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Allodynia and hyperalgesia in diabetic rats are mediated by GABA and depletion of spinal potassium-chloride co-transporters

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Abstract

Diabetic rats show behavioral indices of painful neuropathy that may model the human condition. Hyperalgesia during the formalin test in diabetic rats is accompanied by the apparently paradoxical decrease in spinal release of excitatory neurotransmitters and increase in the inhibitory neurotransmitter GABA. Decreased expression of the potassium-chloride co-transporter, KCC2, in the spinal cord promotes excitatory properties of GABA. We therefore measured spinal KCC2 expression and explored the role of the GABA_A receptor in rats with painful diabetic neuropathy. KCC2 protein levels were significantly reduced in the spinal cord of diabetic rats while levels of NKCC1 and the GABA_A receptor were unchanged. Spinal delivery of the GABA_A receptor antagonist bicuculline reduced formalin-evoked flinching in diabetic rats and also dose-dependently alleviated tactile allodynia. GABA_A receptor-mediated rate-dependent depression of the spinal H reflex was absent in the spinal cord of diabetic rats. Control rats treated with the KCC2 blocker DIOA, mimicked diabetes by showing increased formalin-evoked flinching and diminished rate dependent depression. The ability of bicuculline to alleviate allodynia and formalin-evoked hyperalgesia in diabetic rats is consistent with a reversal of the properties of GABA predicted by reduced spinal KCC2 and suggests that reduced KCC2 expression and increased GABA release contribute to spinally-mediated hyperalgesia in diabetes.

Keywords

Diabetes; neuropathy; pain; spinal cord; GABA; KCC2

1. INTRODUCTION

Neuropathy is the most common of the complications associated with diabetes mellitus and pain is a frequent and debilitating consequence of diabetic neuropathy. Spontaneous pain, allodynia and hyperalgesia occur in diabetic patients and the etiology of these conditions is not well understood. Some animal models of diabetes also show allodynia and hyperalgesia [15, 30] and have been used to investigate the mechanisms underlying these disorders and screen potential new therapies [4]. In short-term diabetic rats, glucose metabolism by aldose reductase appears to contribute to thermal hyperalgesia and hyperalgesia following noxious paw stimulation with formalin [7,9,10,20,37]. However, aldose reductase inhibitors do not

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completely abolish hyperalgesia in the formalin test and have no impact on the tactile allodynia seen in insulin-deficient diabetic rats [7,8], indicating that other mechanisms are likely to be involved.

Paw formalin injection prompts a biphasic nocifensive flinching behavior that is exaggerated in diabetic rats. However, spinal microdialysis studies in unrestrained free-moving rats showed that formalin-evoked levels of the excitatory neurotransmitters glutamate and substance P were paradoxically lower in diabetic animals [11,29]. This suggests that the hyperalgesic behavior of diabetic rats is not driven by increased nociceptive input from primary afferents and that amplification of sensory input takes place beyond the peripheral nerve. Another unexpected finding from the spinal microdialysis studies was that both basal and formalin-evoked levels of the spinal inhibitory neurotransmitter γ -aminobutyric acid (GABA) were elevated in the spinal cord of diabetic rats. Whether this reflects an attempt to compensate for hyperalgesic mechanisms in the spinal cord, such as those driven by increased spinal COX-2 expression [20,37] or to overcome some impairment of descending inhibitory systems, is not known. At least superficially, the elevated spinal GABA appears inconsistent with the allodynic and hyperalgesic state of diabetic rats.

