMP driven transgenically either using "knockin" mice or BAC technology. Again, a variety of such mice are commercially available. A particularly flexible resource is a STOP-Flox-GCaMP6s C57BL/6 mouse available through Jackson laboratories (Stock No. 028866). Here, GCaMP is Cre-dependent, so it can be selectively expressed by crossing the line with any of the extensive list of Cre-lines available, or by using virally induced Cre. In this way, adult expression of GCaMP in subsets of nociceptive neurons or in specific groups of CNS neurons can be achieved.

Monitoring GCaMP Activity in Vivo

Recent and continuing advances in microscopy mean that it is now fairly routine to monitor GCaMP fluorescence in hundreds of individual nerve cells simultaneously. Point scanning confocal or 2-photon systems allow a two- or three-dimensional image to be created by sequentially scanning a tightly focused spot. The limitation for image acquisition depends on the scanning rate of the system and the integration time needed for each pixel. In reality, large fields of view, say 512 × 512 pixels, can be acquired at more than one frame a second. If a three-dimensional volume is to be explored, then multiple frames in the *z*-axis need to be acquired for each image, which therefore cannot be refreshed so rapidly. Images can be acquired faster if multiple points are scanned simultaneously, which can be achieved using so-called light sheet and spinning disk microscopes, which can improve acquisition speeds by 100 times or more (see [42] for review). An important practical point in all real-world systems is the trade-off between improving optical performance with increasing laser light power and the damage and photobleaching that also increases with laser power. For practical use, an animal expressing GCaMP needs to be placed under the confocal

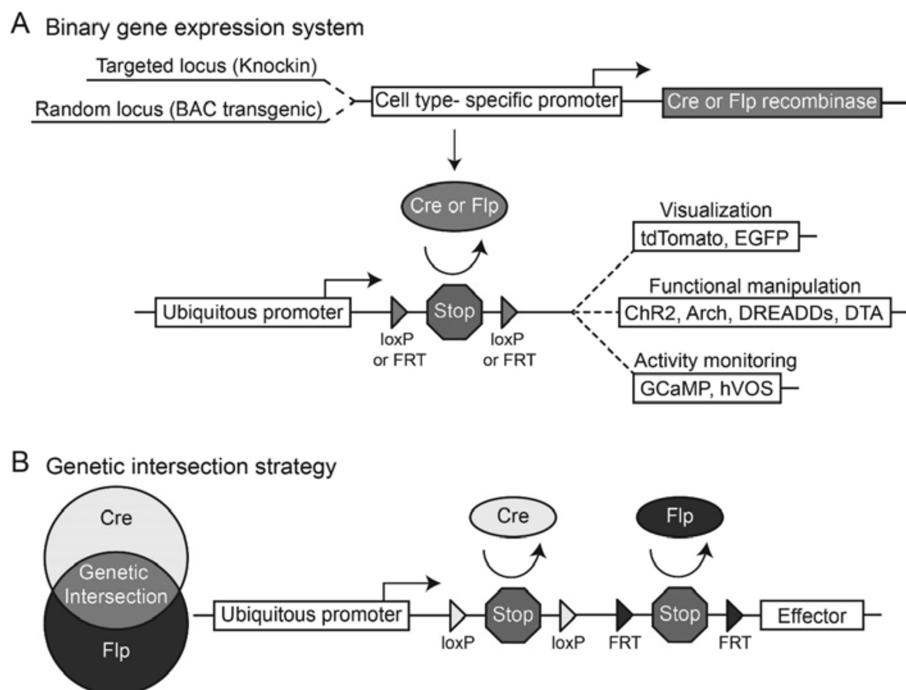

Fig. 3. Cell-type-specific genetic targeting. (A) In the binary gene expression system, a driver mouse line that expresses Cre or Flp recombinase under the control of a cell-type-specific promoter in the endogenous (knockin) or a random gene (BAC transgenic) locus is crossed to an effector mouse line with a transgene for visualization, manipulation, or activity monitoring that is expressed under a ubiquitous promoter and is only expressed when recombination at designated target sites (loxP or FRT) removes a transcription stop signal. (B) A genetic intersection strategy combining Cre and Flp recombinases that are expressed under different cell type-specific promoters can be used to target a more genetically refined neuron population. The effector is only expressed in neurons in which both transcriptional stops are removed. BAC, bacterial artificial chromosome; ChR2, channelrhodopsin-2; Arch, archaerhodopsin; DTA, diphtheria toxin fragment A; hVOS, hybrid voltage sensor.

or 2-photon microscope. This is most easily done if the animal is anesthetized, but there are systems of restraining conscious animals so that the microscope remains focused on the same GCaMP-expressing neurons. For pain research, there are likely to be some further ethical issues around the use of unanesthetized animals. The cells to be imaged have to be accessible to the microscope. In the anesthetized state, surgery to expose the brain, spinal cord, or DRG is necessary. In anesthetized animals, a “thinned-skull” procedure [108] allows imaging of cortex through the cranium, or the chronic implantation of clear “windows” over neuronal structures can be used [25]. Two-photon microscopy has the advantage of being able to image a thin optical plane hundreds of microns within the tissue, while confocal microscopy is limited to more superficial structures but does have some other advantages [11].

