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The spinal cord in diabetic neuropathy.

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Introduction

The spinal cord role in diabetic neuropathy has been described since early 1900 and reviewed in detail in the previous edition [1]. In contrast to the vulnerable peripheral nerves, the spinal cord is exposed to less glycemic stress as a consequence of lower glucose levels in the cerebral spinal fluid than in the plasma [2]. However, the spinal cord is not protected from diabetes-induced injury. Indeed, structural and functional damage are discernable in the spinal cord of diabetic patients with histological evidence of neuropathy, radiculopathy and myelopathy [1] and clear atrophy detectable by magnetic resonance imaging (MRI) scans [3, 4]. Structural damages are also detected in the spinal cord of animal models of diabetes, although not to the extent of structural degeneration seen in human [1]. Rather than merely acting as a passive conduit for the flow of afferent information from the periphery to the higher central nervous system, it is increasingly evident that the spinal cord is a complex sensorimotor processing interface. The spinal cord is the first site of integration of sensory input from the periphery and the last site of descending control of sensory and motor systems, therefore disruption of its function may impede appropriate CNS control systems and contribute to apparent peripheral neuropathy [5].

The number of studies on the function and contribution of the spinal cord to diabetes neuropathy has increased in an almost exponential fashion in the last 15 years (from 15 articles per year in 2006 to 45-50/ year in the last 5 years for spinal cord and diabetic neuropathy in PubMed search). The main progress areas in the last 15 years will be developed in this chapter.

- Inflammation
- Molecular mechanisms
- Spinal disinhibition
- Spinal cord Stimulation

Inflammation

- Microglia activation in type 1 diabetes

Several mechanisms taking place at the spinal cord level may underline the development and maintenance of diabetic peripheral neuropathy. Although diabetic neuropathy is not an inflammatory neuropathy *per se*, among these mechanisms, activation of microglia, and subsequent release of proinflammatory cytokines and reactive oxygen intermediates have been suggested as playing an important role in spinal sensitization. Systemic changes such as hyperglycemia may be involved in microglial activation. Under culture conditions,

hyperglycemia can cause morphological changes indicative of activation of microglia as well as increased release of inflammatory cytokines [6, 7]. However, hyperglycemia was shown to be necessary but not sufficient to induce hyperalgesia, which was associated with increase of extracellular signal-regulated kinase (ERK), cJun N-terminal kinase (JNK) and p38 phosphorylation in the spinal cord and dorsal root ganglia (DRG), more particularly in neurons and microglia of streptozotocin (STZ) diabetic rats [8]. Microglial density increased along with phosphorylated p38 MAPK (a marker of microglial activation) in diabetic rodent spinal cord, correlating with mechanical hyperalgesia [9, 10] and a cannabinoid receptor B2 (CB2) agonist attenuated the neuropathic state, with reduced elevation of microglial density and phosphorylated p38 in diabetic mice [10]. Similarly, exercise, gabapentin and lidocaine attenuated microglia activation, as measured by p38 phosphorylation in diabetic rodents [11-13]. However, in our group, we have shown that differences exist between microglial activation observed in diabetic rats and that observed in nerve-ligated rats (Dr. Lee-Kubli, unpublished). Unlike nerve-injury that caused a large increase in the total number of microglia, diabetes was not associated with a change in microglia number. However, the percentage of microglia with retracted processes was similar in both diabetic and nerve-ligated rats and was consistent with descriptions of morphological activation depicted in the literature [8, 14, 15].

Additionally, the mitogen-activated protein kinases (MAPK)s phosphorylation was not detected in diabetic non-hyperalgesic rats and N-methyl-D-aspartate receptor (NMDAR) activation was required for the MAPK phosphorylation in neurons and microglia and consequent hyperalgesia in the diabetic hyperalgesic rats [8]. The NMDAR contributes to central sensitization in the spinal cord, a phenomenon which comprises various pathophysiological mechanisms responsible for neuropathic pain-like signs in animal models. NMDAR function is modulated by post-translational modifications including phosphorylation, and this is proposed to underlie its involvement in the production of pain hypersensitivity. In particular, increased phosphorylation of NMDAR1 was found in neurons and microglial cells in the spinal cord of STZ-rats developing hyperalgesia. Blockers of MEK (an upstream kinase of ERK) and of NMDAR suppressed hyperalgesia and decreased NMDAR phosphorylation, demonstrating involvement of neuronal and microglial NMDA and ERK in central sensitization and mechanical hyperalgesia in diabetes [16].

Microglia involvement in allodynia in type 1 diabetic rats was further supported by administration of gabapentin or minocycline, which attenuated microglial activation (reduction of expression of the early gene cFos) that correlated with reduced allodynia in STZ diabetic rats [13, 14, 17-19]. Inhibition of microglial activation by minocycline restored normal spinal expression of OX-42, BDNF and DREAM (a

calcium regulated transcriptional repressor) proteins [20]. Increased microglial activation and protein expression of BDNF and DREAM were not present in diabetic rats without painful neuropathy (hypoalgesia after formalin injection) [21].

