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ANESTHESIOLOGY

Molecular Changes in the Dorsal Root Ganglion during the Late Phase of Peripheral Nerve Injury–induced Pain in Rodents: A Systematic Review

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ABSTRACT

The dorsal root ganglion is widely recognized as a potential target to treat chronic pain. A fundamental understanding of quantitative molecular and genomic changes during the late phase of pain is therefore indispensable. The authors performed a systematic literature review on injury-induced pain in rodent dorsal root ganglions at minimally 3 weeks after injury. So far, slightly more than 300 molecules were quantified on the protein or messenger RNA level, of which about 60 were in more than one study. Only nine individual sequencing studies were performed in which the most up- or downregulated genes varied due to heterogeneity in study design. Neuropeptide Y and galanin were found to be consistently upregulated on both the gene and protein levels. The current knowledge regarding molecular changes in the dorsal root ganglion during the late phase of pain is limited. General conclusions are difficult to draw, making it hard to select specific molecules as a focus for treatment.

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Chronic pain is a debilitating disease affecting millions of people worldwide. Currently available pharmacotherapeutic agents are systemically applied and realize their effects in both the peripheral nervous system and central nervous system (CNS). They frequently lack efficacy and can lead to unwanted side effects.[1] The problems encountered in pain treatment and the resulting burden on society are unprecedented.[2] The development of effective treatment is therefore urgently required.

The dorsal root ganglion transmits sensory information, including nociceptive information, from the periphery toward the CNS. It contains the cell bodies of primary sensory neurons, which can be classified by several electrophysiologic characteristics, cellular marker expression profiles, and the peripheral nerve fibers that receive and convey information to these cell bodies (*i.e.*, myelinated A beta and A delta fibers, and unmyelinated C fibers). Dorsal root ganglion neurons undergo various forms of changes in chronic pain conditions, as do immune and glial cells surrounding these neurons. The mechanisms by which these changes contribute to chronic pain are still incompletely understood; however, dorsal root ganglions are widely recognized as a potential focus for treatment.[3] The dorsal root ganglion is a logical structure to target, not only in view of its function in the nociceptive pathway, but also because of its anatomical location outside the CNS, and its accessibility *via* the bloodstream due to the absence of a blood–nerve barrier. Local intervention by targeting the pain-related molecular processes in the dorsal root ganglion (*e.g.*, targeted drug delivery), could therefore potentially be more effective while avoiding undesirable side effects related to systemic treatment.

The molecular and cellular changes in the dorsal root ganglion induce upstream and downstream effects on the spinal dorsal horn and injury site, respectively, *via* its central and peripheral projections.[4] While some of these changes are pathologic, others are related to acute nonpathologic pain signaling, which serves a protective function and is therefore vital to normal functioning of the human body.

The time course and location of these changes are also relevant, since different processes and molecules have been shown to be involved at different timepoints since the onset of chronic pain in both human and animal studies.[5] Molecular processes in the early or later phases of chronic pain are not necessarily the same. This is of special importance when searching for new therapeutic targets, since patients often present themselves in the clinic during the later phases, already experiencing pain for months or even years.

It is therefore crucial to identify the underlying molecular and cellular interactions at the dorsal root ganglion, which are contributing to later phases of chronic pain. We therefore performed a systematic review of the literature investigating quantitative molecular changes that occur in

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the dorsal root ganglion. We specifically focused on preclinical studies using peripheral nerve injury–induced pain models in rodents. We defined the late or "chronic" phase as timepoints from 3 weeks onward after nerve injury, since these represent the overwhelming majority of what has now been described. The main objectives of this review are (1) to provide a systematic overview of quantitative molecular changes (on both the protein and messenger RNA [mRNA] levels) and high-throughput RNA sequencing analysis in the dorsal root ganglion during the late phase of peripheral nerve injury–based pain models; (2) to give a descriptive overview of potential mechanisms involved and evidence supporting their causal link with chronic pain; and (3) to identify gaps of knowledge, which could limit successful clinical translation.

Materials and Methods

Study Protocol

For protocol development, the guidelines of de Vries *et al.*[6] and the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES; Berlin, Germany) were followed. Before preparing our study protocol, the CAMARADES and Prospero databases were searched for studies published or in preparation that were comparable to our protocol. None were found. Finally, the protocol was registered with Prospero (registration ID: CRD42021222586). No deviations from the protocol were encountered during the search for, evaluation of, and entry of the eligible articles.

Populations of Interest, Exposures, Comparators, and Outcomes Statement

In order to clearly formulate the overall objective of this review, we have identified our Populations of Interest, Exposures, Comparators, and Outcomes (PICO) statement, which is part of a framework for the systematic review and integrated assessment of animal studies.[7] Our Populations of Interest, Exposures, Comparators, and Outcomes statement is defined as follows:

(P) Laboratory rodents
(E) Exposure to one of the following peripheral nerve injury models: chronic constriction injury, spinal nerve ligation, sciatic nerve transection, partial sciatic nerve ligation, or a variant of these models. A second necessary condition for inclusion in this review was a survival time of 3 weeks or longer after injury.
(C) The control group consisted of naïve animals, sham-operated animals or, dorsal root ganglions from the contralateral side.
(O) Our main outcome was quantitative data of molecules, on either the protein or the mRNA level, in the dorsal root ganglions of injured animals relative to the control group, at 3 weeks or later timepoints after injury.

Search

An experienced, independent librarian performed a search of the literature in MEDLINE, Embase, Web of Science, Emcare, and Academic search premier databases following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram[8] and focused on the molecular changes in the dorsal root ganglion in peripheral nerve injury–induced pain models in rodents. The search was performed on of July 9, 2020, and included studies with protein data (*e.g.*, immunohistochemistry, Western blot), mRNA data (polymerase chain reaction, *in situ* hybridization) and/or -omics studies (microarray, genomics, proteomics). Our complete search strategy is presented in Supplemental Digital Content 1 (http://links.lww.com/ALN/C759).

Definition of Chronic Phase in This Review

The time of transition from the acute phase to the chronic phase of neuropathic pain is rather arbitrary and is, especially in animal studies, not well defined. We chose to include only studies with quantitative analysis performed at least 3 weeks postoperatively for two reasons. First, many molecules show a peak level around 1 to 2 weeks after injury, after which they either remain elevated or return to preinjury levels, suggesting a distinction between the acute transient molecular changes during the first 2 weeks after nerve injury and the more persistent changes thereafter. Second, articles with a follow-up time of more than 3 weeks are relatively rare. An overview based only on studies with a postoperative analysis performed later than 4 weeks would, therefore, have provided too little information. As such, terms like "chronic" or "late" refer to the definition we determined here. This terminology cannot be simply translated to the term "chronic" in humans, where it is defined as pain lasting for more than 3 to 6 months.

Inclusion and Exclusion Criteria

The selection process of eligible studies for this review was conducted in two steps. First, articles were screened for eligibility based on their study design. Studies were included only if they provided quantitative data regarding molecular changes in dorsal root ganglions of rodents using a peripheral nerve injury–based model to induce pain-like behavior. Quantitative data could be based on either protein level or mRNA level and was required to be relative to a control group (*e.g.*, sham-operated group, naïve group, or contralateral dorsal root ganglion). As such, studies that only provided descriptive data on these topics were excluded. The peripheral nerve injury models used to induce pain-like behavior were chronic constriction injury, spared nerve injury, spinal nerve ligation, sciatic nerve transection, partial sciatic nerve ligation, or a variant of one of these models. Studies regarding cultured dorsal root ganglion neurons, rather than dorsal root ganglion tissue, were excluded.

This review focuses exclusively on molecular changes during the chronic phase, here defined as 3 weeks or more

after injury. Therefore, after exclusion of ineligible studies using the criteria above, the remaining studies were screened on the time after injury, at which quantitative data were collected. Studies were excluded from analysis if they only provided quantitative data at less than 3 weeks after injury, whereas studies containing quantitative data at 3 weeks after injury or any later timepoint were included.

Risk of Bias Estimation

Two independent researchers performed a risk of bias estimation of the different publications studying the same molecules (n = 53). The SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) and Cochrane Risk of bias estimation tool was used.[6,9]

Outcomes

All screened records proved clear and conclusive concerning their content for inclusion or exclusion into categories "follow-up of less than 3 weeks" or "follow-up 3 weeks or more" after injury (fig. 1). Quantitative data of all molecular changes in the dorsal root ganglion were noted in relation to the specific timepoint after the lesion. In addition, the following outcomes and characteristics were noted: animal type, animal breed, sex, age, group size, time of follow-up, type of peripheral nerve injury–induced pain model, method of quantification (*e.g.*, protein level: immunohistochemistry, Western blot, enzyme-linked immunosorbent assay, or mRNA level: polymerase chain reaction, *in situ* hybridization), the anatomical dorsal root ganglion level, and the type of control group (naive, sham, contralateral dorsal root ganglion). The findings of the studies were represented in the tables by different colors, with (dark) green or red signifying (significantly) increased or decreased, respectively, and blue meaning that the molecule was not found to be regulated. Moreover, double or multiple entries of the same PubMed identification or accession number indicate that more than one pain model was investigated, and/or that more than one species or breed was used.

The results section consists of two parts. Part one entails extracted quantitative data for each molecule (on both the protein and the mRNA level) and outcomes from included studies. Data obtained from high-throughput transcriptomics (*e.g.*, RNA sequencing and microarrays) are presented and discussed separately. The top differentially regulated genes and categories of genes (*e.g.*, based on gene ontology analysis) were extracted. We also assessed the overlap between the protein-level studies and the high-throughput sequencing studies.

In part two, a descriptive overview is given on specific cellular localization or distribution of the molecule (*e.g.*, only increased in injured neurons), potential mechanisms, and evidence supporting a causal link with chronic pain, based on the findings of the included studies. For part two, studies were primarily used that looked at changes at the protein level (with or without mRNA data). Studies providing exclusively mRNA data seldom investigated potential mechanisms or causal links with pain. If protein data for the same molecule showed conflicting results between studies, mRNA data are also discussed to provide additional findings of the specific molecule, which could potentially explain the conflicting results.

Results

Our search yielded 1,628 articles. Five articles were found *via* cross-referencing, resulting in a total of 1,633 studies. After excluding 879 articles in the first step of the selection process, a total of 754 studies remained containing quantitative data on molecular changes in the dorsal root ganglion of rodents after peripheral nerve injury. Of these 754 articles, 586 (77.7%) studies only provided quantitative data regarding the acute phase of peripheral nerve injury–induced pain models and were therefore excluded. A total of 168 (22.3%) articles were investigating timepoints at 3 weeks or longer and were therefore used for the final analysis (fig. 1). A complete overview of all the articles that were revealed by our search, and the inclusion process, are provided in Supplemental Digital Content 2 (http://links.lww.com/ALN/C760).