Another option is to mount very lightweight camera systems onto freely moving animals and access GCaMP labeled neurons via implanted optical fibers focused with attached gradient-index (GRIN) lenses. A commercial system of this type is available from Inscopix (https://www.inscopix.com/), which uses a camera weighing only 2 g. Such a system can be readily

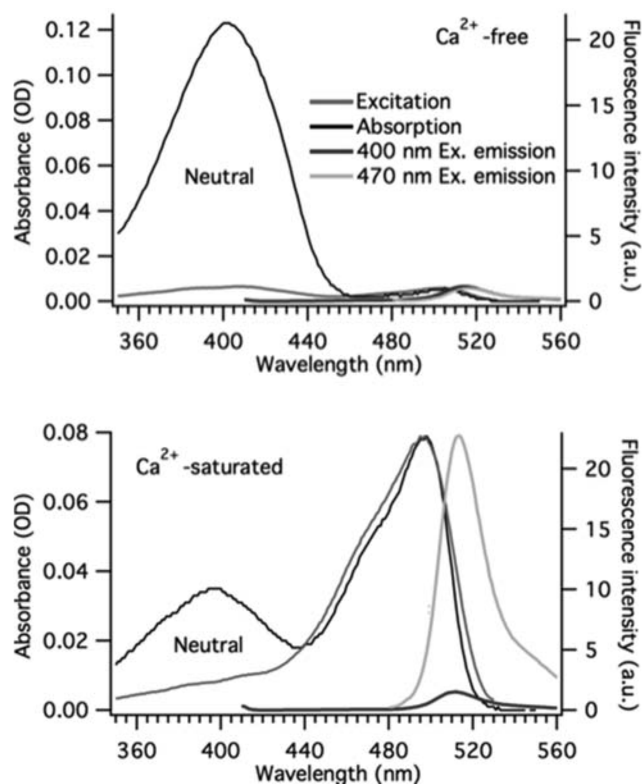

Fig. 4. Absorbance and emission spectra for GCaMP6m in the absence (top) or presence (bottom) of calcium. Note the very large increase in emitted fluorescent light when the GCaMP6m has calcium bound. From [6].

mounted onto the cranium of rats and mice, but, particularly for pain research, it might be adapted to image from spinal or even peripheral neurons.

Examples of Use in Pain Research

The pain field has been rather slow in adopting these in vivo imaging methods. However, multiple reports are now appearing in which pain-related processes have been studied at different levels of the neuroaxis. Peripheral ganglia (DRG) have been the focus of multiple studies because the neurons are suitably located for imaging after laminectomy, and of course, the importance of nociceptors is well recognized. There have also been studies using optical imaging of spinal neurons and cortical networks. Fig. 3 shows some data from one of our recent publications highlighting the utility of the approach. Fig. 3A shows three frames from a video of an L4 DRG imaged in an anesthetized mouse. The first frame represents basal fluorescence, in the absence of any applied stimulation. The second frame shows the fluorescence recorded during a period of 20-Hz electrical stimulation of the sciatic nerve at an intensity to activate only A fibers. A subset of mainly large DRG neurons fluoresce as action potentials propagate into the DRG. The third frame illustrates the responses of the same ganglia during 5-Hz electrical sciatic nerve stimulation at C-fiber strength. Here, the majority of DRG neurons are activated, both large and small. Fig. 3B shows the fluorescent signal recorded from a small number of different cells (each color a different cell) in response to electrical stimulation of the sciatic nerve at different rates. Several important points are apparent. First, most cells give a clear response to a single action potential arising from nerve stimulation. Second, the calcium transients summate with repeated stimulation above 0.5 Hz. This means that for DRG cells at least, the average level of fluorescence reflects the amount of afferent activity. Fig. 3C illustrates the time course of calcium fluorescence associated with a single action potential for two typical cells. The time constant of decay in calcium signal is about half a second, explaining the summation of fluorescence with activity at 1 Hz or above.