Peripheral infiltrated macrophage may also contribute to the spinal cord inflammation as their number increased throughout the development of diabetic neuropathy in STZ mice. Administration of clodronate liposomes to deplete monocyte reduced infiltrated macrophages along with alleviation of tactile allodynia [22].

Similarly, alteration of astrocytes activation may be involved in diabetic neuropathic pain. However, the role of astrogliosis in the hyperalgesia detected in type 1 diabetic rodents is not clear, with report showing decreased GFAP-positive astrocytes [23, 24] while other reported increased activation of astrocytes in addition to microglial activation in the spinal cord of STZ diabetic rats [25]. Reduced activation of microglia and astrogliosis were also observed in the spinal cord of the HFD/STZ diabetes model only at the late stage (after 30 days of diabetes) [26].

- <u>Proinflammatory peptides and cytokines</u>

Various studies have shown increased proinflammatory cytokines levels in diabetic rodent spinal cord. Here we report only recent studies for kinins and TNFα, while studies showing efficacy to reduce microglial activation and cytokines release are reported in Table 1.

Kinins are defined as pro-inflammatory and vasoactive peptides, which act through the activation of two G-protein-coupled receptors denoted as B_1 and B_2 [27, 28]. The pro-nociceptive kinin B1 receptor (B1R) is upregulated on sensory C-fibers, astrocytes and microglia in the spinal cord of STZ-diabetic rat. This upregulation was associated with increased Iba1 staining and reversed by microglia inhibitors (fluorocitrate and minocycline), while improving tactile allodynia [29].

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine that has been implicated as a key pain mediator in the development and maintenance of neuropathic pain conditions. TNFα was significantly increased in STZ diabetic rats that had painful diabetic neuropathy and to a lesser extend in diabetic rats with non-painful neuropathy as determined by their response to von Frey filaments [30]. The production of TNFα in the spinal cord has been shown to be increased in diabetic mice, and attenuation of this overproduction by a selective cannabinoid CB1 receptor antagonist prevented mechanical allodynia [31]. Systemic or intrathecal administration of etanercept, a TNFα

inhibitor, alleviated tactile allodynia in diabetic mice [32]. The increased TNFα levels in diabetic rat spinal cord was also reduced by treatment with a Poly(ADP-ribose)Polymerase-1 (PARP) inhibitor [33]. TNFα has been shown to be elevated in diabetic spinal cord and its modulation improved painful diabetic neuropathy.

Numerous studies have shown activation of microglia and release of proinflammatory cytokines (Il1β, TNFα) in STZ rodents that were reduced by administration of various compounds, extracts and drugs (Table 1).

Modulation of spinal cord inflammation in rodent models of diabetic neuropathy supports the contribution of inflammation to painful diabetic neuropathy and shows it could be a beneficial therapeutical avenue to explore for treatment of diabetic neuropathy.

- Inflammation in type 2 diabetes

In contrast to the type 1 diabetic rodent models, spinal astrocytes, but not microglia, were shown to be activated in type 2 diabetic mice. The astrocyte activation correlated with increased expression of IL1β and increased phosphorylation of NMDAR in spinal dorsal horn neurons of type 2 diabetic mice [34]. In the type 2 diabetic mice, astrocytic

inhibition attenuated allodynia while microglia inhibition had no effect [34].

Phosphorylated ERK was increased in the spinal cord and DRG of type 2 diabetic mice (db/db) [35] and inhibition of MEK (ERK Kinase) attenuated mechanical allodynia, thermal hyperalgesia, and formalin response in these mice [36]. The enhanced ERK phosphorylation was detected in projection sensory neurons and was associated with astrocyte activation, increased NMDAR1 phosphorylation in projection neurons and upregulation of nitric oxide synthase (neuronal and inducible) in interneurons and astrocytes peaking up at 10 weeks of diabetes [35]. Inhibition of NMDAR, MAPK or NOS decreased ERK phosphorylation, NOS upregulation, and reduced astrocytosis and GFAP upregulation in db/db mice spinal cord while inhibiting mechanical allodynia [35]. Increased ERK activation was demonstrated in ZDF rats following pressure of the hindpaw. This was reduced after 7 weeks of treatment with pioglitazone (a peroxisome proliferator-activated receptor γ (PPARγ) ligand) [37]. However, the treatment reversed hypoglycemia, therefore the effect on spinal sensitization cannot be attribute to pioglitazone alone but partially to a reversal of diabetes.