Study Characteristics

Of the 168 included studies, 150 (89.3%) used rats and 22 (13.1%) used mice (table 1). The most commonly used rat strain was the Sprague–Dawley rat, followed by Wistar rats, which were used in 112 (66.7%) and 28 (16.7%) of all studies, respectively. C57/B6 was the most widely used mice breed, found in 11 (6.5%) studies. Male animals were used in 151 (89.9%) studies, while female animals were used exclusively in only 9 (5.4%) of the studies. Eight studies (4.8%) used both male and female animals in their experiments. Different peripheral nerve injury models were used to induce experimental neuropathic pain. The chronic constriction injury model was used most frequently and comprised 56 (33.3%) of the 168 studies. The L5 spinal nerve ligation, sciatic nerve transection, and spared nerve injury models were used in 31 (18.5%), 30 (17.9%), and 30 (17.9%) of the studies, respectively. To quantify molecular changes, 126 (75.0%) of the articles used methods based on protein level, while 88 (52.4%) used mRNA-based quantification methods. Of the protein-based quantification methods, Western blot and immunohistochemistry were most often used, in 63 (37.5%) and 57 (33.9%) of the studies, respectively. mRNA levels were most commonly measured using quantitative polymerase chain reaction, which was performed in 79 (47.0%) studies.

Risk of Bias Estimation

Risk of bias estimation showed that the average score of "unclear bias" was at 77%, the average "clear risk of bias" was at 10%, and "no risk of bias" was at 13% in 52 studies (Supplemental Digital Content 3, http://links.lww.com/ALN/C761).[10] The SYRCLE screening tool has two additional questions related to bias.[9] Screening of the same set

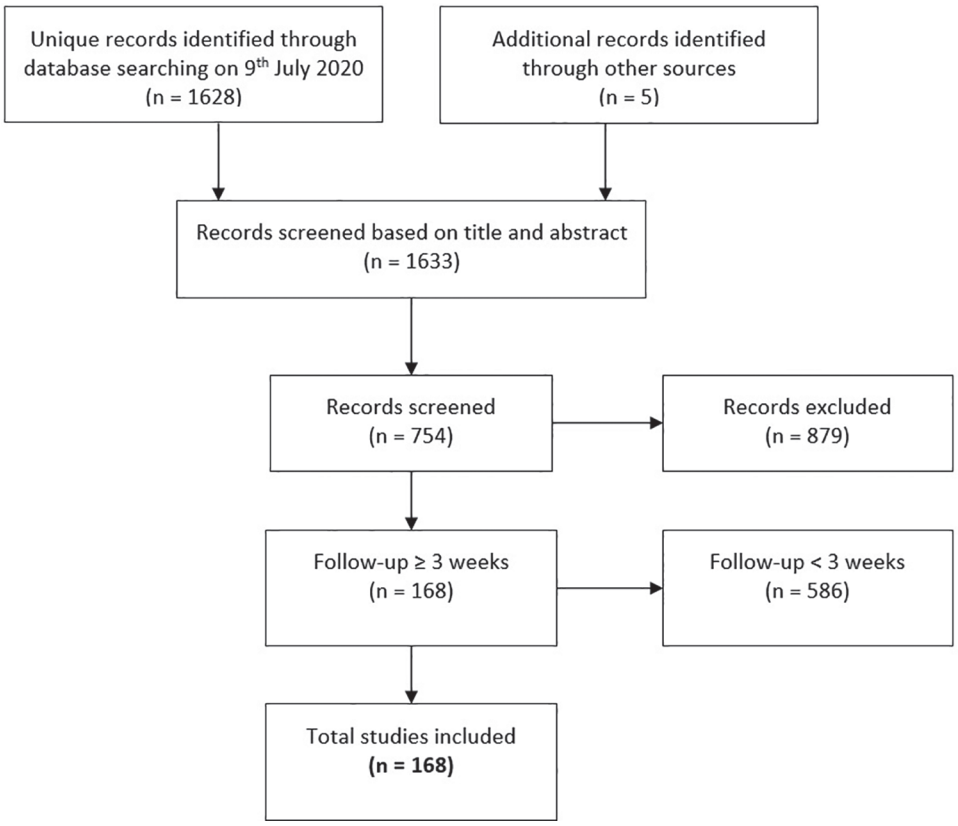

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram, indicating the total number of studies screened based on abstract and title, the total number of eligible studies and the portion of studies focusing on the chronic phase of pain (n = 168).

of studies revealed that with the SYRCLE, tool the average score of "unclear bias" wat at 69%, the average "clear risk of bias" was at 19%, and "no risk of bias" was also at 19%. Note, however, that in the majority of studies, no information on randomization or blinding strategies was provided, and risk of bias was in these cases scored as "unknown." Strictly speaking, these unknowns can be interpreted as "high risk of bias."

Quantitative Molecular Changes in the Dorsal Root Ganglion during the Chronic Phase of Peripheral Nerve Injury–induced Pain Models

A total of 309 molecules were quantified in the 168 studies. Of these, 246 (79.6%) were only quantified in just one study, while quantitative data of only 63 molecules (20.4%) were provided in more than one study. Tables 2, 3, and 4 give an overview of the quantitative changes on the protein level in the dorsal root ganglion during the chronic phase of the peripheral nerve injury–induced pain models (3 weeks or more after injury). On protein level, a total of 99 molecules was found to be significantly increased in at least one study (table 2). The molecules that were most frequently found to be significantly increased on the protein level were activating transcription factor 3 (ATF3; seven times), transient receptor potential cation channel subfamily V member 1 (TRPV1) and glial fibrillary acidic protein (GFAP; six times), major histocompatibility complex class II (MHC-II; five times), and galanin, neuropeptide Y (NPY), p-p38, and caspase 3 (four times). Additionally, of these 99 molecules, only 28 (28.2%) were found to be significantly increased in more than one study. A complete of overview of the raw extracted data from all studies is presented for each molecule in Supplemental Digital Content 4 (http://links.lww.com/ALN/C762).

A total of 30 molecules was found to be significantly decreased on the protein level in at least one study (table 3). The molecules confirmed to be significantly decreased in more than one study were calcitonin gene-related peptide 1 (CGRP; five studies), interleukin-10 (IL-10; three studies), and mu-type opioid receptor (MOR), H(+)/Cl(–) exchange transporter 3 (ClC-3), and substance P (two studies). Some molecules produced conflicting results, showing both (significantly) increased and (significantly) decreased levels in different studies (table 4). More details regarding the individual studies of these molecules are depicted in table 5. Possible explanations for these discrepancies are discussed separately, if possible, for each molecule in "High-throughput Sequencing Studies." Furthermore, 14

Table 1. Characteristics of the Total Number of Studies Focusing on the Chronic Phase of Nerve Injury–induced Pain Models (n = 168), Specified per Subcategory

Category	Number of Studies (% of 168)
Sex	
Male	151 (89.9)
Female	9 (5.4)
Both	8 (4.8)
Age	
Adult (8–12 weeks)	161 (95.8)
Young	7 (4.2)
Old	2 (1.2)
Species	
Rat	150 (89.3)
Sprague–Dawley	112 (66.7)
Wistar	28 (16.7)
Lewis	5 (3.0)
F344	3 (1.8)
Long–Evans	3 (1.8)
Mouse	22 (13.1)
C57BL/6	11 (6.5)
ICR	3 (1.8)
Swiss	3 (1.8)
Balb/c	2 (1.2)
CD1	2 (1.2)
WT-MOP	1 (0.6)
Pain model	
Chronic constriction injury	56 (33.3)
L5 spinal nerve ligation	31 (18.5)
Sciatic nerve transection	30 (17.9)
Spared nerve injury	30 (17.9)
L5/L6 spinal nerve ligation	13 (7.7)
Partial sciatic nerve ligation	9 (5.4)
Saphenous nerve axotomy	1 (0.6)
L4–L6 spinal nerve ligation	1 (0.6)
Sciatic nerve ligation	1 (0.6)
Quantification methods	
Protein level	126 (75.0)
Western blot	63 (37.5)
Immunohistochemistry	57 (33.9)
ELISA	4 (2.4)
Gelatin zymography	1 (0.6)
HPLC	1 (0.6)
mRNA level	88 (52.4)
Polymerase chain reaction	79 (47.0)
In situ hybridization	7 (4.2)
Microarray	2 (1.2)

ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography.

molecules that were investigated showed no difference on protein level during the chronic phase of peripheral nerve injury. These molecules are listed in Supplemental Digital Content 5 (http://links.lww.com/ALN/C763).

Based on mRNA level, the expression of 93 molecules was found to be significantly upregulated in at least one study, of which ATF3, IL-1β, and NPY were most frequently reported. A total of 45 molecules was found to be significantly decreased on mRNA level, most often voltage-gated potassium channel (Kv) subunit Kv7.2 (Kv7.2), voltage-gated sodium channel (Nav) subunit alpha Nav1.8 (Nav1.8), proenkephalin, and tyrosine hydroxylase (TH; Supplemental Digital Content 6, http://links.lww.com/ALN/C764).

The latest timepoints at which significant changes on the protein level were reported in the investigated studies are shown in Supplemental Digital Content 7 (http://links.lww.com/ALN/C776) for each molecule. It is clearly visible that the majority of the studies were concentrated in weeks 3 and 4 after injury. Of the molecules quantified at the protein level, 102 (80.1%) were investigated and found to be regulated at week 3 or 4 after injury. Only 12 molecules were investigated and found to be significantly changed at 10 weeks or more after injury.

High-throughput Sequencing Studies

Our search yielded 57 high-throughput sequencing studies, of which 11 (16.4%) used timepoints at 3 weeks or later after injury. Of these 11 studies, 3 used the same dataset, implying that only 9 individual sequencing studies were performed so far. Study characteristics are depicted in table 6, as well as the top up- or downregulated genes and the functional categories that were found to be differentially expressed most often. All studies looked at mRNA changes at 21 to 30 days after injury, except for one study, which performed sequencing at 20 weeks after L5/L6 spinal nerve ligation. Only one of the nine studies used female animals in their experiment, while seven used male animals. One study did not specify the sex that was used. The most regulated categories of genes that were observed varied between studies, but important functional groups that were found across different sequencing studies are neuropeptides, molecules involved in synaptic function and signal transduction, ion channels, immune-related molecules, and molecules involved in cell–cell or cell–matrix interactions. These are functional categories that were also found in the studies regarding the individual molecules at the protein level. The top up- or downregulated genes were found to vary between high-throughput transcriptomics studies as well. Differences could be explained by heterogeneity between studies in respect to the animals, used model or timepoint after injury, variation in selection of genes for microarray analyses, and application of different significance thresholds. Definitive conclusions on genes that are involved in pain across different studies could therefore not be drawn. The genes that were found to be most consistently regulated between different sequencing studies were NPY, vasoactive intestinal peptide (VIP), and galanin. NPY and galanin were among the molecules that were also upregulated on the protein level most often (table 2).