GABA acts as an excitatory neurotransmitter during development of the spinal cord [22] and studies of neuropathic pain resulting from physical injury to the peripheral nerve of rats indicate that spinal GABA can be revert to an excitatory role in the spinal cord [14]. This switch from inhibitory to excitatory in the mature spinal cord is prompted by decreased expression of the potassium-chloride co-transporter, KCC2, resulting in a shift of the transmembrane anion gradient such that GABA binding to post-synaptic GABA_A receptors no longer causes an influx of chloride ions and membrane hyperpolarization, but rather an outflow of chloride ions and subsequent depolarization [14]. Spinal KCC2 expression is reduced in animals with nerve injury and allodynia, while inhibiting spinal KCC2 in normal animals produces tactile allodynia similar to that seen in nerve-injured animals [14]. Given these findings, and our observations that diabetic rats show both tactile allodynia and increased spinal GABA levels, we investigated the impact of diabetes on spinal KCC2 expression and the possibility that allodynia and hyperalgesia in diabetic rats is promoted by spinal GABA acting as an excitatory rather than inhibitory neurotransmitter.

2. METHODS

2.1. Animals

All studies were performed using adult female Sprague-Dawley rats (Harlan Industries, San Diego CA, USA). Animals were housed 2-3 per cage with free access to food and water and maintained in a vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. All animal studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California San Diego.

2.2. Induction of diabetes

Insulin-deficient diabetes was induced following an overnight fast by a single injection of streptozotocin (STZ, Sigma) at 50mg/kg i.p. freshly dissolved in 0.9% sterile saline. Hyperglycemia was confirmed using a strip-operated reflectance meter in a blood sample obtained by tail prick four days after STZ injection and in another sample collected at the conclusion of the study. Rats were studied 4-8 weeks after onset of hyperglycemia.

2.3. Drugs

Bicuculline, a GABA_A receptor antagonist (TCI America, Portland, OR, USA) and muscimol, a GABA_A receptor agonist (AG Scientific Inc. San Diego, CA, USA) were dissolved in saline

and the selective KCC2 blocker [(dihydroindenyl)oxy] alkanoic acid (DIOA; Alexis Biochemicals, San Diego, CA, USA) was dissolved in saline + 10% DMSO. Drugs or vehicle were injected directly to the spinal cord via an indwelling intrathecal catheter implanted 3-7 days prior to drug delivery as described elsewhere [49].

2.4. Tactile response threshold

Tactile allodynia was assessed using von Frey filaments as described in detail elsewhere [6]. Responses to drug delivery were transformed to percentage maximal effect (%MPE) by designating the pre-drug value for each animal as 0% effect and the upper paw withdrawal threshold (PWT) of 15g as 100% effect, using the formula $\%MPE = (PWT \text{ post-drug} - PWT \text{ pre-drug}) / (15 - PWT \text{ pre-drug})$ [13].

2.5. Thermal response latency

Rats were placed in an observation chamber on top of the thermal testing apparatus (UARD, San Diego, CA, USA) and allowed to acclimate to the warmed glass surface (30°C) and surroundings for 30 minutes. The mobile heat source was maneuvered to below the center of the right hind paw or 1 cm from the tip of the tail and turned on, a process that activates a timer and locally warms the glass surface at a rate of approximately 1°C/second. When the rat withdrew the limb, movement sensors stopped the timer and turned off the heat source. Both hind paws were measured 3 times, with measurements made 5 minutes apart on alternating paws, and the mean of all 6 measurements used as a composite score for each rat, reflecting the symmetrical nature of the peripheral neuropathy induced by diabetes. To avoid tissue damage, the upper cut-off limit was set at 20 seconds. Where group measurements were staggered over separate days, the response latency was converted to the response temperature using a time:floor temperature calibration curve that was constructed each day to allow for day-to-day variations in the surface heating rate [3].

2.6. Formalin-evoked flinching

Rats were restrained manually and formalin (50µl of 0.5% solution) injected sub-dermally into the hindpaw dorsum. Rats were then placed in an observation chamber and flinching behaviors counted in 1 minute blocks every 5 minutes for 1 hour. The sum of flinches was grouped to highlight specific phases of the test [8]. Bicuculline (0.6 µg) or saline was injected intrathecally in control and diabetic rats 15 minutes prior formalin injection. DIOA (30 µg) was injected intrathecally 15 minutes prior to formalin injection followed 5 minutes later by intrathecal injection of bicuculline or saline.