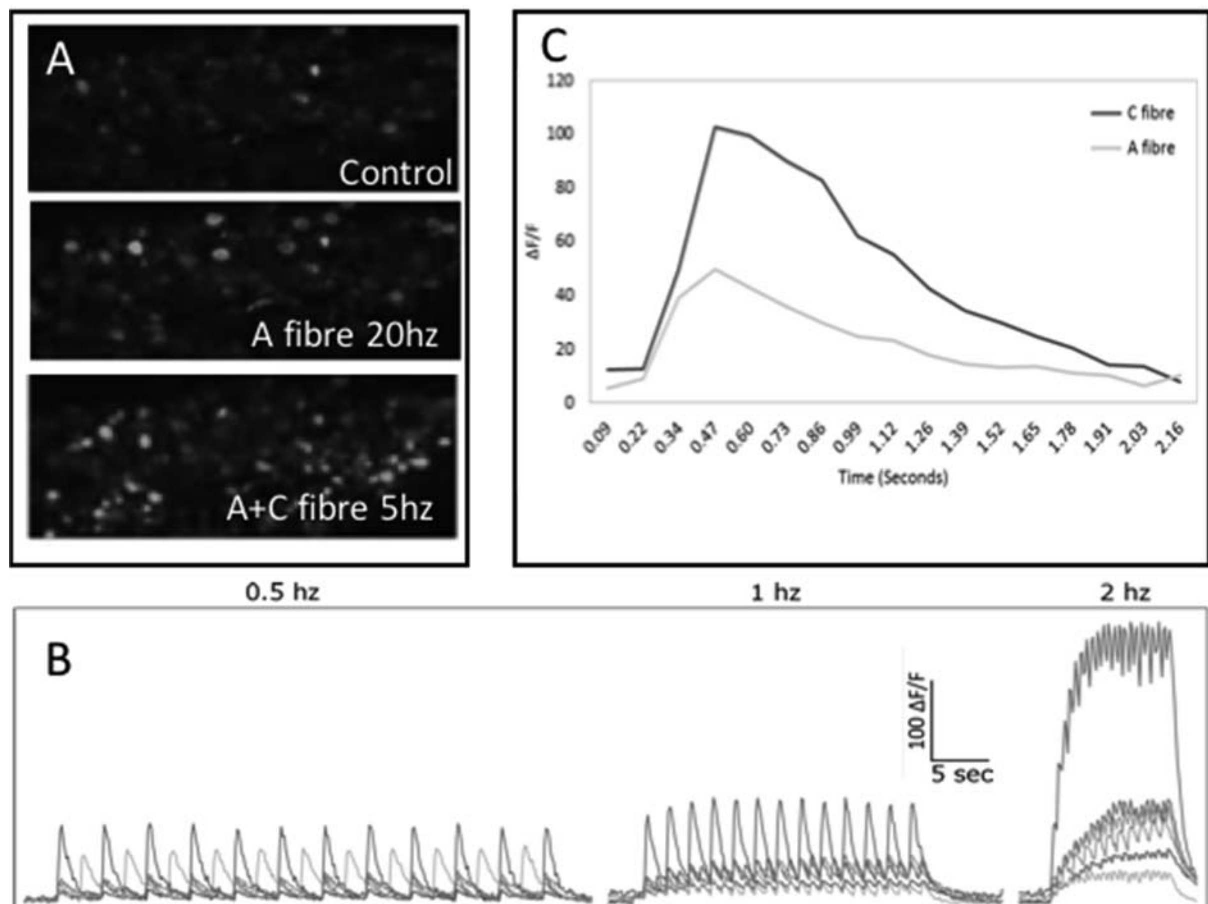

Fig. 5. (A) Fluorescence of L4 dorsal root ganglion (DRG) neurons recorded during stimulation of the sciatic nerve with different intensities. (B) Fluorescent intensity of six typical cells during electrical stimulation of the sciatic nerve at 0.5 to 2 Hz. (C) Time course of calcium transients in DRG neurons with either a myelinated or unmyelinated axon following invasion of a single action potential [11].

Limitations of Use

While powerful, optical imaging has some limitations. It is important to realize that it does not directly measure neuronal excitability, and the relationship between activity and calcium levels will often be unknown and may not be fixed. Another important point is that the calcium transients associated with activity have long time constants, and so the technique has limited temporal resolution irrespective of the technology used to monitor it. GCaMP is a calcium buffer, and so its expression may affect cellular function, especially if it is expressed at high levels. Finally, long-term recording can be compromised by photobleaching, especially if high light levels are employed.

Future Directions

The continuing rapid advances in microscopy are likely to improve the utility of optical imaging. The miniaturization of technology is likely to increase the ease of use of imaging techniques and extend them to monitor activity in freely moving animals in many circumstances. Finally, continuing development of the sensors such as genetically encodable voltage sensors is likely to offer many new possibilities. We are likely to learn much from the continuing use of these techniques (Figs. 4, 5).

References

[1] Abraira VE, Kuehn ED, Chirila A, Springel M, Toliver A, Zimmerman A, Orefice L, Boyle KA, Bai L, Song B, Bashista K, O'Neill T, Zhuo J, Tsa C, Jessica H, Rutlin M, Kus L, Niederkofler V, Watanabe M, Dymecki SM, Nelson S, Heintz N, Hughes D, Ginty DD. The cellular and synaptic architecture of the mechanosensory dorsal horn. Cell 2017;168:295–310.e19.

[2] Akerboom J, Rivera JD, Guilbe MM, Malave EC, Hernandez HH, Tian L, Hires SA, Marvin JS, Looger LL, Schreiter ER. Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. J Biol Chem 2009;284:6455–64.

[3] Agarwal N, Offermanns S, Kuner R. Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 2004;38:122–9.

[4] Akopian AN, Sivilotti L, Wood JN. A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. Nature 1996;379:257–62.