An important advantage of high-throughput sequencing studies is the ability to quantify mRNA changes for thousands of different genes in one experiment. Many up/downregulated genes can be found in the data that were not found in the studies on the protein level. However, an increased mRNA expression of a certain gene does not always translate into increased quantities of protein levels. For example, one of the studies, which performed

Table 2. Molecules Upregulated (*Dark Green*), Molecules Not Significantly Upregulated (*Light Green*) and Mentioned, but Not Regulated (*Blue*) in the Chronic Phase of Nerve Injury–induced Pain Models, with Their Corresponding PubMed Identification Numbers

Molecule	Accession Number							Latest significant timepoint in days
Activating transcription factor 3	25017582	24269493	11275393	29921170	29870694	31918012	31918012	42
Glial fibrillary acidic protein	28125108	27671501	22050959	30152258	31918012	31918012	19307059	56
Major histocompatibility complex-II	22789131	17187959	12044469	12044469	14769352			77
Galanin	8919294	31864679	1,68E+11	1,68E+11	29928003			385
Neuropeptide Y	19879928	24269493	8919294	15047027				168
p-P38	12764087	30632086	30528326	32617100				28
Caspase 3	24269493	30632086	30769782	29870694	29870694			42
CD68	17187959	14769352	14769352	22789131				77
Glial cell line-derived neurotrophic factor	25974189	15698937	16289634	25687543				28
Tumor necrosis factor alpha	27441756	28481389	30632086	26781879				28
Transient receptor potential cation channel A1	25974189	32617100	2E+09	27329106				21
p-Extracellular signal-regulated kinases	28359290	30989502	30632086					28
GDNF receptor alpha-1	25974189	15698937	16289634					28
Transient receptor potential cation channel subfamily M member 8	27329106	32617100	2E+09					28
Voltage-gated sodium channel subunit alpha Nav1.7	27765894	25657691	32468069	22720761				28
Allograft inflammatory factor 1	32424233	31918012	27497321	31918012				42
Brain-derived neurotrophic factor	25687543	10564362						70
Galanin receptor 2	29928003	31864679						28
Voltage-gated sodium channel subunit alpha Nav1.3	22720761	30632086						28
NF-kB	28359290	30632086						28
Nerve growth factor	25687543	28685530						70
p-Jun kinase	30632086	30528326						28
T-cell receptor	17187959	12044469						77
Transient receptor potential cation channel subfamily V member 4	32617100	2E+09						21
Tyrosine Hydroxylase	17672895	32626770						56
Matrix metalloproteinase 9	30632086	18264108						28
Activating transcription factor 2	29921170							21
Beta catenin	26054011							28
Binding immunoglobulin protein	32424233							28
Cystathionine β-synthetase (CBS)	32468069							21
CD3	28837503							28
CD8	17187959							70
CD171 / L1-CAM	17331206							28
p-Cofilin	27216618							21
Cyclo-oxygenase-2	22198006							28

(*Continued*)

Table 2. (Continued)

C-X-C motif chemokine	30076959							28
CXCR2	27145805							21
Cytosolic Cytochrome C	30632086							28
Prostaglandin EP-1 receptor	18786748							548
p-EP-1 Receptor	18786748							548
Prostaglandin EP-4 receptor	18786748							548
Ephrin type-B receptor 1	18321739							28
EphrinB1	18321739							28
ERK	28359290							21
Histone-lysine N-methyltransferase EZH2	26551542							21
Fibroblast growth factor 13	12395088							28
p-Fibroblast growth factor receptor	17905520							28
G9a protein	26551542							21
Glypican-1	2,2E+11							28
Membrane glycoprotein 130	30872136							21
myeloid differentiation antigen Gr-1	28837503							28
Histone H2AX	30632086							28
H3K9me2	26551542							21
H3K27me3	26551542							21
Histone deacetylase 1	26551542							21
Histone deacetylase 2	26551542							21
Histone deacetylase 4	26551542							21
High mobility group protein B1	28359290							21
Heat shock protein beta-1	2,28E+11							49
Isolectin B4-rings	11596053							84
pro-Interleukin-1-beta	28359290							21
Interleukin-6	30872136							21
Interleukin-33	32513089							28
microtubule associated protein 1 light chain 3 (LC3)-II	26021876							21
Leptin	30961664							21
p-LIM kinase	27216618							21
Methyl-CpG-binding protein 2	26448907							28
Matrix metalloproteinase 2	18264108							21
Myeloid differentiation primary response protein MyD88	28359290							21
pNF-kB	28359290							21
Solute carrier family 12 member 2 (NKCC1)	31719257							21
Nitric oxide synthase	2,28E+11							49

(*Continued*)

both genome-wide translational profiling and mRNA sequencing, found that seven genes were transcriptionally upregulated while translationally downregulated.[60] In table 7, we have examined whether the molecules that were significantly changed on the protein level in at least two studies, were also observed in the high-throughput sequencing studies. Sequencing studies that did not provide tables/figures of all genes or only focused on microRNAs (miRNAs) were excluded from this table. Only a small percentage (average 12.5%) of the molecules were found in

Table 2. (Continued)

Nitric oxide synthase 1	12127013							21
NADPH oxidase 1	30632086							28
NADPH oxidase 2	30632086							28
NADPH oxidase 4	30632086							28
Glutamate receptor ionotropic, NMDA 2B	27803647							21
leptin receptor (OB-Rb)	24325936							21
Pannexin-1	25925949							21
Transforming protein RhoA	27216618							21
Synaptoporin	16777346							28
Synaptophysin	27441756							28
TNF receptor-associated factor 6	29885668							21
Translocating chain-associated membrane protein 1	30338452							21
Transient receptor potential cation channel subfamily V member 2	2E+09							21
Transient receptor potential cation channel subfamily V member 4	2E+09							21
Thrombospondin-4	25327416							28
Voltage-gated calcium channel subunit alpha Cav3.2	30872136	27571431						21
Macrophage colony-stimulating factor 1	31918012	31918012						21
C-X-C motif chemokine 11	30076959							
C-X-C motif chemokine 12	26781879							
C-X-C chemokine receptor 4	26781879							
pERK2 / MAPK1	26270581							
microtubule associated protein 1 light chain 3 (LC3)-I	26021876							
LIM kinase	27216618							
suppressor of tumorigenicity 2 (ST2)	32513089							
Transcription factor 4	25963533							

the high-throughput sequencing data, indicating that many pain-related molecules will be missed when only focusing on high-throughput sequencing studies.

Molecular Mechanisms on the Protein Level

In the following section, we discuss the molecules that were studied and provide an overview of the molecular processes that occur in the dorsal root ganglion during the chronic phase (3 or more weeks follow-up) of peripheral nerve injury–induced pain models. We primarily discuss studies which quantified molecules at protein level (tables 2, 3, and 4). Furthermore, only mechanisms and findings directly based on the results of the included articles were used, unless otherwise stated. For clarity purposes, we subdivided the processes underlying peripheral nerve injury–induced pain-like behavior in the dorsal root ganglion into different topics: ion channels, immune system, neuropeptides, intracellular neuronal changes, cell–cell and cell–matrix interactions, and satellite glial cells. A schematic overview of the molecules and mechanisms is depicted in a schematic way in figure 2.

Ion Channels

Sodium. The rapid influx of sodium ions causes the polarity of the plasma membrane to reverse, which initiates the rising phase of action potentials. Sodium channels play an essential role in neuronal transmission. They consist of various subunits, some of which have been associated with neuropathic pain conditions. One of these subunits, Nav1.7, has been associated with the chronic phase (3 or more weeks of survival time) of pain. Nav1.7 was quantified on protein level in four studies using rats.[40,61–63] Of these four studies, two reported an increased expression at week 3 postinjury using the spared nerve injury model, and one study found an increased expression at week 4 using the chronic constriction injury model. In contrast, the fourth study found a downregulation of Nav1.7 around day 4 after L5 spinal

Table 3. Molecules Downregulated (*Red*), Molecules Not Significantly Downregulated (*Light Red*), and Mentioned but Not Regulated (*Blue*) in the Chronic Phase of Nerve Injury–induced Pain Models, with Their Corresponding PubMed Identification Numbers or Accession Numbers

Molecule	Accession Number									Latest significant timepoint in days
Calcitonin gene-related peptide 1	24269493	14769355	31864679	1,68E+11	1,68E+11	8919294	28256957	18786748	38859367	385
Interleukin-10	28481389	31308727	30961664	28011263						32
Mu-type opioid receptor	9483516	21486477	21310637							28
H(+)/Cl(-) exchange transporter 3	27460962	629906834								28
Substance-P	8919294	31864679	18786748							27
Acetyl-H3	26551542									21
Alpha-7 nicotinic receptor	31308727									21
Anandamide (AEA)	23597506									28
Calmodulin-dependent protein kinase type II	25982557									21
Voltage-gated calcium channel subunit alpha Cav2.2	29709527									28
C-X-C motif chemokine 10	30076959									28
GABA-B receptor 1 b	16427742									120
G protein-coupled receptor kinase 6	27145805									21
Hydroxymethylglutaryl-CoA synthase 1	27671501									28
Isolectin B4	14769355									28
Calcium-activated potassium channel subunit alpha-1	26551542									28
Voltage-gated potassium channel subunit Kv1.4	26551542									28
Voltage-gated potassium channel subunit Kv4.2	26551542									28
Voltage-gated potassium channel subunit Kv7.2	26551542									28
Voltage-gated potassium channel subunit Kv9.1	23197740									28
Myelin basic protein	30769782									21
Mu-type opioid receptor 1	26917724									28
Mas-related G-protein coupled receptor (MrgC)	24374082									28
Tyrosine kinase receptor A	8,15E+10									21
Y1R receptor	15047027									28
CD18 / ITGB2	24269493	38859367								42
Anion exchange protein 3	29355942									
Prostaglandin receptor EP-3	18786748									
Hydroxymethylglutaryl-CoA synthase 2	27671501									
Calcium release-activated calcium channel protein 1	21389210									
Stromal interaction molecule 1	21389210									

Similar entries at the same molecules means that this publication investigated the molecule in multiple pain models and/or breeds.

Table 4. Molecules Regulated in the Chronic Phase of Nerve Injury–induced Pain Models with Conflicting Observations: Upregulated (*Green*), Not Significantly Upregulated (*Light Green*), Downregulated (*Red*), and Not Significantly Downregulated (*Light Red*), with Their Corresponding PubMed Identification Numbers or Accession Numbers

Molecule	Accession Number							Latest significant timepoint in days	
Transient receptor potential cation channel subfamily V member 1 (TRPV1)	27329106	25974189	29369297	18786748	32617100	2003967960	2,99401E+11	540	
Interleukin-1-beta	28359290	30632086	31308727	30961664	30769782			32	21
P2X purinoceptor 3	25974189	31261200	30989502	47249259				28	28
Sigma-1 Receptor	32424233	24015960						28	21
Voltage-gated sodium channel subunit alpha Nav1.8	30632086	26005195	22720761					28	28
Interleukin-1 receptor-associated kinase 1	29885668	30859436						21	
C-X-C chemokine receptor type 3	30448292	30076959						21	
Voltage-gated sodium channel subunit alpha Nav1.9	30632086	26005195						28	
CD163 / ED2	17187959	14769352	14769352						

Similar entries at the same molecules means that this publication investigated the molecule in multiple pain models and/or breeds.

nerve ligation, which returned to baseline levels at 3 weeks after injury.[40] This discrepancy suggests that the expression profile and timing of elevated expression of Nav1.7 highly depend on the location of and type of peripheral nerve injury inflicted. The heterogeneity between these studies, caused by the use of different pain models, may therefore underlie the variability in results regarding the regulation of Nav1.7. Remarkably, one study, using the same rat breed (Sprague–Dawley) and pain model (spared nerve injury), found a significant decrease in Nav1.7 expression on mRNA level at 4 weeks after injury. This is in contrast to the above-mentioned report demonstrating increased protein levels.[41] Notably, however, in contrast to the studies investigating protein levels, the study showing decreased mRNA levels used female instead of male rats.[41,61] This sex difference in the expression profile of Nav1.7 might potentially point to a sex-specific response of Nav1.7. Nevertheless, additional studies have to be performed using both sexes to explain this conflicting outcome.