2.7. Rate-dependent depression

The Hoffman (H) reflex was recorded as previously described [23,41]. Briefly, under isoflurane anesthesia, the left hindlimb of the animal was secured and a transcutaneous stimulating needle electrode inserted adjacent to the tibial nerve at the ankle. For recording, a pair of needle electrodes was inserted into the interosseous muscles of the left foot. The tibial nerve was stimulated using bursts of 5×2.5 v, 0.05 ms duration square waves with 40ms intervals between individual stimuli and with bursts repeated every second for 8 seconds. Stimulus generation and recording of M and H waves from the subsequent electromyogram was performed using a Powerlab 4/30 (AD Instruments, Colorado Springs, CO, USA) connected to a computer running Scope software. Rate-dependent depression (RDD) was calculated as the change in amplitude of the H wave evoked by the first of the 5 pulses in the initial burst and the equivalent H wave amplitude evoked by the second burst 1 second later. Measurements were made before (baseline), 5 and 10 min after drug treatment. Bicuculline (0.6 µg) or muscimol (0.1 µg) was injected intrathecally 5 minutes after intrathecal injection of saline or DIOA (30 µg).

2.8. Western blotting

Spinal cords were obtained by hydraulic extrusion after decapitation of anaesthetized rats. Portions of the lumbar enlargement were collected into ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X, protease inhibitor cocktail) and homogenized before centrifugation ($14,000 \times g$). Aliquots of the supernatant incubated 30 min at 37°C in Laemmli LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Ten to 20 µg of total protein was separated on 4-12% SDS-PAGE Bis-Tris gels (Novex, Invitrogen, Carlsbad, CA, USA) and immunoblotted on nitrocellulose. Membranes were incubated with anti-KCC2 (1/1000, Upstate, Temecula, CA, USA), anti-NKCC1 (1/3000, monoclonal antibody developed by Lytle and Forbush [28] and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), anti-GABA_A receptor $\alpha 2$ subunit (1/2500, Abcam Inc., Cambridge, MA, USA) or anti-actin antibodies (1/2000, Sigma, St. Louis, MO, USA), followed by incubation with horseradish peroxidase-linked anti-rabbit secondary antibody (1/10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-mouse secondary antibody (1/10,000, Santa Cruz Biotechnology). Blots were developed using an ECL Western-blotting protocol (Enhanced Chemiluminescence, Lumilight Roche Applied Science, Indianapolis, IN, USA). For sequential analysis of Western blot membranes, previously bound antibodies were removed with stripping buffer (Pierce, Rockford, IL, USA). Quantification of immunoreactivity was performed by densitometric scanning using Quantity One software (BioRad, San Diego, CA, USA). For each animal, band intensities were normalized by calculating the ratio of the intensity of bands corresponding to KCC2, NKCC1 or GABA_A receptor $\alpha 2$ subunit to the intensity of the band corresponding to actin. The actin normalized data for each lane was expressed as a percentage of the group mean of values obtained from all control rats run on the same gel.

2.9. Immunohistochemistry

Rats were anaesthetized and perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). Immediately after perfusion, spinal cords were dissected and post-fixed in 4% paraformaldehyde, cryoprotected by immersion in 0.1M PBS containing 30% sucrose, embedded in OCT medium, and stored at -20°C prior to cryostat sectioning (16 µm sections). KCC2-immunoreactivity was visualized by the ABC method using 3,3'-diaminobenzidine (DAB) as the chromagen. After washing with 0.05M Tris buffer solution (pH 7.6), the sections were blocked with avidin-biotin block solution (Dako Corporation, Carpinteria, CA, USA) and 10% normal goat serum containing 0.3% Triton-X. Sections were then incubated overnight at 4°C with selective rabbit anti-KCC2 antibodies (1/800, Upstate, Temecula, CA, USA) in Tris buffer containing 5% normal goat serum and 0.3% Triton-X. Sections were then incubated with biotinylated goat anti-rabbit antibody (1/200: Vector Laboratories, Burlingame, CA, USA), followed by StreptABC complex/HRP (DakoCytomation, Carpinteria, CA, USA). The immunoreactive products were visualised with DAB substrate kit (Vector Laboratories). To minimize variability in stain intensity, tissues from control and diabetic rats were stained side by side and evaluation of non-specific staining was performed by omission of the primary antibody. Sections were evaluated by light microscopy.