[5] Aresh B, Freitag FB, Perry S, Blümel E, Lau J, Franck MCM, Lagerström MC. Spinal cord interneurons expressing the gastrin-releasing peptide receptor convey itch through VGLUT2-mediated signaling. Pain 2017;158:945–61.

[6] Barnett LM, Hughes TE, Drobizhev M. Deciphering the molecular mechanism responsible for GCaMP6m's Ca^{2+}-dependent change in fluorescence. PLoS One 2017;12:e0170934.

[7] Branda CS, Dymecki SM. Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. Dev Cell 2004;6:7–28.

[8] Carr FB, Zachariou V. Nociception and pain: lessons from optogenetics. Front Behav Neurosci 2014;8:1–6.

[9] Cavanaugh DJ, Chesler AT, Bráz JM, Shah NM, Julius D, Basbaum AI. Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. J Neurosci 2011;31:10119–27.

[10] Chan KY, Jang MJ, Yoo BB, Greenbaum A, Ravi N, Wu WL, Sánchez-Guardado L, Lois C, Mazmanian SK, Deverman BE, Gradinaru V. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. Nat Neurosci 2017;20:1172–80.

[11] Chisholm KI, Khovanov N, Lopes DM, La Russa F, McMahon SB. Large scale in vivo recording of sensory neuron activity with GCaMP6. eNeuro 2018;5(1).

[12] Chou SJ, Babot Z, Leingärtner A, Studer M, Nakagawa Y, O'Leary DDM. Geniculocortical input drives genetic distinctions between primary and higher-order visual areas. Science 2013;340:1239–42.

[13] Cieślik M, Chinnaiyan AM. Cancer transcriptome profiling at the juncture of clinical translation. Nat Rev Genet 2018;19:93–109.

[14] Copits BA, Pullen MY, Gereau RW. Spotlight on pain: optogenetic approaches for interrogating somatosensory circuits. Pain 2016;157:2424–33.

[15] Crews KR, Gaedigk A, Dunnenberger HM, Leeder JS, Klein TE, Caudle KE, Haidar CE, Shen DD, Callaghan JT, Sadhasivam S, Prows CA, Kharasch ED, Skaar TC; Clinical Pharmacogenetics Implementation Consortium. Clinical Pharmacogenetics Implementation Consortium guidelines for cytochrome P450 2D6 genotype and codeine therapy: 2014 update. Clin Pharmacol Ther 2014;95:376–82.

[16] Dana H, Mohar B, Sun Y, Narayan S, Gordus A, Hasseman JP, Tsegaye G, Holt GT, Hu A, Walpita D, Patel R, Macklin JJ, Bargmann CI, Ahrens MB, Schreiter ER, Jayaraman V, Looger LL, Svoboda K, Kim DS. Sensitive red protein calcium indicators for imaging neural activity. Elife 2016;5.

[17] Daou I, Tuttle AH, Longo G, Wieskopf JS, Bonin RP, Ase AR, Wood JN, De Koninck Y, Ribeiro-da-Silva A, Mogil JS, Seguela P. Remote optogenetic activation and sensitization of pain pathways in freely moving mice. J Neurosci 2013;33:18631–40.

[18] Ding YQ, Xiang CX, Chen ZF. Generation and characterization of the PKCgamma-Cre mouse line. Genesis 2005;43:28–33.

[19] Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN. The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J Physiol 2003;550:739–52.

[20] Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X, Ross SE, Lowell BB, Wang Y, Goulding M, Ma Q. Identification of spinal circuits transmitting and gating mechanical pain. Cell 2014;159:1417–32.

[21] Ecker JR, Geschwind DH, Kriegstein AR, Ngai J, Osten P, Polioudakis D, Regev A, Sestan N, Wickersham IR, Zeng H. The BRAIN Initiative Cell Census Consortium: lessons learned toward generating a comprehensive brain cell atlas. Neuron 2017;96:542–57.

[22] Emery EC, Luiz AP, Sikandar S, Magnusdottir R, Dong X, Wood JN. In vivo characterization of distinct modality-specific subsets of somatosensory neurons using GCaMP. Sci Adv 2016;2:e1600990.

[23] Fagoe ND, Eggers R, Verhaagen J, Mason MR. A compact dual promotor adeno-associated viral vector for efficient delivery of two genes to dorsal root ganglion neurons. Gene Ther 2014;21:242–52.

[24] Fagoe ND, Eggers R, Verhaagen J, Mason RJ. Gene delivery to neurons of the dorsal root ganglia using adeno-associated viral vectors. Neuromethods 2015;98:175–90.

[25] Farrar MJ, Bernstein IM, Schlafer DH, Cleland TA, Fetcho JR, Schaffer CB. Chronic in vivo imaging in the mouse spinal cord using an implanted chamber. Nat Methods 2012;9:297–302.

[26] Feil R, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun 1997;237:752–7.

[27] Geller AI, Breakefield XO. A defective HSV-1 vector expresses Escherichia coli beta-galactosidase in cultured peripheral neurons. Science 1988;241:1667–9.