The mechanism by which Nav1.7 may be upregulated during the chronic phase after peripheral nerve injury is not fully understood. Of the studies included in this review, some attempted to elucidate these mechanisms. For example, Tian *et al.* showed that the increased expression of Nav1.7 and enhanced excitability after spared nerve injury could be partially reversed by inhibiting cystathionine-β-synthase, an enzyme that was also increased in the dorsal root ganglion at week 3 after spared nerve injury.[63] The authors suggested that increased cystathionine-β-synthase levels upregulated the expression of Nav1.7 *via* the p-MEK/p-ERK pathway, as the increase in Nav1.7 expression normally seen after injury was also reversed by inhibiting cystathionine-β-synthase (fig. 2). Another study suggested that small noncoding microRNAs may be involved in the regulation of Nav1.7 expression after peripheral nerve injury. Shao *et al.* indicated that the microRNA miR-30b was able to inhibit Nav1.7 expression by preventing transcription of the ion-channel subunit (fig. 2).[61]

Other sodium channel subunits that have been extensively studied in the context of pain are the Nav1.3 and Nav1.8. Nav1.3 was found to be significantly increased at the protein level after L5 spinal nerve ligation and chronic constriction injury in rats, both at week 4 after injury.[24,40] In one of these two studies,[40] a downregulation of Nav1.8 was found at the same timepoint. The authors suggested, therefore, that peripheral nerve injury leads to an increased expression of Nav1.3 with a concomitant decrease of Nav1.8 expression in injured neurons. A different article, also reporting a downregulation of Nav1.8 (3 weeks after chronic constriction injury), supported this statement.[64] However, another study from our literature search showed an increased expression of Nav1.8 instead, at 4 weeks after chronic constriction injury (table 5).[23] When looking at studies with mRNA data, a concomitant increase of Nav1.3 and decrease of Nav1.8 was also found at week 4 after spared nerve injury in female rats (Supplemental Digital Content 6, http://links.lww.com/ALN/C764).[41] Moreover, mRNA levels of Nav1.8 returned to baseline levels by day 90 after spared nerve injury. Concluding from these studies, it appears that the

Table 5. Additional Data on Studies that Had Conflicting Results

TRPV1							Protein											mRNA									
Accession #	Author Year	model	animal	breed	sex	method																					
27329106	Koh 2016[11]	L5/6 SNL	Rat	SD	M	WB										28											
25974189	Shi 2015[12]	CCI	Rat	SD	M	IHC		2				6	7	10	14												
29369297	Parisi 2017[13]	CCI	Mice	Swiss	M	WB	1				5					21											
18786748	Ma 2010[14]	pSNL	Rat	SD	M	IHC/WB												540									
32617100	Zhang 2020[15]	CCI	Rat	SD	M	WB										21											
2003967960	Zhang 2019[16]	CCI	Rat	SD	M	WB				4			7		14	21											
2,99401E+11	Szigeti 2012[17]	SNT	Rat	Wistar	M	WB/PCR			3						14			30			3			14			30	
19446956	Staaf 2009[18]	SNI	Rat	SD	M	PCR																4		14				84
11376855	Macdonald 2001[19]	SNT	Rat	Wistar	M	PCR													1				7	14		28		
32012798	Cernit 2020[20]	pSNL	Rat	SD	M	PCR																			21			
354224824	Jankowski 2009[21]	SphT	Mice	Swiss	M	PCR																				28		70
IL-1beta																												
28359290	Liu 2017[22]	CCI	Rat	SD	M	WB	1	3	7	14	21																	
30632086	Yang 2019[23]	CCI	Rat	SD	M	WB						28																
31308727	Wang 2019[24]	SNI	Rat	SD	M	WB		3	7	14	21																	
30961664	Sanchez 2019[26]	CCI	Rat	LE	M	IHC							32															
30769782	Kwan 2019[27]	L5 SNL	Rat	SD	M	WB/PCR					21														21			
27941941	Jeanson 2016[28]	CCI	Rat	SD	M	PCR																				28		
27856286	Geis 2017[29]	CCI	Mice	C57B6	M	PCR													1		4	7		21				
22525520	Urtikova 2012[30]	CCI	Rat	SD	M	PCR																		21				
15597059	Lee 2004[31]	CCI	Rat	SD	M	PCR													1	3		7	14	21	28			
31918004	Noor 2020[32]	CCI	Rat	LE	F	PCR																			28			
P2X3																												
25974189	Shi 2015[12]	CCI	Rat	SD	M	WB	1		6		10	14	21															
31261200	Fu 2019[33]	CCI	Rat	SD	M	WB								28														
47249259	Zhou 2007[34]	ScNL	Rat	SD	FM	IHC		3		7		14	21															
30989502	Guo 2021[35]	pSNL	Rat	SD	M	WB/PCR							21						1		7		21					
11275393	Tsuzuki 2001[36]	SNI	Rat	SD	M	ISH														3	7	14		28				
354224824	Jankowski 2009[21]	SphT	Mice	Swiss	M	PCR															7	14	21	28	70			
Sigma-1 Receptor																												
32424233	Shin 2020[37]	SNI	Rat	SD	M	WB		28																				
24015960	Bangaru 2013[38]	L5/6 SNL	Rat	SD	M	IHC/WB/PCR	21												21									
Nav 1.8 / SCN10A																												
30632086	Yang 2019[23]	CCI	Rat	SD	M	WB						28																
26005195	Li 2015[39]	CCI	Rat	SD	M	WB/PCR	3		7	14	21								3	7	14	21						
22720761	Cheng 2012[40]	L5 SNL	Rat	SD	M	WB		4	7	14	21	28																
25698112	Casalz-Diaz 2015[41]	SNI	Rat	SD	F	PCR														7			28	90				
IRAK1																												
29885668	Wang 2018[42]	CCI	Rat	SD	M	IHC/WB/PCR	1	3		7		14	21						1	3	7	14	21					
30859436	Yin 2019[43]	CCI	Rat	SD	M	WB	1	3	5	7	10	14	21															
CXCR3																												
30448292	Chen 2019[44]	CCI	Rat	SD	M	WB	1		3	5	7	10	14	21														
30076959	Piotrowska 2018[45]	CCI	Rat	Wistar	M	WB/PCR		2			7		14		28				2	7	14	28						
Nav 1.9 / SCN11A																												
30632086	Yang 2019[23]	CCI	Rat	SD	M	WB					28																	
26005195	Li 2015[39]	CCI	Rat	SD	M	WB/PCR	3	7	14	21									3		7	14	21					
23045676	Shankarappa 2012[46]	SNI	Rat	SD	M	PCR														5					60			
25698112	Casalz-Diaz 2015[41]	SNI	Rat	SD	F	PCR															7			28		90		
CD163 / ED2																												
17187959	Hu 2007[47]	CCI	Rat	SD	M	IHC		7		70																		
14769352	Hu 2003[48]	SNT	Rat	Wistar	F	IHC	3	7	28		77																	
		L5 SNL	Rat	Wistar	F	IHC		7	28		77																	

The colors of the boxes mean significantly increased (*dark green*), not significantly increased (*light green*), not regulated (*blue*), significantly decreased (*dark red*), and not significantly decreased (*light red*). The number represents the day after injury at which the data were quantified.

CCI, chronic constriction injury; F, female; IHC, immunohistochemistry; ISH, *in situ* hybridization; LE, Long–Evans; M, male; PCR, polymerase chain reaction; pSNL, partial sciatic nerve ligation; ScNL, sciatic nerve ligation; SD, Sprague–Dawley; SNI, spared nerve injury; SNL, spinal nerve ligation; SNT, sciatic nerve transection; SphT, saphenous nerve transection; WB, Western blot.

direction of expression of Nav channels is highly dependent on timing of investigation after peripheral nerve injury. It is therefore not possible to definitely state that Nav1.3 is significantly increased and Nav1.8 is significantly decreased during the chronic phase in pain models. Moreover, the returning to baseline of Nav1.8 mRNA

levels at later timepoints may suggest Nav1.8 to play a time-locked role in the adaptation of the sensory system after peripheral nerve injury. Regarding potential mechanisms of Nav1.3 upregulation, one study reported the microRNA miR-96 to be significantly decreased at week 3 after chronic constriction injury. Subsequent intrathecal administration of miR-96 decreased Nav1.3 immunoreactivity in dorsal root ganglions of chronic constriction injury rats, suggesting a downregulating effect of miR-96 on Nav1.3[65] (fig. 2).

Another sodium channel subunit that has been mentioned in the context of peripheral nerve injury–induced pain models is the Nav1.9 subunit. Data on Nav1.9 expression during the chronic phase of pain have been rather conflicting as Nav1.9 was found to be significantly increased on the protein level in one study 4 weeks after chronic constriction injury.[23] In contrast, another study, using the same species, breed, and sex, found a decrease of Nav1.9 expression, which was only significant on the mRNA level (table 5).[64] The discrepancy was noticed by the authors, but a potential explanation was unfortunately not given. Another study investigating Nav1.9 on the mRNA level also found Nav1.9 to be decreased at 28 and 90 days after spared nerve injury.[41] The relevance of Nav1.9 during the chronic phase of peripheral nerve injury–induced pain models remains therefore uncertain.

Potassium. In contrast to the depolarizing effect of sodium channels, potassium channels play an important role in neuronal transmission *via* repolarization of the membrane potential. Like sodium channels, they consist of different subunits. Our search revealed that five potassium channel subunits were quantified on the protein level in two different studies, all of which were found to be significantly decreased during the chronic phase of these models.[66,67] For example, Kv1.4, Kv4.2, Kv7.2, and KCNMA1 were significantly downregulated at 3 weeks after L5/L6 spinal nerve ligation.[66] One study found the nerve injury–induced downregulation of these potassium channels to be associated with altered histone modification, in particular an increase in the enrichment of G9a-dependent H3K9me2, which is involved in epigenetic modification of histone proteins.[66] Inhibition of G9a restored the expression levels of Kv1.4, Kv4.2, Kv7.2 ,and calcium-activated potassium channel subunit alpha-1 (KCNMA1) in injured neurons and largely restored the diminished Kv currents[66] (fig. 2). Furthermore, mice lacking G9a in dorsal root ganglion neurons failed to reduce the expression of these potassium channels after peripheral nerve injury. It was therefore concluded that G9a is crucial for nerve injury–induced potassium channel gene silencing and reduction in Kv currents. Interestingly, inhibition of G9a also normalized the expression of other genes that were upregulated or downregulated after peripheral nerve injury, suggesting an important role of G9a in many downstream processes related to neuropathic pain.[66]

In dorsal root ganglions of naïve rodents, the Kv9.1 subunit is robustly expressed in medium- to large-diameter neurons (mean ± SE, 97.2 ± 0.7% of $NF200^+$ neurons), which give rise to Aβ and Aδ fibers. This subunit is rarely expressed in small-diameter neurons (mean ± SE, 3.5 ± 1.0% and 0.8 ± 0.6% of $CGRP^+$ and isolectin B4 ($IB4^+$) neurons, respectively).[67] In one study, at 4 weeks after L5 spinal nerve ligation, Kv9.1 was found to be significantly downregulated.[67] Kv9.1k knockdown in naïve rats, using small interfering RNA (siRNA) and L5 spinal nerve ligation, produced mechanical hyperalgesia and significantly increased spontaneous activity and stimulus-evoked activity on *ex vivo* intracellular recordings of the dorsal root. Notably, Kv9.1 knockdown of Kv9.1 did not produce heat hyperalgesia. This suggests that diminished Kv9.1 function and the subsequent lower firing threshold and spontaneous activity in dorsal root ganglion neurons are involved in mechanical hypersensitivity, but not in thermal hypersensitivity behavior.[67]