2.10. Statistical analysis

Statistical analysis was performed using *t* tests or one-way ANOVA followed by appropriate post hoc tests as indicated. Data are reported as group mean $\pm$ SEM.

3. RESULTS

3.1. Diabetes

All streptozotocin-injected animals that were entered into the diabetic group exhibited hyperglycemia (blood sugar $>15\text{mmol/l}$) 4 days after injection and at sacrifice. Physiological and behavioral assays were carried out between 4 and 8 weeks of diabetes.

3.2. KCC2, NKCC1 and GABA_A receptor protein in rat spinal cord

Quantification of protein levels by Western blot indicated a significant decrease of KCC2 in spinal cord to $76\pm3\%$ of control values (Figure 1A). KCC2-immunoreactivity was present in the dorsal horn and in cell bodies of the ventral horn and this distribution of KCC2-immunoreactivity was unchanged by diabetes (data not shown). Protein levels of NKCC1 and the $\alpha 2$ subunit of the GABA_A receptor in the spinal cord were not affected by diabetes (Figure 1B,C).

3.3. Effect of GABA agonist and antagonist on responses to light touch

All diabetic rats showed marked allodynia, as defined by a 50% response threshold below 5g force (cohort median = 2.4g, $n = 36$), whereas control rats were unresponsive to light touch (cohort median = 15.0g, $n = 10$). Intrathecal delivery of saline or 0.3 μg muscimol did not alter tactile response thresholds of diabetic rats over the subsequent 6 hours (30 minutes after injection, group median = 2.8g and 2.4g, respectively, $n=6$; Figure 2A). Rats receiving 0.6 μg or higher of muscimol exhibited a transient loss of muscular tone of the lower half of their body with dragging or clenching of their hind paw that subsided 4-5 hours after injection but which made them unsuitable for behavioral testing. This observation is consistent with prior reports of depressed motor tone within this dose range [17, 40]. Intrathecal injection of 0.3 μg bicuculline alleviated tactile allodynia in diabetic rats with peak efficacy occurring within 30 minutes after injection (group median 50% PWT = 10.7 g; Figure 2A). Bicuculline showed a dose-dependent efficacy against tactile allodynia in diabetic rats measured 30 minutes after delivery that was maximal at 0.6 μg and declined at the dose of 1 μg (Figure 2B). Administration of 0.6 μg bicuculline to control rats did not affect tactile response thresholds (30 minutes after injection, group median = 15g, $n=7$). No changes in motor function, general physiology or behavior were observed in response to intrathecal bicuculline at any dose in either control or diabetic rats.

3.4. Effect of GABA agonist and antagonist on responses to thermal stimulation

Diabetes did not significantly alter the temperature of withdrawal for the paw (control = 41.5 ± 0.6 ; diabetic = 41.9 ± 0.8 °C, data are group mean of $n = 8-11$ animals) or tail (control = 44.3 ± 0.5 ; diabetic = 44.8 ± 1.0 °C, data are group mean of $n = 8-11$ animals) after 6-8 weeks of hyperglycemia. Intrathecal injection of bicuculline did not acutely alter paw thermal response time measured 30 minutes after delivery at any dose tested in control or diabetic rats, whereas muscimol significantly ($p<0.05$ vs baseline value by paired t test) increased the paw thermal response time at the dose of 0.3 μg in diabetic but not control rats (Figure 3A). The tail flick response was also not significantly altered by intrathecal delivery of saline or 0.3 μg bicuculline in control or diabetic rats when measured 30 minutes after drug delivery, whereas muscimol significantly ($p<0.05$ vs baseline by paired t test) increased response times in both groups (Figure 3B). Similar findings were noted at times up to 120 minutes after drug delivery (data not shown).