Molecular mechanisms

- Mitochondrial function

Mitochondrial respiratory chain activity is reduced in heart, kidney, skeletal muscle and DRG of patients with type 2 diabetes and/or diabetic rodents (reviewed in [38]) and contributes to impaired bioenergetics due to alteration of the AMP-activated protein kinase (AMPK)/sirtuin (SIRT)/peroxisome proliferator-activated receptor γ coactivator α (PGC1α) pathway. In contrast to the peripheral nerves and the brain [38-40], little is known about mitochondria dynamics, distribution and function in the diabetic spinal cord. Mitochondrial fission was increased, resulting in reduction of mitochondria in neuronal axons and was accompanied by an abnormal distribution of mitochondria away from the axons towards the cell bodies in the spinal cord neurons of type 1 diabetic rats and in ventral spinal cord neurons in culture in high glucose condition [41]. The mitochondria also appeared fragmented. Another recent study showed reduction in mitochondrial mass in the STZ diabetic rat spinal cord [42, 43].

More studies on spinal cord mitochondria are required to fully characterize the role of the spinal cord in diabetic neuropathy.

- Synaptic plasticity

In contrast to the spinal mitochondria, synaptic plasticity has been extensively studied in the last 15 years.

Imaging of the spinal cord of type 1 diabetic rats by BOLD functional MRI demonstrated a decreased blood-oxygen-level-dependent activity, indicative of changes in synapses between primary afferents and second order neurons [44]. Synaptic and dendritic alterations were detected after established diabetes in type 1 diabetic rats with reduced axonal stabilization and dendritic structures associated with decreased expression of ILK-PINCH proteins levels in the spinal cord [45]. In STZ diabetic rats, alterations in dendritic spine morphology have been shown in the dorsal spinal cord and were associated with indices of neuropathic pain [46]. Morphologic changes were not observed after 1 week of diabetes when hyperglycemia was present without evidence of pain [46]. Additionally, evoked responses to brush and low force von Frey filaments, but not higher force static von Frey stimulation, were recorded from wide dynamic range (WDR) neurons in STZ diabetic rat spinal cord while, in the genetic BB/Wor rat model, responses to brush and von Frey stimulation were not affected, corresponding to their lack of tactile hypersensitivity [47].

In neuronal cells, RhoA, a member of the small molecular G proteins family, Ras superfamily (see review [48]), is involved in the guidance and extension of axons as well as the development and structural

plasticity of dendrites and dendritic spines [49, 50]. Several studies have suggested that RhoA regulates the stability of dendritic branches and spines in neurons [49, 51]. High glucose levels can induce the activation of RhoA through multiple mechanisms mediated by PKC, cyclic guanosine monophosphatase (cGMP)-dependent protein kinase G (PKG) and reactive oxygen species [52]. It was also shown that RhoA and its downstream kinase, Rho kinase (ROCK) play important roles in the development and/or maintenance of chronic pain [53, 54] and the direct activation of ROCK is involved in diabetic painful neuropathy. The expression of cleaved product of ROCK was increased in rats fed a high-fructose diet [55]. Furthermore, ROCK inhibitors have shown efficacy in STZ diabetic rat with attenuation of mechanical allodynia and thermal responses, and reduction of WDR neuron hyperxactability [46, 56]. Other intracellular pathways and proteins play a role in the altered synaptic plasticity in diabetes. APPL1, a neuronal adaptor protein, affects synaptic plasticity, but its defined role in the pathogenesis of painful diabetic neuropathy is under investigation. APPL1 expression was decreased in neurons and microglia of diabetic rats that presented mechanical and thermal hyperalgesia. Reduced APPL1 was associated with activation of mTor, reduction of AMPK phosphorylation and regulation of dendritic spines. APPL1 deficiency exacerbated the hyperalgesia in STZ-rats and restoration of APPL1 levels ameliorated painful diabetic neuropathy [57].

Sirtuins also play a role in synaptic plasticity and pain. Sirtuin levels were shown to be decreased along with enhanced expression of structural synaptic plasticity (PSD95, GAP 43 and synaptophysin) in spinal dorsal horn of diabetic neuropathic rats and db/db mice [58] [59]. Activator of Sirtuin 1 relieved pain behavior and inhibited the enhanced structural synaptic plasticity in diabetic rats and db/db mice while silencing Sirtuin 1 induced pain behavior in normal rats [58].

Preclinical work in animal models of diabetes demonstrated alteration of synaptic plasticity at the spinal cord levels contributing to painful diabetic neuropathy, that corroborated with human data, where MRI imaging showed spinal cord atrophy as an early process only in patients with clinically detectable diabetic painful neuropathy [4].

- Receptors and ion channels

Pain, and more particularly here, diabetic painful neuropathy, is linked to aberrant, exaggerated or reduced receptors and ion channels activity. Preclinical models of painful diabetic neuropathy have allowed studies of the pathological modulation of ion channels at the spinal cord level and the development of therapies.