Basolateral Na-K-Cl symporter (NKCC1) was reported by one study to be significantly increased on the protein level in small- and medium-sized neurons at 3 weeks after chronic constriction injury in rats.[68] Based on the findings of the study, the upregulation was suggested to contribute to thermal hypersensitivity through an increase of intracellular chloride concentrations, causing an increase in action potential frequency.[68] However, while intracellular chloride concentrations were increased at day 7 and day 14 after chronic constriction injury, they were back to baseline levels at day 21. These data suggest that this cotransporter may be more relevant in the early stages after peripheral nerve injury and contributes less to the chronic phase of peripheral nerve injury–induced pain behavior.[68]

Calcium. We found 3 studies that investigated calcium-channel expression at timepoints greater than 3 weeks after peripheral nerve injury.[69–71] Voltage-gated calcium channel subunit alpha Cav3.2 (Cav3.2), a subunit of the T type calcium channel, was found to be significantly increased in the dorsal root ganglion of rats at 3 weeks after L5 spinal nerve ligation in one study.[69] However, another study found no change in Cav3.2 expression at 4 weeks after sciatic nerve transection in mice. Rather, they found a small decrease at day 2 that was not significant and that quickly returned to baseline levels at day 7.[70] This discrepancy was also noticed by the authors, and might be explained by the use of different nerve injury models. Unfortunately, no other sciatic nerve transection studies have quantified Cav3.2 in the dorsal root ganglion at timepoints longer than 3 weeks in the context of peripheral nerve injury–induced pain models. However, during the acute phase, increased expression of Cav3.2 is consistently found in several other peripheral nerve injury models.[72–76] The one study that showed an increased expression of Cav3.2 during the chronic phase found Cav3.2 to be colocalized with neurons labeled for IL-6. This cytokine appeared also to be upregulated at 3 weeks after peripheral nerve injury.[69] Based on this observation and additional *in vitro* studies on cultured primary sensory neurons, the authors suggested Cav3.2 to be upregulated *via* IL-6, through the

Table 6. Overview of High-throughput Sequencing Studies Performed at 3 Weeks or Later after Peripheral Nerve Injury

	Database No.	Model	Species	Sex	Latest Timepoint	Method	Threshold	Most Regulated Functions/Processes	Upregulated	Downregulated	Top Upregulated	Top Downregulated	Study
1	BG 662484-BG 673712	Sciatic nerve transection	Rat	Male	28 d	Microarray	2-fold change	Neuropeptides, growth-associated proteins. receptors, channels, synaptic proteins, and signal transduction	56	335	Cck, Gal, Npy, Vip	Arf1	Xiao 2002[49]
2	GSE2636	Partial sciatic nerve ligation/CCI	Rat	Male	21 d	Microarray	1.5-fold change	Not mentioned	138/80	80/93	—	—	Barclay 2007[50]
3	Not given	L5/L6 spinal nerve ligation	Rat	Not specified	19–21 d	Microarray	3-fold change	Neuropeptides, inflammatory proteins, DRG-specific pain targets	207	130	VIP, C3, NPY, Map4k1, HT018	Nav1.9, 5HT3, Kv9.1, GluR5-2, Kcnd3, Sst	Levin 2008[51]
4	Not given	L5/L6 spinal nerve ligation	Rat	Male	20 wk	Microarray	2-fold change	Cell–cell communication, synaptogenesis, immune molecules	14	40	NPY, VIP, fibronectin, leucine zipper protein, MRP14	Pcna, Cytb5, Cyp, p21, CD44	Kim 2009[52]
5	GSE24982	L5 spinal nerve ligation	Rat	Male	28 d	Microarray	1.5-fold change	—	—	—	—	mir-204-5p, mir-519d-3p, mir-20b-5p, mir-6838-5p	von Schack 2011[53]
	GSE24982	L5 spinal nerve ligation	Rat	Male	28 d	Microarray	1.5-fold change	Transmembrane transport, synaptic transmission, regulation membrane potential, sensory pain perception	123	—	Dpp10, 5HT3b, Pvalb, MX2, chrnb4	—	Gao 2018[54]
	GSE24982	L5 spinal nerve ligation	Rat	Male	28 d	Microarray	2-fold change	Cell cycle, complement cascade, FoxO signaling, synaptic vesicle cycle	173	257	Ctnnb, Irs1, Cdk6, Mdm2, IL6st	Cav1, Nrg1, Stim1, Jak1, Vcl	Chen 2017[55]
6	GSE60033	Sural spared nerve injury *vs.* tibial spared nerve injury	Rat	Male	23 d	Microarray	$P < 0.05$	Voltage-dependent sodium channels, voltage-dependent calcium channels	2	7	—	miR-133b, miR-143, miR-335-5p, miR-1	Norcini 2014[56]
7	GSE59043	L5/L6 spinal nerve ligation	Rat	Male	21 d	RNA sequencing	2-fold change	Neuronal system, potassium channels, ECM organization, cytokine–cytokine receptor interaction	1,684	1,039	—	—	Garriga 2018[57]
8	Not given	Spared nerve injury	Mouse	Female	30 d	RNA sequencing	1.5-fold change	Transcription regulator, enzyme, kinase, transporter	144	33	Sprr1a, Npy, Fgf3, Cckbr, Gpr151	Mobp, Olig2, Neurod2, Opalin, Pou3f3	Uttam 2018[58]
9	GSE107180	Spared nerve injury	Mouse	Male	28 d	RNA sequencing	3-fold change	Response to biotic stimulus, defense response, immune response, epidermis development, cAMP-mediated signaling	254	55	sprr1b, Gm5152, Sh2d1b2, Taf7l, Sprr1a	Scgb1c1, Calb2, Ostn, Uts2, Arx	Chen 2020[59]

A total of 11 studies was found, of which 3 studies used the same dataset.

cAMP, cyclic adenosine monophosphate. Top upregulated genes: 5HT3b, 5-hydroxytryptamine receptor 3B; C3, complement C3; Cck, cholecystokinin; Cckbr, cholecystokinin B receptor; Cdk6, cyclin dependent kinase 6; Chrnb4, cholinergic receptor nicotinic beta 4 subunit; Ctnnb, catenin beta 1; Dpp10, dipeptidyl peptidase like 10; Fgf3, fibroblast growth factor 3; Gal, galanin and GMAP prepropeptide; Gm5152, predicted gene 5152; Gpr151, G protein–coupled receptor 151; HT018, semaphorin 6A; IL6st, interleukin 6 cytokine family signal transducer; Irs1, insulin receptor substrate 1; Map4k1, mitogen-activated protein kinase kinase kinase kinase 1; Mdm2, MDM2 proto-oncogene; MRP14, S100 calcium binding protein A9; MX2, MX dynamin like GTPase 2; Npy, neuropeptide Y; Pvalb, parvalbumin; Sh2d1b2, SH2 domain containing 1B2; Sprr1a, small proline rich protein 1A; Sprr1b, small proline rich protein 1B; Taf7l, TATA-box binding protein associated factor 7 like; Vip, vasoactive intestinal peptide. Top downregulated genes: 5HT3, 5-hydroxytryptamine receptor 3A; Arf1, ADP ribosylation factor 1; Arx, aristaless related homeobox; Calb2, calbindin 2; Cav1, caveolin 1; Cyp, peptidylprolyl isomerase G; Cytb5, cytochrome b5; GluR5-2, glutamate ionotropic receptor kainate type subunit 1; Jak1, Janus kinase 1; Kcnd3, potassium voltage-gated channel subfamily D member 3; Kv9.1, potassium voltage-gated channel modifier subfamily S member 1; Mobp, myelin associated oligodendrocyte basic protein; Nav1.9, sodium voltage-gated channel alpha subunit 11; Neurod2, neuronal differentiation 2; Nrg1, neuregulin 1; Olig2, oligodendrocyte transcription factor 2; Opalin, oligodendrocytic myelin paranodal and inner loop protein; Ostn, osteocrin; p21, cyclin dependent kinase inhibitor 1A; Pcna, proliferating cell nuclear antigen; Pou3f3, POU class 3 homeobox 3; Scgb1c1, secretoglobin family 1C member 1; Sst, somatostatin; Stim1, stromal interaction molecule 1; Uts2, urotensin 2; Vcl, vinculin.

Table 7. Overlap between Molecules that Were Found to Be Significantly Regulated in Studies on the Protein Level and Significantly Regulated Genes Found in the Sequencing Studies

	12060780	18160218	19307059	30233637	29180893*	30906902
Activating transcription factor 3						
Glial fibrillary acidic protein						
Major histocompatibility complex class II						
Galanin						
Neuropeptide Y						
p-P38						
Caspase 3						
CD68 / ED1						
Glial cell line-derived neurotrophic factor						
Tumor necrosis factor alpha						
Transient receptor potential cation channel A1						
p-ERK						
GDNF receptor alpha 1						
Transient receptor potential cation channel M8						
Voltage-gated sodium channel Nav1.7						
Allograft inflammatory factor 1						
Brain-derived neurotrophic factor						
Galanin receptor 2						
Voltage-gated sodium channel Nav1.3						
NF-kB						
Nerve growth factor						
p-Jun-kinase						
T-cell receptor						
Transient receptor potential cation channel 4						
Tyrosine Hydroxylase						
Transient receptor potential cation channel 1						
Interleukin-1-beta						
P2X purinoceptor 3						
Calcitonin gene-related peptide						
Interleukin-10						
Mu-type opioid receptor						
H(+)/Cl(-) exchange transporter 3						
Substance-P						
Sigma-1 Receptor						
Voltage-gated sodium channel Nav1.8						
Total: 35	**7 (20.0%)**	**6 (17.1%)**	**4 (11.4%)**	**1 (2.9%)**		**4 (11.4%)**

Green cells represent significantly upregulated genes, and *red cells* represent significantly downregulated genes.

*This study looked at changes in gene expression in the uninjured dorsal root ganglion in L5 spinal nerve ligation model.