3.5. Effect of GABA and KCC2 antagonists on formalin-evoked behavior

Intrathecal delivery of saline 15 minutes prior to injection of 0.5% formalin into the hindpaw of control rats induced minimal flinching during the 60-minute observation period, whereas

saline pre-treated diabetic rats exhibited increased flinching (Figures 4A and B). Intrathecal pre-treatment with 0.6µg bicuculline 15 min prior to formalin injection did not affect flinching of control rats but significantly ($p < 0.01$, ANOVA followed by Bonferroni's post hoc test) reduced flinching in diabetic rats during phase 2, representing flinching between minutes 15 and 60, (Figures 4A and B). Fifteen-minute pre-treatment of control rats with the KCC2 blocker DIOA (30µg) significantly ($p < 0.01$) increased flinching during phase 2 compared to saline treated control rats (Figure 4C and D). Intrathecal injection of 0.6µg bicuculline 5 minutes after delivery of DIOA significantly ($p < 0.01$, ANOVA followed by Bonferroni's post hoc test) reduced flinching in DIOA-treated control rats during phase 2 (Figure 4C and D).

3.6. Rate-dependent depression

Rate-dependent depression (RDD) of the H-reflex amplitude was measured as another index of a spinal GABA-mediated physiological response [26]. In control rats, there was an approximately 40% reduction in H wave amplitude between the first and second stimulation trains that was maintained over the following 6 stimulations (Figure 5A). After 8 weeks of diabetes, RDD was attenuated so that the decline between the first and second stimulations was significantly ($p < 0.001$ by one-way ANOVA) less than that of control rats (Figure 5A and B). M-waves were unchanged by stimulation in both control and diabetic rats (data not shown). A significant attenuation ($p < 0.001$ by one-way ANOVA) of RDD was also observed when control rats received an intrathecal injection of DIOA (30µg given 5 minutes before stimulation: Figure 5A and B).

Intrathecal delivery of 0.6µg bicuculline resulted in significant attenuation of RDD in control rats that was evident 5 and 10 minutes after delivery (Figure 6), indicating that RDD was mediated by GABA_A receptors. In contrast, intrathecal delivery of 0.6µg bicuculline restored RDD of the H-wave in diabetic rats and also in control rats treated with DIOA (Figure 6). M-wave amplitudes were not changed after injection of 0.6µg bicuculline in either control or diabetic rats (data not shown). Intrathecal delivery of 0.1µg muscimol did not affect RDD in control animals (% change from baseline after 2nd pulse: saline = 50 ± 10 , muscimol = 48 ± 3 , $n = 4-8$) or in DIOA treated rats (% change from baseline after 2nd pulse: DIOA = 110 ± 6 , DIOA + muscimol = 114 ± 8 , $n = 7-11$).

4. DISCUSSION

These studies were prompted by observations that diabetic rats exhibiting tactile allodynia and exaggerated formalin-evoked hyperalgesia do not show a corresponding increase in stimulus-evoked spinal release of excitatory neurotransmitters, but rather an unanticipated increase in basal and evoked spinal GABA levels [11,29]. GABA participates in the inhibitory tone of the spinal cord in adult animals [16] but there have been reports that GABA, acting via GABA_A receptors, assumes an excitatory role following physical nerve injury that is mediated by decreased expression and activity of the potassium-chloride co-transporter KCC2 [14,35,46]. The reduction in spinal KCC2 protein levels seen in diabetic rats echoes that reported in the spinal cord of adult rats after physical nerve injury and both models also develop tactile allodynia [8,14]. A similar measurement of reduced spinal KCC2 protein in diabetic rats has recently been made known to us (Dr's Isaura Tavares and Carla Morgado, personal communication). This deficit is not part of a generalized reduction of spinal cord proteins, as neither NKCC1 nor the alpha-2 subunit of the GABA_A receptor were depleted by diabetes. As KCC2 activity is largely regulated by protein expression rather than the phosphorylation/dephosphorylation cycles that regulate NKCC1 activity [19], it is likely that KCC2 activity is also reduced in the spinal cord of diabetic rats.