    - Glutamatergic receptors

mRNA for subunits of glutamatergic NMDA and AMPA receptors are increased in the spinal cord of diabetic rodents [60]. More specifically, NMDA R1 subunit levels were increased in the spinal dorsal horn of STZ diabetic rats which were hyperalgesic/allodynic and magnesium, a voltage-dependent blocker of NMDA, prevented the increased of phosphorylated NR1 (activation) and thermal and tactile allodynia [16, 61]. Similar results were obtained with the NMDA antagonist MK-801 [62]. Additionally, higher expression of phosphorylated spinal NR2B subunit was accompanied by tactile allodynia and increased nociceptive response in the formalin test in diabetic rats and was reduced by intrathecal treatment with ifenprodil, an atypical non-competitive NMDA R2B antagonist [63]. Expression of spinal NMDA R2B was reduced along with TRPV1 expression in diabetic mice after treatment with Ginger extracts and was associated with partial alleviation of allodynia and thermal hyperalgesia [64]. Activation of NMDA R was also demonstrated in prediabetes and type 2 diabetes. Spinal NMDA R2 expression and activation were increased in high fat diet fed mice at 24 weeks when tactile allodynia and thermal hyperalgesia occurred [65]. Furthermore, inhibition of NMDA R2B alleviated tactile allodynia but not thermal hypoalgesia in the prediabetic mice [65]. In type 2 diabetic mice (db/db), NMDA inhibition by intrathecal MK801 reduced mechanical allodynia along with reduction of pERK, pAKT, TNFα and IL6 levels in the spinal cord [66].

Synaptic calcium-permeable AMPA receptors are increased in the spinal dorsal neurons of diabetic rats and blocking them reduced nociceptive hypersensitivity [67]. Increased metabotropic glutamate receptors (mGluR5) in spinal cord of STZ diabetic rats may also contribute to increased glutamatergic input and nociceptive transmission in diabetic neuropathic pain [68].

- Adrenergic receptors

Adrenergic receptors are G protein-coupled receptors, with 2 main groups, α and β, which activation can generate analgesic effect or contribute to chronic neuropathic pain [69]. The increased amplitude of glutamatergic excitatory postsynaptic current in spinal cord slides from STZ diabetic rats were inhibited by a specific α2-adrenoceptor agonist, that concurrently alleviated hyperalgesia. The electrophysiological study suggested an up-regulation of α2-adrenoceptors activity in the spinal cord horn of STZ diabetic rats and a somewhat contrastive antinociceptive effect of the α2 adrenoceptor agonist that resulted in attenuation of glutamatergic transmission in the spinal dorsal horn [70]. In allodynic db/db mice, decreased levels of neuronal α2 adrenergic receptor protein were detected during period of mechanical allodynia, which was alleviated by systemic administration of

dexmedetomidine, a selective α2 adrenergic receptor agonist [71], possibly via the Wnt10a/β catenin signaling pathway [72].

- Ligand-gated channels

Transient receptor potential (TRP) channels transduce extracellular stimuli into neuronal responses through influx of calcium [73] and when dysfunctional, may contribute to painful diabetic neuropathy. Protein expression of TRP vanilloid 1 (TRPV1) was increased at an early stage in the spinal cord, but not the DRG, of STZ rats developing allodynia. The increased TRPV1 levels were detected in small size CGRP neurons and intrathecal administration of TRPV1 antagonists alleviated allodynia [74]. Similarly, increased expression of TRPV1 receptor in the spinal cord of STZ diabetic rats was reduced by treatment with the antidepressant mirtazapine [75]. Recently, in contrast, TRPV1 and CGRP expressions were shown to be reduced in the spinal cord of diabetic rats and further decreased with administration of Ropivacaine, a commonly used clinical anesthetic, dramatically affecting NCV of the sciatic nerve [76], suggesting greater precaution when planning surgery for patients with diabetes.

Spinal TRP ankyrin 1 (A1) channels, on central terminals of primary afferent nerve fibers, were shown to play an important role in maintenance of mechanical hypersensitivity and contribute to cutaneous neurogenic inflammation while cutaneous TRPA1 channels

contributed to mechanical hypersensitivity in STZ rats [77, 78]. Methylglyoxal, a reactive glucose metabolite, was reported to facilitate painful diabetic neuropathy via sensitization of TRPA1-adenylyl cyclase type 1 (AC1) signaling cascade in type 2 diabetic db/db mice [79].

- Voltage-gated sodium channels

Voltage-gated sodium channels (Na_v) play a critical role in controlling cellular excitability and have gain interest for their role in painful diabetic neuropathy particularly Nav 1.3, 1.7 and 1.8 among the 10 sodium channel α subunits ($Na_v1.1$-1.9 and Na_vX).

Intrathecal administration of Nav1.7 and Nav1.8 antagonists alleviated thermal hyperalgesia in diabetic mice but not in control mice, although changes in the spinal sodium channels proteins were not different between control and diabetic mice [80].

- Voltage-gated calcium channels

Voltage-gated calcium channels of L, N and T type are widely studied for their role in neuropathic pain, with the T-type channel $Ca_v3.2$ particularly studied in painful diabetic neuropathy, mainly in DRG sensory neurons [81]. Limited numbers of studies have indirectly shown a role of dorsal horn T-channels in neuropathic pain responses with intrathecal administrations of T-channel blockers rapidly reducing

neural excitability and pain responses [82, 83]. Only one study has demonstrated that specific inhibition of $Ca_v3.2$ T channels in superficial dorsal horn neurons suppressed spontaneous excitatory synaptic transmission in diabetic rats to a greater extent than in healthy age-matched animals [84].