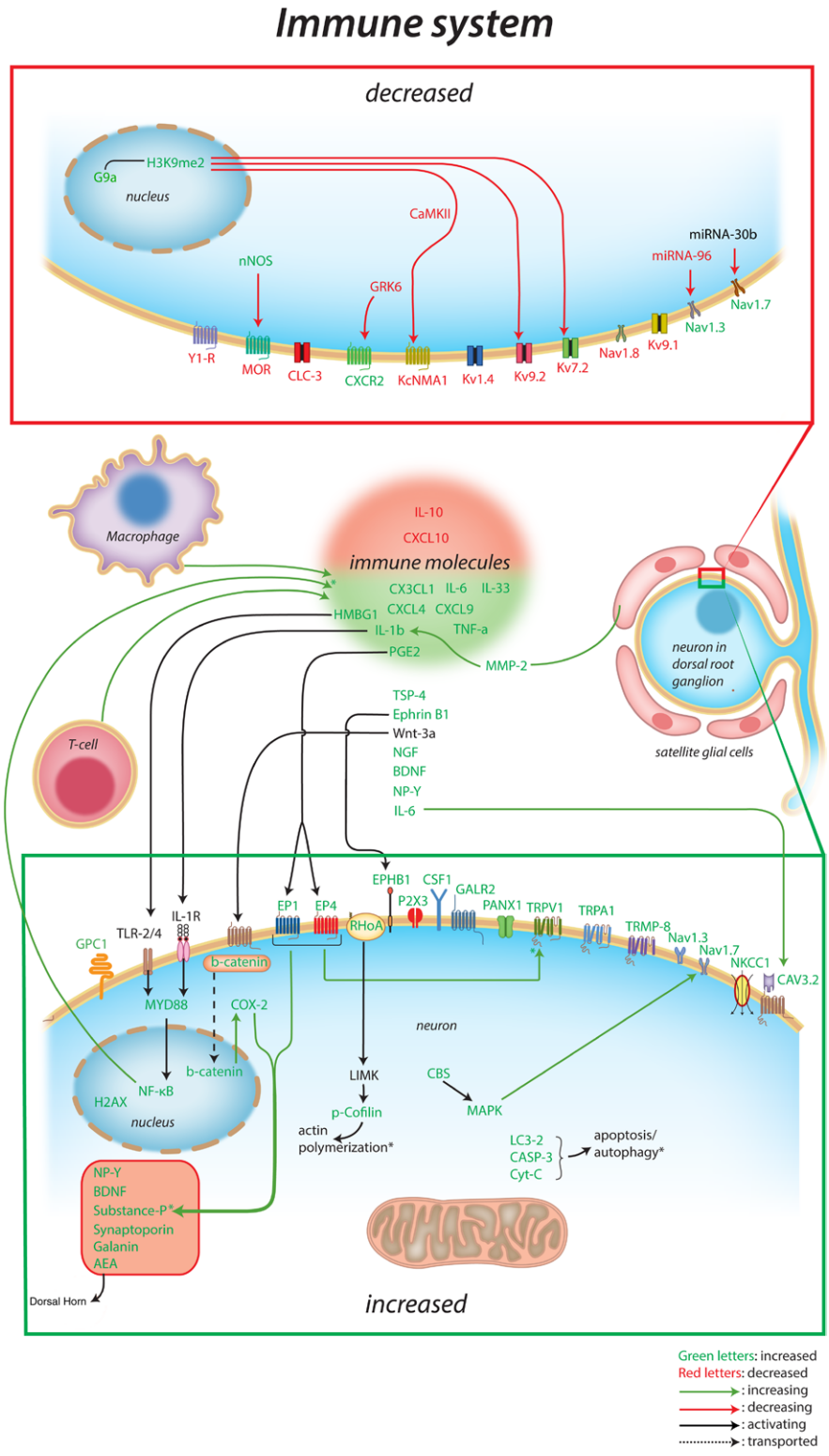

Fig. 2. Schematic overview of the molecules studied and regulated at the dorsal root ganglion in the chronic phase of nerve injury–induced pain models, based on results of the included studies. Names in *red* indicate those molecules that are downregulated in the chronic phase of nerve injury–induced pain models. *Green* names represent molecules that are increased in the chronic phase of nerve injury–induced pain models. *Red and green arrows* indicate decreasing or increasing processes and/or molecular interactions respectively. *Black arrows* indicate transporting or activating pathways. Pathways or molecules with an *asterisk* (*) are inferred from the study as hypothetical and not actually demonstrated. (*Continued*)

Fig. 2. (*Continued*) AEA, anandamide; BDNF, brain-derived neurotrophic factor; CaMKII, calmodulin-dependent protein kinase type II; CASP-3, caspase 3; CAV3.2, voltage-gated calcium channel subunit alpha Cav3.2; CBS, cystathionine β-synthetase; CLC-3, H(+)/Cl(–) exchange transporter 3; CSF1, macrophage colony-stimulating factor 1; CXCL, C-X-C motif chemokine; CXCR2, C-X-C chemokine receptor type 2; Cyt-C, cytochrome C; EPHB1, ephrin type-B receptor 1; G9a, G9a protein; GALR2, galanin receptor type 2; GPC1, glypican 1; GRK6, G protein–coupled receptor kinase 6; H2AX, histone H2AX; HMBG1, high mobility group protein B1; IL-1b, interleukin-1b; IL-1R, interleukin-1R; IL-6, interleukin-6; IL-10, interleukin-10; IL-33, interleukin-33; KcNMA1, calcium-activated potassium channel subunit alpha-1; Kv1.4, voltage-gated potassium channel subunit Kv1.4; Kv7.2, voltage-gated potassium channel subunit Kv7.2; Kv9.1, voltage-gated potassium channel subunit Kv9.1; Kv9.2, voltage-gated potassium channel subunit Kv9.2; LC3-2, microtubule associated protein 1 light chain 3-2; LIMK, LIM kinase; MAPK, mitogen-activated protein kinase; miRNA-30b, microRNA-30b; miRNA-96, microRNA-96; MMP-2, matrix metalloproteinase 2; MOR, mu-type opioid receptor; MYD88, myeloid differentiation primary response protein MyD88; Nav1.3, voltage-gated sodium channel Nav1.3; Nav1.7, voltage-gated sodium channel Nav1.7; Nav1.8, voltage-gated sodium channel Nav1.8; NGF, nerve growth factor; NKCC1, basolateral Na-K-Cl symporter; NF-κB, nuclear factor kappa B; NP-Y, neuropeptide Y; nNOS, neuronal nitric oxide synthase; PANX1, pannexin-1; P2X3, P2X purinoceptor 3; PGE2, prostaglandin E2; RHoA, transforming protein RhoA; TLR-2/4, Toll-like receptor 2/4; TNF-a, tumor necrosis factor alpha; TRPA1, transient receptor potential cation channel A1; TRPV1, transient receptor potential cation channel subfamily V member 1; TSP-4, thrombospondin-4; Y1-R, Y1R receptor.

sIL-6R/gp130 pathway (fig. 2).[69] In line with their reasoning, *in vivo* suppression of the sIL-6R/gp130 pathway with an inhibitor reversed mechanical hypersensitivity behavior and increased Cav3.2 protein levels after spinal nerve ligation.[69]

Another calcium channel investigated in the context of pain is voltage-gated calcium channel subunit alpha Cav2.2 (Cav2.2). Cav2.2, an N-type calcium channel, was found to be significantly decreased in injured dorsal root ganglions at 3 days after L5 spinal nerve ligation and remained low until 4 weeks after injury, which was the latest timepoint investigated in this study.[71] In contrast, the expression of Cav2.2 was found to be significantly increased in the uninjured L4 dorsal root ganglion at those timepoints. Blockage of Cav2.2 at day 7 after injury (in the same study) alleviated mechanical allodynia and inhibited the hyperexcitability of uninjured L4 dorsal root ganglion neurons induced by L5 spinal nerve ligation. Since the observed changes of Cav3.2 and Cav2.2 persisted during the chronic phase after injury, these findings support the idea that a delicate balance in the expression of calcium channels exists in both injured and uninjured dorsal root ganglions. This balance, or disbalance induced by peripheral nerve injury, may underly processes related to the "chronification" of pain.

Other Ion Channels. Besides sodium channels, potassium channels and calcium channels, some other ion channels have been associated with the chronic phase of peripheral nerve injury–induced pain models. Significant downregulation of ClC-3, a potassium-chloride exchange transporter, was observed in rat dorsal root ganglions by two studies, starting from day 3 and lasting until day 28 after spared nerve injury, which was the latest investigated timepoint.[77,78] Restoration of ClC-3 expression, using adenovirus harboring ClC-3, prevented mechanical hypersensitivity behavior in rats subjected to spared nerve injury, while knockdown of ClC-3 in naïve rats resulted in a decreased mechanical paw withdrawal threshold.[78] In one of the two studies, ovariectomized female rats were used. Interestingly, 17β-estradiol replacement *via* subcutaneous injections resulted in both a partial reversal of cold hypersensitivity behavior and restoration of ClC-3 protein expression.[77] These data suggest that estrogen levels contribute to endogenous analgesic mechanisms *via* regulation of ClC-3.[77] This by itself is an interesting finding, as chronic pain in humans has been more frequently reported in female individuals.[79]

Pannexin-1 (Panx1), a large-pore membrane channel involved in the release of adenosine triphosphate and other signaling mediators, was found to be significantly upregulated in dorsal root ganglions of rats after L5/L6 spinal nerve ligation, starting from day 5 until at least day 21 after injury.[80] Notably, *in vivo* inhibition of Panx1 at 3 weeks after injury *via* intrathecal administration of siRNA significantly attenuated tactile allodynia and mechanical hypersensitivity induced by the nerve injury.[80]

TRPV1, a nonselective cation channel also known as the capsaicin receptor, is one of the most frequently quantified molecules in the dorsal root ganglion related to studies investigating the chronic phase of pain models. Nevertheless, literature on this molecule showed highly conflicting results (table 5). For example, protein expression of TRPV1 was consistently found to be elevated in the dorsal root ganglions of both rats and mice at 3 to 4 weeks after chronic constriction injury.[12,13,15] Also, in the L5/L6 spinal nerve ligation and partial sciatic nerve ligation models, TRPV1 protein levels were found to be increased in the dorsal root ganglions at 4 weeks and 18 months after injury, respectively.[11,14] On the mRNA level, TRPV1 expression was found to be increased at week 3 and week 10 after partial sciatic nerve ligation and saphenous nerve axotomy, respectively.[20,21] In contrast, one study found protein expression of TRPV1 in dorsal root ganglions of rats to be decreased at day 30 after sciatic nerve transection.[17] Also, on the mRNA level, TRPV1 was found to be significantly downregulated at 30 days after sciatic nerve transection, and 12 weeks after spared nerve injury.[17,18] One study found no differences on the mRNA level at 4 weeks after sciatic nerve transection.[19] Interestingly, an increase in TRPV1 mRNA expression levels has been reported in noninjured dorsal root ganglions at 4 weeks after L5 spinal nerve ligation.[81] Summarizing, TRPV1 levels in the

dorsal root ganglion during the chronic phase of peripheral nerve injury–induced pain models show conflicting results that vary with the pain model used. The role of TRPV1 in pain is complex, and studies revealed both pronociceptive and antinociceptive properties.[82] For example, increases in TRPV1 mRNA expression and peripherally directed axonal transport of TRPV1 protein have been demonstrated to be associated with neuropathic pain states and inflammation.[83] Conversely, knockdown of the TRPV1 gene prevents the development of inflammatory hyperalgesia in the rat.[84,85] Based on the inconsistent results of TRPV1 expression levels during the chronic phase, likely depending on the animal model used, one must be cautious in drawing conclusions regarding a universal role of TRPV1 for different chronic pain conditions. Ideally, significant findings should be validated in different pain models.

P2X purinoceptor 3 (P2X3), which belongs to the family of purinoceptors for adenosine triphosphate, was found to be significantly increased at the protein level at 3 to 4 weeks after chronic constriction injury, partial sciatic nerve ligation, spared nerve injury, and saphenous nerve transection.[12,21,33,35,36] In contrast, one study reported a significant decrease in P2X3 levels at week 4 after sciatic nerve ligation.[34] These conflicting results could indicate that P2X3 has different roles in peripheral nerve injury–induced pain behavior, depending on the type of nerve lesion that is made.