Regulation of intracellular chloride concentration is achieved by NKCC1 which maintains a high internal chloride concentration [36] and by KCC2 which maintains a low internal chloride

concentration. KCC2 is absent from primary afferents [14] but present in post-synaptic membranes in the dorsal horn and also in ventral motor neuron cell bodies [21]. Changes in activity of either or both of these pumps can regulate sensory processing in the spinal cord, but only KCC2 expression was altered by diabetes. Diabetes did not change the distribution of KCC2 suggesting that there was not total ablation of KCC2 expression at one site, although we cannot exclude the possibility of a differential loss between the two sites. The decrease in KCC2 expression has the potential to increase internal chloride concentrations and make the opening of the GABA_A chloride channel a depolarizing event [47]. Given our prior observation that basal extracellular GABA levels are increased in the spinal cord of diabetic rats [29], and the present finding of a reduction in spinal KCC2 protein, we speculated that a GABA_A antagonist would alleviate behavioral indices of painful neuropathy in diabetic rats rather than enhance nociception, as occurs in control rats [40,43,48]. Our finding that the GABA_A antagonist bicuculline dose-dependently alleviated allodynia in diabetic rats is consistent with GABA acting via GABA_A receptors in diabetic rats to maintain tactile allodynia. It is noteworthy that the efficacy of bicuculline disappeared at doses above 0.6μg, which may reflect reports of agitation after spinal application of GABA and glycine receptor antagonists [40, 42].

GABA levels are not only increased at rest in the spinal cord of diabetic rats, but also rise further during formalin-evoked stimulation of the paw and are accompanied by an increase in flinching behavior indicative of hyperalgesia [29]. We therefore extended our studies to address whether GABA_A receptor activation/inhibition also contributed to this disorder in diabetic rats and found that the spinal antagonist bicuculline again reduced hyperalgesic behavior. Moreover, inhibiting KCC2 in control rats by spinal delivery of DIOA induced increased flinching during phase 2 of the formalin test and this was reduced by bicuculline, in a manner similar to that seen in diabetic rats. Inhibiting KCC2 in control rats thus mimicked the diabetic phenotype and supports the role of KCC2 and excitatory GABA in diabetes-induced spinally-mediated hyperalgesia. It is notable that the efficacy of bicuculline was not seen against phase 1 behavior, suggesting that the response to the initial afferent barrage was not altered, and also that efficacy of bicuculline was concentrated in the early periods of phase 2. This may simply reflect washout of bicuculline or else some mechanistic divergence between different portions of phase 2, as suggested by others [31]. At the dose used, bicuculline did not completely ablate formalin-evoked flinching during phase 2. Spinal delivery of COX-2 inhibitors also partially suppresses phase 2 hyperalgesic behavior in diabetic rats [20,37]. Whether combined COX-2 and GABA_A receptor inhibition produces an additive effect or corrects the same component of the hyperalgesic mechanism remains to be established.