[28] Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992;89:5547–51.

[29] Goswami SC, Mishra SK, Maric D, Kaszas K, Gonnella GL, Clokie SJ, Kominsky HD, Gross JR, Keller JM, Mannes AJ, Hoon MA, Iadarola MJ. Molecular signatures of TRPV1 lineage neurons revealed by RNA-Seq of mouse and rat transcriptomes. J Pain 2014;15;12:1338–59.

[30] Goswami SC, Thierry-Mieg D, Thierry-Mieg J, Mishra SK, Hoon MA, Mannes AJ, Iadarola MJ. Itch-associated peptides: RNA-Seq and bioinformatic analysis of natriuretic precursor peptide B and gastrin releasing peptide in dorsal root and trigeminal ganglia, and the spinal cord. Mol Pain 2014;10:44.

[31] Grieger JC, Soltys SM, Samulski RJ. Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. Mol Ther 2016;24:287–97.

[32] Grimes WN, Seal RP, Oesch N, Edwards RH, Diamond JS. Genetic targeting and physiological features of VGLUT3+ amacrine cells. Vis Neurosci 2011;28:381–92.

[33] Hafko R, Villapol S, Nostramo R, Symes A, Sabban EL, Inagami T, Saavedra JM. Commercially available angiotensin II At_2 receptor antibodies are nonspecific. PLoS One 2013;1;8:e69234.

[34] Han C, Yang Y, Te Morsche RH, Drenth JP, Politei JM, Waxman SG, Dib-Hajj SD. Familial gain-of-function Nav1.9 mutation in a painful channelopathy. J Neurol Neurosurg Psychiatry 2017;88:233–40.

[35] Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, Kim Y, Nie H, Qu L, Patel KN, Li Z, McNeil B, He S, Guan Y, Xiao B, Lamotte RH, Dong X. A subpopulation of nociceptors specifically linked to itch. Nat Neurosci 2013;16:174–82.

[36] Hansen RD, Christensen AF, Olesen J. Family studies to find rare high risk variants in migraine. J Headache Pain 2017;18:32.

[37] Häring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lönnerberg P, La Manno G, Sharma N, Borgius L, Kiehn O, Lagerström MC, Linnarsson S, Ernfors P. Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. Nat Neurosci 2018;21:869–80.

[38] Harris JA, Hirokawa KE, Sorensen SA, Gu H, Mills M, Ng LL, Bohn P, Mortrud M, Ouellette B, Kidney J, Smith KA, Dang C, Sunkin S, Bernard A, Oh SW, Madisen L, Zeng H. Anatomical characterization of Cre driver mice for neural circuit mapping and manipulation. Front Neural Circuits 2014;8:1–16.

[39] Hastie E and Samulski RJ. Adeno-associatedvirus at 50: a golden anniversary of discovery, research and gene therapy success—a personal perspective. Hum Gene Ther 2015;26:257–65.

[40] He M, Tucciarone J, Lee SH, Nigro MJ, Kim Y, Levine JM, Kelly SM, Krugikov I, Wu P, Chen Y, Gong L, Hou Y, Osten P, Rudy B, Huang ZJ. Strategies and tools for combinatorial targeting of GABAergic neurons in mouse cerebral cortex. Neuron 2016;91:1228–43.

[41] Heather JM, Chain B, The sequence of sequencers: the history of sequencing DNA. Genomics 2016;107:1–8.

[42] Hillman EM, Voleti V, Patel K, Li W, Yu H, Perez-Campos C, Benezra SE, Bruno RM, Galwaduge PT. High-speed 3D imaging of cellular activity in the brain using axially-extended beams and light sheets. Curr Opin Neurobiol 2018;50:190–200.

[43] Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, Ladle DR, Arber S. A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS Biol 2005;3:e159.

[44] Huang H, Kuzirian MS, Cai X, Snyder LM, Cohen J, Kaplan DH, Ross SE. Generation of a NK1R-CreER knockin mouse strain to study cells involved in neurokinin 1 receptor signaling. Genesis 2016;54:593–601.

[45] Iadarola MJ, Berman KF, Zeffiro TA, Byas-Smith MG, Gracely RH, Max MB, Bennett GJ. Neural activation during acute capsaicin-evoked pain and allodynia assessed with PET. Brain 1998;121:931–47.

[46] Iadarola MJ, Sapio MR, Mannes AJ. A new splice of life for the μ-opioid receptor. J Clin Invest 2015;26:2558–61.

[47] Iyer SM, Montgomery KL, Towne C, Lee SY, Ramakrishnan C, Deisseroth K, Delp SL. Virally mediated optogenetic excitation and inhibition of pain in freely moving nontransgenic mice. Nat Biotechnol 2014;32:274–8.

[48] Johannssen HC, Helmchen F. In vivo Ca^{2+} imaging of dorsal horn neuronal populations in mouse spinal cord. J Physiol 2010;588:3397–402.