Immune-related Molecules

Neuroinflammatory process are known to contribute to pain immediately after peripheral nerve injury (*i.e.*, nerve injury hurts). Nevertheless, little is known about the contribution of the immune system to chronic pain in humans and the late phase (3 weeks or more) of animal models investigating peripheral nerve injury–induced pain-like behaviors. In dorsal root ganglions of naïve rodents, a small population of resident macrophages is present. During the chronic phase, an influx of immune cells from the blood into the dorsal root ganglion, mainly macrophages and T cells, was observed.[47,48,86,87] While the peak amount of immune cell infiltration was observed around 1 to 2 weeks after injury, significant increased levels were still apparent months later after chronic constriction injury, sciatic nerve transection, and spinal nerve ligation.[47,48] Some immune cells started penetrating the glial sheath of neurons and formed ringlike structures around neuronal cell bodies. The numbers and types of immune cells encircling neuronal cell bodies were found to differ between different peripheral nerve injury models.[47] The exact role of immune cells in the pain process is not clear, but it is suggested they contribute to nerve injury–induced pain-like behavior in animals by producing proinflammatory molecules, which can have pronociceptive effects on the excitability of neurons.[48]

The cytokine IL-1β is such a proinflammatory molecule, which has been frequently investigated in the pain field. During the chronic phase, IL-1β protein in three studies was found to be significantly upregulated in dorsal root ganglions at 3 to 4 weeks after chronic constriction injury.[22,23,26] Another study, which performed spared nerve injury lesions in rats, confirmed increased IL-1β protein expression at 3 weeks after injury.[24] Interestingly, one study showed increased mRNA levels but decreased protein levels at 3 weeks after L5 spinal nerve ligation injury, suggesting differences in IL-1β levels between peripheral nerve injury–induced pain models (table 5).[27] Regarding the mechanism by which IL-1β could potentially contribute to the pain process, it was hypothesized by Liu *et al.* that IL-1β might cause upregulation of other cytokines *via* binding on the IL-1 receptor. IL-1 binding to its receptor may activate the myeloid differentiation primary response protein MyD88 (Myd88)-pathway with concomitant phosphorylation and activation of nuclear factor-Kβ p65 and extracellular signal-regulated kinase (ERK; fig. 2).[22] In the same study, high mobility group protein B1 (HMGB-1) was found to be increased as well at 3 weeks after chronic constriction injury. It was suggested to activate Myd88 and nuclear factor-Kβ p65/ERK *via* the Toll-like receptor (TLR)2/4 receptors, based on the fact that Myd88, nuclear factor-Kβ p65, and ERK were also upregulated in the dorsal root ganglions, and that this upregulation was reverted by a Myd88 inhibitor.[22] Additional evidence is needed to confirm this mechanism with more certainty.

IL-1β is activated by cleavage, which can be done by matrix metalloproteinases. Peripheral nerve injury induced an upregulation of matrix metalloproteinase 2 (MMP2) protein and mRNA levels in the dorsal root ganglions of rats at days 7, 10, and 21 after L5 spinal nerve ligation.[88] This upregulation in expression was found to be present in satellite glial cells in the dorsal root ganglion.[88] This was in contrast to matrix metalloproteinase 9 (MMP9), which showed a rapid and transient upregulation in expression the first day after injury, suggesting a different role between MMP9 and MMP2 in initiation and maintenance of nerve injury–induced pain-like behavior in animals, respectively.[27,88] Inhibition of MMP2 alleviated established pain-like hypersensitivity behavior, which was also accompanied with an inhibition of caspase 3 activity.[27] It was hypothesized that MMP2 could potentially contribute to chronic pain by activating IL-1β (fig. 2).[88]

Besides cytokines, C-X-C motif chemokines (CXCL) CXCL4 and CXCL9 were found to be upregulated on the protein level in the dorsal root ganglion of rats at 4 weeks after chronic constriction injury, while chemokine receptor CXCR3 showed no significant changes.[45] However, another study reported CXCL10 and CXCR3 to be increased in the dorsal root ganglion at 3 weeks after chronic constriction injury (table 5).[44] Interestingly, they were colocalized in neurons, suggesting a role of chemokines in neuron–neuron interactions.[44] An explanation for the discrepancy of CXCR3 between the two studies is difficult to provide, since both used the same type of animals, breed, and pain model.

Another chemokine receptor, CXCR2, was also found to be increased significantly on the protein level at 3 weeks

after chronic constriction injury. This was suggested to be caused by a downregulation of G protein–coupled receptor kinase 6 (GRK6), since the upregulation of CXCR2 was suppressed by overexpression of GRK6 using lentiviral injections (fig. 2).[89] Furthermore, overexpression of GRK6 by lentiviral injections attenuated chronic constriction injury–induced pain responses.[89]

Last, the percentage of immunoreactive neurons for prostaglandin E2 receptor subtypes EP-1 and EP-4 was found to be significantly increased in the dorsal root ganglions of rats at 18 months after partial sciatic nerve ligation.[14] It was suggested that injury-derived prostaglandin E2, *via* the EP-1 and EP-4 receptors, was involved in the synthesis of nociceptive molecules, such as substance P, since the upregulation of these molecules was suppressed by administration of a cyclooxygenase-2 (COX-2) inhibitor (fig. 2).[14]

Neurotrophic Factors and Neuropeptides

Neurons and activated satellite glial cells inside the dorsal root ganglion produce and secrete neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF). BDNF was shown to be upregulated on the protein level at weeks 6 and 10 after partial sciatic nerve ligation, and at week 4 after sciatic nerve transection.[90,91] Interestingly, the increased BDNF expression was not generalized, but rather confined mainly to medium- and large-diameter neurons.[91] In naïve animals, BDNF is mostly expressed in small neurotrophic tyrosine kinase receptor type (Trk) $TrkA^+$ neurons, but after partial sciatic nerve ligation, larger $TrkB/C^+$ neurons started to express BDNF.[91] Additionally, BDNF immunoreactivity was observed in some fiber bundles that formed pericellular baskets, which were most prominent around small- to medium-sized cells.[91]

NPY has a very low constitutive expression in the dorsal root ganglion of naive animals, but was found be increased in the dorsal root ganglion at weeks 3, 4, 6, and 24 after injury, preferentially in medium- to large-sized neurons.[92,93] This increased expression was observed in different peripheral nerve injury models, including spared nerve injury, sciatic nerve transection, and L5 spinal nerve ligation.[92,94–96] The average size of NPY expressing neurons was found to increase over time, from 666 μm^2 at 2 weeks to 817 μm^2 at 24 weeks after spared nerve injury.[92] The expression pattern of NPY shifted from Aδ to Aß fiber neurons, which also correlated with a shift toward a more mechanical type of hypersensitivity in the rats.[92] This is in congruence with the theory that Aß neurons are involved in mechanical allodynia.[92] The authors therefore hypothesized that the upregulation of NPY seems to play a role in mechanical hypersensitivity, but not in thermal hypersensitivity.[92]

Galanin, another neuropeptide, was found to be increased on the protein level by four studies during the chronic phase in different pain models, namely sciatic nerve transection, spared nerve injury, L5 spinal nerve ligation, and chronic constriction injury, of which all were statistically significant except chronic constriction injury.[95,97–99] A significant increase of its receptor, galanin receptor type 2, was also found at 4 weeks after chronic constriction injury and spared nerve injury, mainly in the medium- to large-sized neurons.[97,99] The latest timepoint at which galanin was investigated was 55 weeks after injury, in a study that used two pain models (sciatic nerve transection and L5 spinal nerve ligation).[98] Interestingly, besides $galanin^+$ neurons, they also reported the presence of pericellular axonal rings containing galanin in the dorsal root ganglions after injury, which were not identified in naïve or contralateral dorsal root ganglions.[98] Unlike TH^+ rings and $CGRP^+$ rings, which were also identified, the $galanin^+$ rings encircled both large- and small-diameter neurons. Although the upregulated galanin expression was gradually lost with time, both galanin and CGRP expression remained elevated for over a year after the injury.[98] Both the changes in peptide expression and the appearance of rings were more dramatic and occurred sooner after L5 spinal nerve ligation compared to sciatic nerve transection.[98] The functional significance of galanin and the perineuronal rings in dorsal root ganglions after nerve injury remains to be elucidated.

After sciatic nerve transection and spared nerve injury, substance P was found to be significantly downregulated, mainly in small-sized neurons.[95,97,100] Interestingly, *de novo* expression of substance P was reported in large-sized neurons.[100] The same observation was made with CGRP, which is normally expressed only in small-sized neurons.[100] In this way, it was suggested that substance P and CGRP are increasingly transported to the dorsal horn by small-sized neurons *via* their central projections, contributing to central sensitization. On the other hand, the local *de novo* expression of substance P and CGRP by large-sized neurons may contribute to changes in Aß neurons, which then potentially could lead to spontaneous pain and mechanical allodynia.[101]

Intracellular Neuronal Changes

The transforming protein RhoA/LIM kinase/Cofilin pathway was indicated, by one study, to be activated in the dorsal root ganglion during the chronic phase, since all three subcomponents were upregulated at week 3 after chronic constriction injury.[102] It was also observed that the membrane/cytosol ratio of RhoA, which exerts its biologic function by translocating from the cytoplasm to the plasma membrane, increased significantly in response to chronic constriction injury[102] Inhibition of the RhoA/LIMK/Cofilin pathway attenuated pain-related behavior and the increased membrane translocation of RhoA, induced by chronic constriction injury, was reversed.[102] The RhoA/LIMK/Cofilin pathway is known to be involved in cytoskeleton regulation and actin depolymerization. The authors hypothesized that this change in the cytoskeleton after chronic constriction injury could potentially serve as a scaffold for trafficking of nociceptive signaling factors toward the central and/or peripheral axons, leading to neuropathic

pain (fig. 2).[102] Additional experiments in future studies are necessary in order to confirm this hypothesis.

Caspase 3, a marker for apoptosis, was found to be significantly increased from day 1 after both spinal nerve ligation and sciatic nerve transection by the same study.[103] However, caspase 3 levels returned to baseline at 21 days after spinal nerve ligation, while remaining significantly increased after sciatic nerve transection at the same timepoint. The same was found for ATF3, a marker for neuronal damage. The time course of the protein expression of these two markers was dependent on the severity of nerve injury, suggesting that sciatic nerve transection leads to more damage than spinal nerve ligation.[103] Various other intracellular pathways are involved in pain processes, such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinases (JNK), of which the exact mechanisms still have to be elucidated during the chronic phase.

Cell–Cell and Cell–Matrix Interactions

β-Catenin, an important cell–cell adhesion molecule, was found to be significantly increased in dorsal root ganglion neurons at 4 weeks after chronic constriction injury.[39] It was further noted that the β-catenin was located more in the cytosol and nucleus in the ipsilateral dorsal root ganglion, while β-catenin in the contralateral dorsal root ganglion was observed more on the membrane.[39] It was proposed that β-catenin increase and nuclear accumulation could promote the biosynthesis and release of substance P through induction of COX-2, thereby contributing to peripheral neve injury–induced pain-like behavior (fig. 2).[39] On the other hand, glypican-1 (a cell surface heparan sulfate proteoglycan), which was upregulated 4 weeks after sciatic nerve transection, showed a shift in localization from a predominantly nuclear to cytoplasmic and membrane-associated expression.[104] Furthermore, glypican-1 expression, both constitutively and peripheral nerve injury–induced, was present in small- and large-sized neurons.[104] Unfortunately, mechanisms regarding the role of glypican-1 in the processes underlying peripheral nerve injury–induced pain behavior were not investigated by the article.