The implied involvement of excess endogenous spinal GABA in amplifying nocifensive behaviors in diabetic rats was not universal. Thermal hyperalgesia is frequently [25,27] but not universally [5], reported in insulin deficient diabetic rats. We did not detect thermal hyperalgesia in either the paw or tail after 8 weeks of diabetes, suggesting that the elevated spinal GABA levels [29] do not modify thermal nociception at this time. The absence of any effect of bicuculline on thermal response latencies in control or diabetes rats is consistent with previous studies in control rats [40,50] and indicates that normal thermal responses are not modulated by endogenous spinal GABAergic inhibitory tone. In contrast, spinal muscimol induced paw thermal hypoalgesia in diabetic, but not control, rats. A similar dichotomy has been observed in nerve-injured mice [38]. Muscimol also had hypoalgesic effects on tail thermal responses of both control and diabetic rats, which is consistent with a prior study [40] and the effect was greater in diabetic rats. We did not detect any change in protein levels of the GABA_A receptor $\alpha 2$ subunit in the spinal cord of diabetic rats that might imply increased GABA receptor density, while decreased rather than increased GABA_A receptor function has been reported in the brain stem of hyperglycemic rats [1]. The thermal hypoalgesic properties

of muscimol in the spinal cord of diabetic rats further emphasizes that not all aspects of sensory and spinal nociceptive function are equally disrupted by diabetes and KCC2 depletion.

To test the extent to which diabetes-induced KCC2 depletion and increased spinal GABA levels might alter other spinal properties that involve GABA-mediated inhibition, we measured the impact of diabetes, DIOA and bicuculline on rate-dependent depression (RDD) of the electromyogram H-wave following repetitive peripheral nerve stimulation. Loss of spinal GABAergic inhibitory systems has been associated with diminished RDD and spasticity in rats following spinal cord injury [26]. We confirmed that RDD in control rats involved spinal GABA_A receptors, as it was diminished by intrathecal injection of bicuculline. That diabetes completely ablated RDD coincident with reduced spinal KCC2 expression is consistent with a loss of stimulus-evoked GABA_A-mediated inhibitory capacity in the spinal cord. Moreover, the KCC2 inhibitor DIOA also diminished RDD in control rats, indicating that RDD requires KCC2 activity, and extending the observation that KCC2 inhibition in control rats leads to a diabetic-like phenotype of tactile allodynia [14], and hyperalgesia during phase 2 of the formalin test. Bicuculline restored RDD in both diabetic and DIOA-treated control rats, implying that GABA_A receptors are actively involved in the loss of RDD under conditions of reduced spinal KCC2, although the GABA agonist muscimol had no effect on RDD in either control or DIOA-treated rats. Taken together, these observations support the suggestion that reduced spinal KCC2 in diabetic rats contributes to spinally-mediated hyperalgesia. The impact of diabetes on KCC2 expression in other regions of the nervous system remains to be identified, as does the pathogenesis of reduced KCC2 expression in the spinal cord of diabetic rats. While hyperglycemia and subsequent glucose metabolism by aldose reductase have been linked to hyperalgesia and increased expression of COX-2 protein in the spinal cord [37], insulin deficiency is also recognized as having detrimental effects on sensory function independent of glycemic regulation. Insulin receptors are present and functional in the spinal cord [12,44] and insulin prevents tactile allodynia in diabetic rats at doses that do not reduce hyperglycemia [12,24]. It is plausible that both hyperglycemia and hypoinsulinemia associated with type-1 diabetes contribute to reduced spinal KCC2 expression. There is also an emerging appreciation that altered expression of KCC2 is inversely regulated by the neurotrophic factor BDNF [33, 39]. Axonal transport of endogenous BDNF to the periphery is reduced in diabetic rats [34], but there is also an increase in BDNF mRNA in the dorsal root ganglia of the same animals [18]. Should increased mRNA lead to increased export of BDNF to central terminals of primary afferents in the spinal cord and subsequent activity-driven release [32,45], a mechanism driving local suppression of spinal KCC2 protein expression and hyperalgesia can be envisaged, particularly as local BDNF can also increase spinal release of GABA [2]. These possibilities and the therapeutic potential of intervening in such pathogenic pathways to prevent painful diabetic neuropathy remain to be explored.