[49] Khan N, Muralidharan A, Smith MT. Attenuation of the infiltration of angiotensin ii expressing $CD3^+$ T-cells and the modulation of nerve growth factor in lumbar dorsal root ganglia - a possible mechanism underpinning analgesia produced by EMA300, an angiotensin II type 2 (AT_2) receptor antagonist. Front Mol Neurosci 2017;21;10:389.

[50] Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat Genet 1994;8:148–54.

[51] van Keuren ML, Gavrilina GB, Filipiak WE, Zeidler MG, Saunders TL. Generating transgenic mice from bacterial artificial chromosomes: transgenesis efficiency, integration and expression outcomes. Transgenic Res 2009;18:769–85.

[52] Kim YS, Anderson M, Park K, Zheng Q, Agarwal A, Gong C, Saijilafu, Young LA, He S, LaVinka PC, Zhou F, Bergles D, Hanani M, Guan Y, Spray DC, Dong X. Coupled activation of primary sensory neurons contributes to chronic pain. Neuron 2016;91:1085–96.

[53] Krashes MJ, Shah BP, Madara JC, Olson DP, Strochlic DE, Garfield AS, Vong L, Pei H, Watabe-Uchida M, Uchida N, Liberles SD, Lowell BB. An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature 2014;507:238–42.

[54] Koboldt DC, Steinberg KM, Larson DE, Wilson RK, Mardis ER. The next-generation sequencing revolution and its impact on genomics. Cell 2013;155:27–38.

[55] LaPaglia DM, Sapio MR, Burbelo PD, Thierry-Mieg J, Thierry-Mieg D, Ramsden CE, Iadarola MJ, Mannes AJ. RNA-Seq investigations of human post-mortem trigeminal ganglia. Cephalalgia 2018;38:912–32.

[56] Lau J, Minett MS, Zhao J, Dennehy U, Wang F, Wood JN, Bogdanov YD. Temporal control of gene deletion in sensory ganglia using a tamoxifen-inducible Advillin-Cre-ERT2 recombinase mouse. Mol Pain 2011;7:100.

[57] Luan H, Peabody NC, Vinson CR, White BH. Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. Neuron 2006;52:425–36.

[58] Luo L, Callaway EM, Svoboda K. Genetic dissection of neural circuits. Neuron 2008;57:634–60.

[59] Luo L, Callaway EM, Svoboda K. Genetic dissection of neural circuits: a decade of progress. Neuron 2018;98:256–81.

[60] Lieberman J. Tapping the RNA world for therapeutics. Nat Struct Mol Biol 2018;25:357–64.

[61] Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, McCarroll SA. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 2015;161:1202–14.

[62] Mason MR, Ehlert EM, Eggers R, Pool CW, Hermening S, Huseinovic A, Timmermans E, Blits B, Verhaagen J. Comparison of AAV serotypes for gene delivery to dorsal root ganglion neurons. Mol Ther 2010;18:715–24.

[63] Metzger D, Clifford J, Chiba H. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci USA 1995;92:6991–5.

[64] Milstein AD, Bloss EB, Apostolides PF, Vaidya SP, Dilly GA, Zemelman B V, Magee JC. Inhibitory gating of input comparison in the CA1 microcircuit. Neuron 2015;87:1274–89.

[65] Mishra SK, Tisel SM, Orestes P, Bhangoo SK, Hoon MA. TRPV1-lineage neurons are required for thermal sensation. EMBO J 2011;30:582–93.

[66] Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. Visualizing an olfactory sensory map. Cell 1996;87:675–86.

[67] Nakagawa H, Fujita M. Whole genome sequencing analysis for cancer genomics and precision medicine. Cancer Sci 2018;109:513–22.

[68] Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. Nat Biotechnol 2001;19:137–41.

[69] Nguyen MQ, Wu Y, Bonilla LS, von Buchholtz LJ, Ryba NJP. Diversity amongst trigeminal neurons revealed by high throughput single cell sequencing. PLoS One 2017;12:e0185543.

[70] O'Donovan KJ, Ma K, Guo H, Wang C, Sun F, Han SB, Kim H, Wong JK, Charron J, Zou H, Son Y-J, He Z, Zhong J. B-RAF kinase drives developmental axon growth and promotes axon regeneration in the injured mature CNS. J Exp Med 2014;211:801–14.

[71] 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;6:1–26.

[72] Pfeiffer BD, Ngo TTB, Hibbard KL, Murphy C, Jenett A, Truman JW, Rubin GM. Refinement of tools for targeted gene expression in Drosophila. Genetics 2010;186:735–55.

[73] Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. Nature 2015;9;520:186–91.

[74] Ran C, Hoon MA, Chen X. The coding of cutaneous temperature in the spinal cord. Nat Neurosci 2016;19:1201–9.

[75] Raithel SJ, Sapio MR, LaPaglia DM, Iadarola MJ, Mannes AJ. Transcriptional changes in dorsal spinal cord persist after surgical incision despite preemptive analgesia with peripheral resiniferatoxin. Anesthesiology 2018;128:620–35.