Neural cell adhesion molecule L1 (L1-CAM), a cell adhesion molecule of the L1 protein family, which is normally localized in the cytoplasm of C-fiber neurons, was upregulated on protein level at 4 weeks after sciatic nerve transection. Furthermore, it changed its localization to the cell membrane, forming L1-CAM-ir ring structures around injured small- to medium-sized neurons.[105]

Concluding from these results, it is apparent that interactions of neurons and glia, with each other and with the extracellular matrix, are involved during the chronic phase of peripheral nerve injury–induced pain behavior.

Satellite Glial Cells

Satellite glial cells are glial cells in the dorsal root ganglion that are thought to have similar roles as astrocytes in the CNS. They surround cell bodies of sensory neurons and have important regulatory properties on neuronal behavior. Their exact role in neuropathic pain is still poorly understood. Especially during the chronic phase, only a limited number of studies regarded the contribution of satellite glial cells in peripheral neve injury–induced pain-like behavior. When activated, satellite glial cells show morphologic changes and different expression profiles. Increased expression of GFAP is one hallmark that is associated with the activation of satellite glial cells and astrocytes. GFAP protein levels were examined in six studies, five of which showed significantly increased levels days 21, 30, and 42 after chronic constriction injury, and days 28 and 56 after L5 spinal nerve ligation.[104,106–109]

In naïve dorsal root ganglions, satellite glial cells wrap all types of neurons in a dispersed manner. However, 12 h after spinal nerve ligation, activated satellite glial cells surrounded small- to medium-sized neurons. As time passed, the satellite glial cells wrapped more around large neurons and less around small-sized neurons.[109] Since it is hypothesized that large Aβ-fiber dorsal root ganglion neurons participate in mechanical hypersensitivity behavior in spinal nerve ligation rats, this may indicate that satellite glial cells are activated and contribute to pain-like behavior after nerve injury.[109] However, there exists a discrepancy between studies on whether satellite glial cells play predominantly a role during the early or late phase of allodynia after peripheral nerve injury. One study suggested satellite glial cells to play a role in the early maintenance phase of allodynia after L5 spinal nerve ligation, since usage of a glial metabolism inhibitor resulted in significant decreased hypersensitivity behavior to tactile stimuli at 7 days after spinal nerve ligation, but not at later timepoints.[109] However, another study reported that antisense GFAP treatment reversed tactile allodynia at 6 weeks after injury, but not at 2 weeks after injury.[52]

GFAP expression levels were not unanimous using different pain models, namely the chronic constriction injury and the spared nerve injury model.[107] At 42 days after injury, both chronic constriction injury and spared nerve injury rats showed increased GFAP levels in the dorsal root ganglion, but the total percentage of GFAP signal was significantly higher in spared nerve injury than in chronic constriction injury animals.[107] To conclude, satellite glial cell activation has been shown to be significantly upregulated in various pain models, and its role in the pain process is well established. However, the exact mechanism underlying GFAP-mediated maintenance of pain-like behavior remains elusive.

Discussion

We reviewed the current literature regarding quantitative molecular changes in the dorsal root ganglion during the late phase (3 weeks or more) of peripheral nerve injury–induced pain-like behavior in rodents. One hundred sixty-eight studies were found, in which changes of a total of 309 molecules were described. Nine high-throughput

sequencing studies were performed. Several conclusions can be drawn.

We found evidence of various types of molecular changes, potentially involved in chronic neuropathic pain. The main classes of molecules found to be quantitatively changed include neuropeptides (galanin, NPY, GDNF, BDNF, NGF, substance P), sodium channels (Nav1.7, Nav1.3, Nav1.8), potassium channels (Kv1.4, Kv4.2, Kv7.2), TRP channels (TRPV1, TRPA1, TRPM8), and immune-related molecules (MHC-II, CD68, TNF-α, IL-1β, IL-10). NPY and galanin were found to be most consistently regulated in sequencing studies, which might have potential relevance for local treatment at the dorsal root ganglion level. For some of the identified molecules, the possible causal link with pain and potentially involved mechanisms were clarified more than for others.

Furthermore, we observed a clear lack of focus on the late phase of molecular changes in the dorsal root ganglions of peripheral nerve injury–induced pain models. It appeared that only 22% of the 754 studies provided quantitative data on molecular changes in the dorsal root ganglion at 3 weeks or later after injury, and only 16% of the 57 sequencing studies used these timepoints.

These findings show that the basis of our current understanding regarding molecular changes in the dorsal root ganglion during the late phase of peripheral nerve injury–induced pain models is actually limited. This observation is concerning, since treatment of chronic pain in humans is effectively needed months or even years after its initiation, as this is when patients often present themselves in the clinic.

As of yet, there exists no widely accepted definition of the chronic phase of pain in animals. Studies used the term "late phase," "maintenance phase," or "chronic phase" inconsistently.[107,109–111]

The second and third weeks after injury, however, were mentioned several times as the critical period during which acute pain transitions into chronic pain.[88,109,112–114] We would like to emphasize that our cutoff point of 3 weeks is not based on factors intrinsic to the pain process, but is rather a reflection of the field in regards to the time period after injury mostly focused on and most often described in terms of "chronic," "late," or "maintenance" phase.

It is evident from the preclinical literature that the underlying processes of chronic pain are not static but change over time.[115] It is likely that these underlying processes do not keep changing indefinitely, but reach a permanent "steady pain-state." However, whether such a state really exists is unknown. Moreover, the underlying molecular mechanisms responsible for such a state will not be brought to light if attention in the field stays mainly focused on the first 2 weeks after injury.

An important objective of preclinical studies is to discover new molecular targets for the treatment of chronic pain patients. Targets that are observed in the first week of preclinical models could be ineffective when they are not present during the later phases at which patients present themselves. Long survival times (*i.e.*, up to months after injury) in animal models investigating pain-like features are, therefore, indispensable to making a proper translation to the clinical profile of patients who present themselves with persistent pain up to months after the initial surgery or disease occurred.

Another notable finding is the fact that the vast majority of studies exclusively used male animals, indicating a clear gap of knowledge regarding pain-like behavior in female animals. Only 11.3% of the molecules were identified in studies using female animals. Furthermore, in the studies that used both male and female animals, the presence or absence of sex differences was rarely analyzed or reported. This is remarkable, since pathophysiologic processes underlying pain-like behavior have been shown to differ greatly between male and female animals.[116] A 2019 RNA sequencing study of rats at 2 weeks after chronic constriction injury found 1,513 genes to be differentially expressed between sexes.[117] Similar observations have been made in human studies.[118] Various sex-specific nociceptive mechanisms have already been identified and suggested in the literature.[116] This can have important implications in regards to successful translation of preclinical research into effective treatment strategies, especially when recognizing that chronic pain prevalence has been consistently found to be higher in women.[119] Future experimental studies should include both sexes and analyze sex-specific changes and mechanisms. This could potentially lead to the discovery of sex-specific targets in the treatment of chronic pain.

Many molecules have been quantified in the dorsal root ganglion, but comparing results from different studies in order to make a conclusive overview is limited by three factors. First, there was a lack of homogeneity between studies regarding animal model and type of animal/strain that was used. The four most frequently used pain models (chronic constriction injury, spared nerve injury, sciatic nerve transection, spinal nerve ligation) were applied in roughly equal numbers. Different models can produce different types of pain behavior with distinct underlying molecular mechanisms.[120] The same applies for studies comparing certain species or strains.[121,122] As a consequence, outcomes of studies investigating different animals or models cannot simply be compared or pooled.

Second, one must be cautious with comparing molecular changes that are quantified at different timepoints, for instance, 3 weeks *versus* 6 months after injury. Chronic pain is not a static process, and different molecular processes are involved at different stages.[123] Whether specific molecular processes eventually reach a steady state, and the timepoint at which such a state is realized, are not yet known. Comparing results between studies using different survival times is therefore complex.

Third, the methods that were used to quantify molecules in the dorsal root ganglion differed between studies. This can have implications when interpreting and comparing results. For example, immunohistochemistry is an excellent method to detect molecules with high sensitivity, but is less optimal for quantification compared to Western blots. Also, the method by which molecules are quantified (*e.g.*, counting cells or signal intensity) changes how the results should be interpreted. Methods on protein level often require the use of validated antibodies, which biases the analyses toward well-researched proteins. A technique like quantitative real-time polymerase chain reaction is very sensitive for detection of mRNA changes, but the correlation between protein and mRNA levels can be low, depending on the cell type and state.[124] These limitations, which are often inherent to the quantification methods, make it more complicated to interpret and compare results between studies.

The schematic overview of pathways and mechanisms of the molecules we provided should be viewed keeping these three limiting factors in mind (fig. 2). Not all mechanisms are simultaneously present in every model at all timepoints, and one has to be aware of potential sources of heterogeneity.

This review has several limitations. The number of high-throughput sequencing studies appeared to be very small. The assessment of the overlap with protein studies could therefore not be extensive. Sequencing studies give the ability to quantify thousands of molecules simultaneously, and *post hoc* analysis can identify relevant patterns and sets of genes. They also do not have the drawback of studies looking at individual molecules, which are often based on hypotheses with intrinsic biases. However, we showed that the number of high-throughput sequencing studies is very limited and that overlap between data on the gene level and protein level is small. Also, studies focusing on the protein level more often provided evidence for a causal link with the pain process. On the other hand, high-throughput studies enable the discovery of larger patterns of expression and analysis of functional links and relations between different genes, which is not possible with studies looking at individual molecules. Especially single-cell RNA sequencing enables identification of gene expression changes at the cellular level instead of at the dorsal root ganglion level. Recently, this technique revealed different cell types in the dorsal root ganglion after injury based on gene expression.[125] Unfortunately, no single-cell RNA sequencing studies have been performed at timepoints of 3 weeks or later as of yet. In short, we see merits in both high-throughput sequencing studies and studies on the protein level, but additional high-throughput (single-cell) sequencing studies have to be conducted since there still is a clear gap of knowledge.

Another limitation may appear to be that our review primarily focused on peripheral nerve injury–based models, which have been frequently used in the context of chronic pain. However, there are numerous other types of animal models mimicking clinical practice, like diabetes, cancer, or chemotherapy-based models.[126] Pain involves numerous molecular changes, some of which may overlap between models, but others may not. However, in the studies we reviewed, heterogeneity between models was already making it difficult to compare results and draw conclusions. Including other types of models, therefore, would have had little additional value.

One must also realize, when studying nerve injury–induced pain models, that many molecular processes are related to cell death or regeneration, and not with pain. This is why in the Results section, we reviewed all evidence that supports a potential causal link with the pain process. However, pain-like behavior is mostly studied using evoked responses, which measure changes in nociceptive thresholds, but do not measure spontaneous pain. Measuring pain in animals is a general problem, which also has to be kept in mind when interpreting pain studies.

Conclusions

In conclusion, we performed a systematic review of the literature regarding molecular changes in the dorsal root ganglion during late phase after peripheral nerve injury and have identified several classes of molecules that were significantly changed. Neuropeptide Y and galanin were found to be upregulated on the protein level, and their genes were found to be most consistently regulated. We observed a mismatch between preclinical and clinical research, which could hamper clinical translation between the two. For the setup of future studies, we encourage the use of both male and female animals, longer survival times, and application of high-throughput analysis methods in order to make translation into the clinics more fruitful.

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Competing Interests

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