Aberrant regulation of nociceptive receptors and ion channels in the dorsal horn of diabetic rats contribute to increased pronociception via ion channels in sensory neurons. Modulation of spinal receptors and channels may lead to novel therapies for painful diabetic neuropathy.

- Oxidative stress

Reactive oxygen species (ROS) are produced by physiological functions and scavenged by enzymatic and non-enzymatic antioxidant system. In diabetes, chronic hyperglycemia leads to an imbalance of the oxidative status [85] and increased oxidative stress occurs in peripheral and central tissues [86]. Several studies have demonstrated the role that oxidative stress plays at the level of the spinal cord to contribute to diabetic neuropathic pain, mainly by demonstrating the beneficial effect of antioxidants.

Poly(ADP-ribose)Polymerase-1 (PARP) is an nuclear enzyme that cleaves nicotinamide adenine dinucleotide (NAD+), contributes to

NAD+ deletion and energy failure, impaired signal transduction and apoptosis [87]. Enhanced activation of PARP was demonstrated in diabetic rat spinal cord, with increased nitrotyrosine levels in neurons, oligodendrocytes and astrocytes. A PARP inhibitor prevented sciatic nerve and spinal PARP activation, nitrotyrosine accumulation and ameliorated the neuropathy in the STZ rats [33].

Curcumin is a natural polyphenol with multiple properties, the most recognized being antioxidant and antiinflammatory [88]. Curcumin was shown to ameliorate diabetic neuropathy by suppression of oxidative stress in brain and sciatic nerves of diabetic rats [89] and recently was shown to reduce oxidative stress markers in the spinal cord of STZ-diabetic rats via inhibition of the spinal NADPH oxidases [90]. Consistently with its antiinflammatory properties, curcumin was shown to reduce expression of TNFα and TNFα receptor 1 in the dorsal horn of STZ diabetic rats [91].

Increased oxidative stress damage and neuronal activation (Fos) in the spinal cord neurons were normalized by 10 weeks of preventative treatment of STZ rats with an antioxidant (epigallocatechin-gallate) [92], by mexiletine, a sodium channel blocker with antioxidative properties [93] and by treatment with ozone and/or insulin [94].

Increased oxidative stress has been demonstrated in diabetic neuropathy and the ability to scavenge ROS ameliorates diabetic neuropathy in rodents.

- Serotonin/noradrenaline reuptake inhibitors

Among the currently 4 FDA approved drugs to improve painful diabetic polyneuropathy [95], 2 are serotonin/noradrenaline reuptake inhibitors (SSRI/SNRI), duloxetine and fluoxetine, although fluoxetine is supported by only a small study. Duloxetine was the first SSRI prescription drug approved by FDA for the management of pain associated with diabetic neuropathy [96] and is the most recommended and prescribed treatment for painful diabetic neuropathy while studies continued to define its mechanism(s) of action. Duloxetine exerted its anti-allodynic effect in diabetic rats predominantly via indirect activation of 5HT2A receptors in the spinal cord [97]. Duloxetine is a balanced serotonin/noradrenaline reuptake inhibitor and its efficacy may also depend on modulation of noradrenaline, as it reduced noradrenergic signals in the spinal cord by inhibiting the norepinephrine transporters [98]. Other mechanisms may play a role in the analgesic effect of duloxetine. In diabetic mice, duloxetine treatment for 4 weeks alleviated allodynia and thermal hyperalgesia and this was associated with reduction of microglia and

astrocytes expression [99], and reduced overexpression of TLR4-MYd88 dependent pathway [100]. Other antidepressants, milnacipan (SNRI), paroxetine and fluvoxamine (SSRI) [101] and ammoxetine (SNRI) [102] alleviated allodynia in STZ diabetic rats after intrathecal administration. Despite the influence of serotonin in pain modulation, SSRIs are less effective than tricyclic antidepressants. 5HT2A receptor density was not affected in the spinal cord of diabetic rats [97, 103] but the PDZ domains of PSD-95 that interacts with 5HT2A receptors was upregulated. This interaction PDZ/5HT2AR might contribute to the resistance of SSRI-induced analgesia in painful diabetic neuropathy, as disrupting this interaction enhanced fluoxetine antihyperalgesic effect [103].

Aberrant regulation of nociceptive receptors and ion channels in the dorsal horn of diabetic rats contribute to increased pronociception via ion channels in sensory neurons. Modulation of spinal receptors and channels may lead to novel therapies for painful diabetic neuropathy.