[76] Ramsden CE, Domenichiello AF, Yuan ZX, Sapio MR, Keyes GS, Mishra SK, Gross JR, Majchrzak-Hong S, Zamora D, Horowitz MS, Davis JM, Sorokin AV, Dey A, LaPaglia DM, Wheeler JJ, Vasko MR, Mehta NN, Mannes AJ, Iadarola MJ. A systems approach for discovering linoleic acid derivatives that potentially mediate pain and itch. Sci Signal 2017;22;10(493).

[77] Ray P, Torck A, Quigley L, Wangzhou A, Neiman M, Rao C, Lam T, Kim JY, Kim TH, Zhang MQ, Dussor G, Price TJ. Comparative transcriptome profiling of the human and mouse dorsal root ganglia: an RNA-seq-based resource for pain and sensory neuroscience research. Pain 2018;159:1325–45.

[78] Ren W, Centeno MV, Berger S, Wu Y, Na X, Liu X, Kondapalli J, Apkarian AV, Martina M, Surmeier DJ. The indirect pathway of the nucleus accumbens shell amplifies neuropathic pain. Nat Neurosci 2015;19:220–2.

[79] Rice ASC, Dworkin RH, McCarthy TD, Anand P, Bountra C, McCloud PI, Hill J, Cutter G, Kitson G, Desem N, Raff M, EMA401-003 study group. EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: a randomised, double-blind, placebo-controlled phase 2 clinical trial. Lancet 2014;10;383:1637–47.

[80] Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ, Greenberg ME. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron 2010;65:886–98.

[81] Saloman JL, Scheff NN, Snyder LM, Ross SE, Davis BM, Gold MS. Gi-DREADD expression in peripheral nerves produces ligand-dependent analgesia, as well as ligand-independent functional changes in sensory neurons. J Neurosci 2016;36:10769–81.

[82] Sapio MR, Goswami SC, Gross JR, Mannes MJ, Iadarola, MJ. Transcriptomics analyses of genes and tissues in inherited sensory neuropathies. Exp Neurol 2016;283:375–95.

[83] Sapio MR, Neubert JK, LaPaglia DM, Maric D, Keller JM, Raithel SJ, Rohrs EL, Anderson EM, Butman JA, Caudle RM, Brown DC, Heiss JD, Mannes AJ, Iadarola MJ. Pain control through selective chemo-axotomy of centrally-projecting TRPV1+ sensory neurons. J Clin Invest 2018;2;128:1657–70.

[84] 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 behavior. Cell Rep 2018;22:2216–25.

[85] Sekiguchi KJ, Shekhtmeyster P, Merten K, Arena A, Cook D, Hoffman E, Ngo A, Nimmerjahn A. Imaging large-scale cellular activity in spinal cord of freely behaving mice. Nat Commun 2016;7:11450.

[86] Shalek AK, Benson M. Single-cell analyses to tailor treatments. Sci Transl Med 2017;20;9(408).

[87] Shendure J, Balasubramanian S, Church GM, Gilbert W, Rogers J, Schloss JA, Waterston RH. DNA sequencing at 40: past, present and future. Nature 2017;550:345–53.

[88] Shepherd AJ, Copits BA, Mickle AD, Karlsson P, Kadunganattil S, Haroutounian S, Tadinada SM, de Kloet AD, Valtcheva MV, McIlvried LA, Sheahan TD, Jain S, Ray PR, Usachev YM, Dussor G, Krause EG, Price TJ, Gereau RW 4th, Mohapatra DP. Angiotensin II triggers peripheral macrophage-to-sensory neuron redox crosstalk to elicit pain. J Neurosci 2018;38:7032–57.

[89] Shrivats Mohan Iyer, Kate L Montgomery, Chris Towne, Soo Yeun Lee, Charu Ramakrishnan, Karl Deisseroth, Scott L Delp. Virally mediated optogenetic excitation and inhibition of pain in freely moving non-transgenic mice. Nat Biotechnol 2014;32:274–8.

[90] Sieber M, Storm R, Martinez-de-la-Torre M, Müller T, Wende H, Reuter K, Vasyutina E, Birchmeier C. Lbx1 acts as a selector gene in the fate determination of somatosensory and viscerosensory relay neurons in the hindbrain. J. Neurosci 2007;27:4902–9.

[91] Smith RH, Levy JR, Kotin RM. A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. Mol Ther 2009;17:1888–96.

[92] Stirling LC, Forlani G, Baker MD, Wood JN, Matthews EA, Dickenson AH, Nassar MA. Nociceptor-specific gene deletion using heterozygous Na_V1.8-Cre recombinase mice. Pain 2005;113:27–36.

[93] Storek B, Reinhardt M, Wang C, Janssen WG, Harder NM, Banck MS, Morrison JH, Beutler AS. Sensory neuron targeting by self-complementary AAV8 via lumbar puncture for chronic pain. Proc Natl Acad Sci USA 2008;105:1055–60.