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Figure 1. KCC2, NKCC1 and GABA_A receptor protein level in rat spinal cord. Densitometric quantification and representative blot for (A) KCC2 (140kDa), (B) NKCC1 (145 kDa) and (C) GABA_A receptor α 2 subunit (53 kDa) in spinal cord of control and 8-week STZ-diabetic rats. C = control, D = diabetic. Data are expressed as mean±SEM, n=7-10/group and compared to control values using unpaired *t* test, *p<0.05.

Figure 2.

Effect of bicuculline and muscimol on tactile allodynia. **(A)** Tactile responses, represented as % MPE, for 8-week diabetic rats after intrathecal delivery of saline (open square), 0.3 μ g bicuculline (closed square) or 0.3 μ g muscimol (closed triangle). Data are expressed as mean $\pm$ SEM, $n=6-13$ /group. **(B)** Dose-dependent efficacy of bicuculline against tactile allodynia 30 min after injection. Data are expressed as mean $\pm$ SEM, $n=6-13$ /group and were compared to saline (0) using one-way ANOVA followed by Dunnett's post hoc test, * $p<0.05$, ** $p<0.01$.

Figure 3.

Effect of bicuculline and muscimol on paw thermal and tail flick responses for control and diabetic rats. **(A)** Paw thermal response represented as % change from baseline 30 min after injection of bicuculline or muscimol. **(B)** Tail flick response represented as % change from baseline 30 min after injection of saline, 0.3µg bicuculline or 0.3µg muscimol, white bar: control, black bar: diabetic. Data are mean+SEM, n=8-11/group and are compared to baseline (saline or 0) data using paired *t* test, **p*<0.05, ***p*<0.01.

Figure 4.

Effect of bicuculline and DIOA on formalin-evoked hyperalgesia. **(A)** Formalin-evoked flinching over 1 hr following injection of 0.5% formalin. Control and diabetic rats were injected with saline or 0.6 μ g bicuculline 15 minutes prior to formalin. Data are mean $\pm$ SEM. **(B)** Sum of flinches during phase 1 (0-10) and phase 2 (15-60 min). Data are mean $\pm$ SEM, $n=6-16$ /group and compared using one-way ANOVA followed by Bonferroni's post hoc test. ** $p<0.01$, *** $p<0.001$. **(C)** Formalin-evoked flinching over 1 hr following injection of 0.5% formalin. Control rats were injected 15 min prior to formalin with saline or 30 μ g DIOA followed 5 min later by saline or 0.6 μ g bicuculline. Data are mean $\pm$ SEM. **(D)** Sum of flinches during phase 1 (0-10) and phase 2 (15-60 min) of the formalin test. Data are mean $\pm$ SEM, $n=7-16$ /group and compared using one-way ANOVA followed by Bonferroni's post hoc test, ** $p<0.01$.

Figure 5.

Effect of diabetes or DIOA on rate-dependent depression. H-wave amplitudes were measured for control, control + DIOA and diabetic rats. DIOA (30 μ g) was injected intrathecally 5 min prior to RDD measurement. **(A)** H-wave amplitude over 8 consecutive 5 Hz bursts of stimulation expressed as percent of baseline. Data are expressed as mean $\pm$ SEM. **(B)** Change in H-wave amplitude between the first and second bursts expressed as percent change. Data are expressed as mean $\pm$ SEM, n=17-26/group and compared to control data using one-way ANOVA followed by Bonferroni's post hoc test. *** p<0.001.

Figure 6.

Effect of bicuculline on rate-dependent depression in control, DIOA-treated or diabetic rats. Saline or DIOA (30 μ g) were injected intrathecally 5 minutes before bicuculline. Bicuculline (0.6 μ g) was injected intrathecally and H-wave amplitude to 8×5 Hz stimulations measured after 5 and 10 min. Change in H-wave amplitude between the first and second bursts are expressed as percent change. Data are mean+SEM, n=10-22/group and compare to baseline (for time 0 data) or time 0 (for post-drug measurements) using one-way ANOVA followed by Bonferroni's post hoc test. * p<0.05 versus their respective time 0, # p<0.01 versus baseline.