Spinal disinhibition

Depending on the physiological and pathophysiological state, processing in the spinal cord dorsal horn may dampen down or enhance output from nociceptive projection neurons. Several possible mechanisms of spinally mediated enhancement of ascending nociceptive drive including wind-up, long-term potentiation, glial

inflammation, altered descending pain modulation and spinal disinhibition have been implicated in centrally mediated pain. The following section will focus on the accumulating evidence suggesting that spinal disinhibition plays an important role in pain generation in diabetic neuropathy.

The major inhibitor transmitters in the spinal cord are γ-aminobutyric acid (GABA) and glycine. GABA acts via GABA-A and GABA-B to produce inhibition either at pre-synaptic terminals, thereby reducing transmitter release (i.e. pre-synaptic inhibition) or on the post-synaptic dendrites (i.e post-synaptic inhibition). GABA-A mediated inhibition is ionotropic resulting from the passage of ions through voltage gated channels [104]. The main ion that permeates the pore of the GABA-A receptor is chloride (Cl-), the concentration of which is much greater in the extracellular space. The polarity of the GABA-A response is determined largely by the intracellular Cl^- concentration. In the post-developmental nervous system, the low intracellular Cl^- concentration is controlled by the potassium (K+)/chloride (Cl^-) co-transporter KCC2 which extrudes intracellular Cl^- [105]. Following GABA-A activation, Cl^- enters the cell according to its electrochemical concentration gradient causing inhibitory post-synaptic potentials (IPSP) leading to phasic hyperpolarization [104]. It is increasingly recognized that subtypes of GABA-A also occur at sites other than the synapse. These

extrasynaptic GABA-A receptors, which are activated by GABA derived from glial cells and ‘circulating’ GABA [106], produce tonic hyperpolarisation and consequently tonic inhibition of spinal cord cells [107, 108] and primary afferents [109].

GABA-B mediated inhibition is, in contrast, metabotropic. The GABA-B receptor is G-protein coupled and the binding of GABA triggers a second messenger cascade [110, 111]. In the post-synaptic cell, this results in activation of G protein-activated inwardly-rectifying K+ (GIRK) channels which are highly co-localized with GABA-B receptors [110-112]. The resulting increase in K+ conductance promotes K+ efflux from the cell and hyperpolarisation. In contrast to the fast inhibitory signalling mediated by GABA-A receptors, GABA-B receptor mediated hyperpolarisation is slow and prolonged [111].

Inhibitory interneurons in the spinal cord are intimately associated with nociceptive circuits [113]. The effect of this inhibition is to reduce the output of projection neurons in response to noxious peripheral stimulation. Accordingly, in normal rodents, spinal application of GABA or glycine antagonists result in behavioral indices of hypersensitivity and pain to both noxious and innocuous tactile stimulation [114-117]. Conversely, enhancement of spinal GABA elevates nociceptive thresholds and reverses behavioral indices of pain in rodent pain models [118-121]. A reduction in this tonic GABAergic/glycinergic

inhibition of ascending nociceptive transmission, a phenomenon termed disinhibition, is a putative mechanism of central sensitization and could potentially result from loss of inhibitory interneurons or attenuation of inhibitory neurotransmitter storage/release [122, 123]. Indeed, a reduction in GABAergic tone in the dorsal horn of the spinal cord has been demonstrated in models of peripheral nerve injury [124, 125]. However, this is not the case in the spinal cord of diabetic rats exhibiting behavioral indices of pain. In the STZ rat model of type 1 diabetes, concentrations of GABA in the spinal cord are increased in both basal state as well as evoked responses in the formalin-induced pain model. In contrast, in the same study, levels of the excitatory neurotransmitter glutamate are diminished [126]. Given that spinal cord GABA-A receptor protein levels are not significantly altered in STZ-rats [127] the paradoxical co-existence of increased GABA and increased nociceptive drive suggest that there is impaired/altered post-synaptic responsiveness to GABA.

Alterations in the post-synaptic responsiveness to GABA were first described in a peripheral nerve injury model of pain in non-diabetic rats [128]. Increasing evidence indicates that the dysfunctional spinal inhibitory processing seen in STZ-rats results from a similar mechanism. Allodynia and other behavioral indices of pain in STZ-rats are associated with down-regulation of post-synaptic KCC2 in the

dorsal, although not ventral, spinal cord [127, 129]. This causes GABA, acting via spinal GABA-A receptors, to become pro-nociceptive rather than inhibitory. Accordingly, spinal GABA-A blockade, which under normal circumstances is pro-nociceptive, reverses allodynia in STZ-rats [127]. The mechanism, like that seen in the peripheral nerve injury model, also involves BDNF. Spinal administration of BDNF in normal rats phenocopies the findings in STZ-rats with the emergence of allodynia and reduced spinal KCC2 expression [129]. Furthermore, sequestration of spinal BDNF in STZ-rats reverses allodynia [129]. The source of the BDNF in STZ-rats is currently not clear, although, interestingly, BDNF expression is elevated in the dorsal root ganglia of STZ-rats raising the possibility that it may derive from the primary afferent fibers [130].