[94] Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single "self-cleaving" 2A peptide-based retroviral vector. Nat Biotechnol 2004;22:589–94.

[95] Tallini YN, Ohkura M, Choi BR, Ji G, Imoto K, Doran R, Lee J, Plan P, Wilson J, Xin HB, Sanbe A, Gulick J, Mathai J, Robbins J, Salama G, Nakai J, Kotlikoff MI. Imaging cellular signals in the heart in vivo: cardiac expression of the high-signal Ca2? indicator GCaMP2. Proc Natl Acad Sci USA 2006;103:4753–8.

[96] Taniguchi H, He M, Wu P, Kim S, Paik R, Sugino K, Kvitsani D, Fu Y, Lu J, Lin Y, Miyoshi G, Shima Y, Fishell G, Nelson SB, Huang ZJ. A Resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. Neuron 2011;71:995–1013.

[97] Tedeschi A, Dupraz S, Laskowski CJ, Xue J, Ulas T, Beyer M, Schultze JL, Bradke F. The calcium channel subunit Alpha2delta2 suppresses axon regeneration in the adult CNS. Neuron 2016;19;92:419–34.

[98] Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, Petreanu L, Akerboom J, McKinney SA, Schreiter ER, Bargmann CI, Jayaraman V, Svoboda K, Looger LL. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Methods 20019;6:875–81.

[99] 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;5:52–68.

[100] Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat. Neurosci 2015;18:145–53.

[101] Verhaagen J, Hobo B, Ehlert EME, Eggers R, Korecka JA, Hoyng SA, Attwell CL, Harvey AR, Mason MRJ. Small scale production of recombinant adeno-associated viral vectors for gene delivery to the nervous system. Methods Mol Biol 2018;1715:3–17.

[102] Verma R, Sharma PC. Next generation sequencing-based emerging trends in molecular biology of gastric cancer. Am J Cancer Res 2018;1;8:207–25.

[103] Wager TD, Atlas LY, Lindquist MA, Roy M, Woo CW, Kross E. An fMRI-based neurologic signature of physical pain. N Engl J Med 2013;11;368:1388–97.

[104] Wang H, Zylka MJ. Mrgprd-expressing polymodal nociceptive neurons innervate most known classes of substantia gelatinosa neurons. J Neurosci 2009;29:13202–9.

[105] Webborn N, Williams A, McNamee M, Bouchard C, Pitsiladis Y, Ahmetov I, Ashley E, Byrne N, Camporesi S, Collins M, Dijkstra P, Eynon N, Fuku N, Garton FC, Hoppe N, Holm S, Kaye J, Klissouras V, Lucia A, Maase K, Moran C, North KN, Pigozzi F, Wang G. Direct-to-consumer genetic testing for predicting sports performance and talent identification: consensus statement. Br J Sports Med 2015;49):1486–91.

[106] Weir GA, Middleton SJ, Clark AJ, Daniel T, Khovanov N, McMahon SB, Bennett DL. Using an engineered glutamate-gated chloride channel to silence sensory neurons and treat neuropathic pain at the source. Brain 2017;140:2570–85.

[107] Xu Y, Lopes C, Qian Y, Liu Y, Cheng L, Goulding M, Turner EE, Lima D, Ma Q. Tlx1 and Tlx3 Coordinate specification of dorsal horn pain-modulatory peptidergic neurons. J Neurosci 2008;28:4037–46.

[108] Yang G, Pan F, Parkhurst CN, Grutzendler J, Gan WB. Thinned-skull cranial window technique for long-term imaging of the cortex in live mice. Nat Protoc 2010;5:201–8.

[109] Yarmolinsky DA, Peng Y, Pogorzala LA, Rutlin M, Hoon MA, Zuker CS. Coding and Plasticity in the MammalThermosensory System. Neuron 2016;92:1079–92.

[110] Yu YQ, Barry DM, Hao Y, Liu XT, Chen ZF. Molecular and neural basis of contagious itch behavior in mice. Science 2017;355:1072–6.

[111] Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci USA 1997;94:3789–94.

[112] Zhao J, Nassar M, Gavazzi I, Wood JN. Tamoxifen-inducible NaV1.8-CreERT2 recombinase activity in nociceptive neurons of dorsal root ganglia. Genesis 2006;44:364–71.

[113] Zhao Y, Araki S, Wu J, Teramoto T, Chang YF, Nakano M, Abdelfattah AS, Fujiwara M, Ishihara T, Nagai T, Campbell RE. An expanded palette of genetically encoded Ca^{2+} indicators. Science 2011;333:1888–91.

[114] Zurborg S, Piszczek A, Martínez C, Hublitz P, Al Banchaabouchi M, Moreira P, Perlas E, Heppenstall PA. Generation and characterization of an Advillin-Cre driver mouse line. Mol Pain 2011;7:66.

Correspondence to: Stephen B. McMahon, PhD, King's College London, Wolfson Centre for Age-Related Diseases, Guy's Campus, London SE1 1UL, United Kingdom. Email: stephen.mcmahon@kcl.ac.uk.