Inhibitory function in the spinal cord can be assessed using rate-dependent depression (RDD) of the Hoffmann-reflex (H-reflex) [131]. The H-reflex is a trans-spinal reflex that is elicited by electrical stimulation of a mixed motor and sensory nerve trunk. The H-reflex can be measured in mammalian species including rodents and humans using a simple modification of traditional nerve conduction studies [132-135]. When a second H-wave is elicited with an inter-stimulus interval less than 2s following the initial stimulation, the H-wave

amplitude decreases relative to the first response. The measure of the change in amplitude is referred to as H-reflex RDD - also known as paired-pulse depression or frequency-dependent depression [136-141].

The plausibility of RDD as a mechanistic biomarker of loss of GABAergic inhibition due to GABA reversal, spinal disinhibition and resultant pain-related phenomena in painful diabetic neuropathy has been demonstrated in a number of studies in rodents. STZ rats exhibiting mechanical hypersensitivity/allodynia and reduced KCC2 in the dorsal horn show attenuation of RDD [127]. Furthermore, spinal delivery of either the KCC2 blocker DIOA or BDNF to control rats, interventions that mechanistically recapitulate the spinal disinhibition phenotype in STZ rats, are also associated with impaired RDD [127, 129]. In keeping with the underlying mechanism of GABA reversal, both the behavioral indices of pain and loss of RDD in all these models are reversed by spinal administration of GABA-A antagonists [127] - a pharmacological intervention that both induces hypersensitivity and impairs RDD in control rats. RDD and allodynia in STZ rats are also reversed by the carbonic anhydrase inhibitor acetazolamide, which mitigates the effects of reduced KCC2 and Cl- dysregulation by decreasing bicarbonate efflux through GABA-A receptors [142].

Spinal disinhibition and loss of RDD in painful diabetic neuropathy are not just dependent on synaptic GABA-A. For example, altered responsiveness of the α5 subunit-containing GABA-A receptor is also implicated in allodynia and impaired RDD in STZ rats [143]. Normal levels of RDD can also occur in the presence of GABA-A blockade. As we have already discussed, spinal disinhibition and impaired RDD are reversed by GABA-A blocking drugs in normal rats treated with KCC2 antagonist DOIA or BDNF as well as in diabetic rats [127]. This implies that other inhibitory mechanisms can also contribute to RDD in the spinal cord of STZ rats. Further evidence for this is revealed in studies investigating the time course of RDD and spinal disinhibition in STZ-rats which indicate that the development of spinal disinhibition in diabetes is a dynamic time-dependent process involving GABA-A and GABA-B mediated responses [142]. Impaired RDD due to GABA-A reversal is initially compensated by GABA-B mediated inhibition. However, over time, due to increasing GABA-A reversal or attenuation of GABA-B inhibitory function, impaired RDD and the full spinal disinhibition phenotype becomes evident. The role of glycinergic inhibition in spinal disinhibition in diabetic rats has not been studied extensively although glycine release in the spinal cord of STZ rats are mildly reduced [126].

The disclosure of spinal disinhibition as a putative pain mechanism in STZ rats raises the question as to whether similar mechanisms occur in clinical populations with painful diabetic neuropathy. This is of potential clinical importance as it may lead to mechanistically targeted drug discovery or individualized therapy in patient populations. However, this would require identification of populations or sub-populations of patients with painful diabetic neuropathy in whom spinal disinhibition is a major pain mechanism. Currently it is not possible to measure levels of dorsal horn GABA or KCC2 in humans. However, RDD is conserved across mammalian species including humans raising the possibility that results obtained in rodents may be translated to diabetic patients.

To assess the translational potential of the pre-clinical findings, we measured the magnitude of H-reflex RDD in patients with type 1 diabetes and painful or painless neuropathy [144]. In a sub-set of patients with painful diabetic neuropathy, there was loss of RDD compared to both healthy controls and patients with painless diabetic neuropathy. Importantly, the impairment of RDD was independent of measures of both large and small fiber neuropathy, indicating that is not merely a reflection of severity of neuropathy. Loss of RDD was also independent of glycemic control. These findings support the hypothesis that impaired RDD may serve as clinical biomarker in a sub-set of

patients where pain arises primarily from spinal disinhibition. We have also recently demonstrated that impaired RDD is also seen in patients with type 2 diabetes and painful neuropathy (unpublished observations). Like the findings in type 1 diabetes, not all subjects with type 2 diabetes and neuropathic pain demonstrated impairment of RDD. Approximately 60% of patients with diabetes will develop neuropathy, 30% of those with neuropathy will develop neuropathic pain and, from our exploratory study, 40% of those will show RDD deficits. In contrast, diabetic rodents exhibit much more homogeneous neuropathy, neuropathic pain and impaired RDD phenotypes [131]. Whilst this may reflect a more complex aetiopathogenesis of painful diabetic neuropathy in humans, it is also plausible that this heterogeneity can be used to enable definition of abnormal values and predict therapeutic response to medications that target spinal inhibition, one of these drugs being duloxetine. The selective serotonin-norepinephrine re-uptake inhibitor duloxetine, used to treat painful diabetic neuropathy [145], alleviates the tactile allodynia and also restores RDD in STZ-rats in a spinal 5HT2A receptor dependent manner [97, 144] and diabetic patients with impaired spinal disinhibition seemed to find duloxetine to be an efficacious treatment for their neuropathic pain [146].

To summarize, there is accumulating pre-clinical evidence that loss of RDD and indices of neuropathic pain share a common pathogenic mechanism involving spinal KCC2 depletion and disinhibition caused by inversion of GABA-A receptor function. Although the exact neural circuitry has yet to be defined, both RDD and behavioral indices of neuropathic pain also exhibit common responses to spinally acting analgesics. RDD status, which is easily measurable in clinical populations, may be a viable biomarker for identifying the dominant generator site in individual patients with painful diabetic neuropathy and for predicting efficacy of therapeutic strategies that alleviate spinal disinhibition.

Spinal cord stimulation

Spinal cord stimulation (SCS) is proposed to relieve chronic intractable pain by stimulating nerve fibers in the spinal cord. The resulting impulses in the fibers may inhibit the conduction of pain signals to the brain, according to the pain gate theory proposed by Melzack and Wall [147] and block the sensation of pain. In general, SCS is part of an overall treatment strategy and is used only after the more conservative treatments have failed (review in [148]). SCS is an effective therapy for different chronic painful conditions that has gained interest for painful peripheral diabetic neuropathy within the last 15 years. In addition to the pain gate theory, several other

mechanisms have been proposed for the beneficial effect of SCS in diabetes (review in [149]). SCS may produce peripheral vasodilatation via stimulation of the sympathetic system and release of neurotrophic factors [150, 151], may involve small diameter nociceptive afferents expressing TRPV1 receptor which stimulation induces release of calcitonin gene-related peptide (CGRP) and nitric oxide [152] and activation of ERK [153]. Vasodilatation was observed after SCS in STZ diabetic rats, however hyperglycemic conditions attenuated the blood flow responses to SCS [154], possibly due to the decreased CGRP levels [155, 156], or reduced TRPV1 containing C fibers [152]. Another mechanism may involve alteration of spinal neurochemistry, such as increased GABA and acethylcholine after SCS resulting in activation of inhibitory interneurons with reduction of allodynia in neuropathic pain models [157, 158]. This plausible mechanism has not been studied in diabetic models and may not apply to efficacy of SCS in diabetes. GABA has been shown to be excitatory in diabetic rats due to impairment in spinal KCC2, resulting in spinal inhibitory dysfunction (see Spinal disinhibition section above and [126, 127, 146])

Mechanisms by which SCS improves neuropathic pain, particularly in diabetes, are not clearly understood. However, studies in diabetic patients have shown efficacy. Neuropathic pain was significantly reduced after 6 months of SCS in diabetic patients with chronic pain in their lower limbs that was not responsive to conventional treatment

and this was not associated with changes in microcirculatory perfusion [159]. SCS showed pain relief of more than 50% in 50-77% of patients [160, 161] and up to 5 years [162].

Optimization of SCS parameters has shown improvement over conventional SCS (30-120Hz). One of these optimization is High Frequency SCS (150-500 Hz). In a preclinical rodent model of type 1 diabetes, alleviation of mechanical hypersensitivity was independent of the frequency of stimulation applied [163]. However, it was shown that high frequency SCS demonstrated delayed effect after cessation of SCS on mechanical allodynia in STZ diabetic rats [164].

Recently, another paradigm of stimulation has been developed, burst SCS (five pulses at 500 Hz, delivered 40 times per second). Burst SCS reduced pain further than tonic stimulation (30-120 Hz) with reduced local paresthesia [165]. 58% of the patients experienced significant additional pain reduction [166].

SCS has improved in the last 15 years with High Frequency and burst protocols applied to diabetes-induced neuropathic pain not responding to current treatments. However, little is known in diabetic conditions and the mechanisms by which SCS is effective in diabetic patients remain unknown. Further studies are needed to understand how SCS

works in diabetes and to predict which patients may benefit from SCS, possibly patients with evidence of spinal disinhibition detected by RDD.

Summary/Conclusions

It is clear, particularly from the preclinical rodent studies, that diabetes is a multifactorial disease. Many mechanisms take place in the spinal cord, contributing to neuropathy and painful neuropathy that can be alleviated in animal models by a multitude of drugs. However, little has been translated to human with many failed clinical trials. Nevertheless, progress has been made with spinal cord stimulation to benefit intractable pain, 4 FDA-approved drugs are now available and a new method of detection of pain generation site may facilitate physicians' prescription ability of the current drugs. The detection of pain generation site by RDD may also enhance clinical trial design and patient selection for central versus peripheral effectors, and therefore contribute to more successful clinical trials.

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