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ALTERED RATE-DEPENDENT DEPRESSION OF THE SPINAL H-REFLEX AS AN INDICATOR OF SPINAL DISINHIBITION IN MODELS OF NEUROPATHIC PAIN

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Keywords

H-reflex; spinal disinhibition; diabetic neuropathy; pain; taxol neuropathy; BDNF

1. Introduction

Neuropathic pain is a common health care problem with low positive treatment outcomes [2; 15; 49]. One contributing factor is that the etiology of neuropathic pain is varied, and may even differ between patients with the same predisposing condition [11]. Putative mechanisms of neuropathic pain include peripheral sensitization, spinal sensitization and spinal disinhibition [11]. With many different potential etiologies, it is no surprise that front line drugs show infrequent and unpredictable efficacy against neuropathic pain [16]. The development of diagnostic techniques to identify specific mechanisms contributing to neuropathic pain may be valuable for developing and refining treatment options for individual patients.

Rate-dependent depression (RDD) is a measure of the decline in amplitude of the spinal Hoffman reflex (H-reflex) over consecutive stimulations that can be assessed in humans and rodents [26; 32; 36; 41]. The magnitude of RDD is indicative of the function of spinal inhibitory systems and, in rodents, is dependent on $GABA_A$ receptor-mediated inhibition [29]. RDD is impaired in rat models of spinal cord injury in which inhibitory interneurons are selectively lost [30; 40] and also in humans with spinal cord injury [5; 26; 50]. This suggests that measurement of RDD in animal models can be directly applicable to the human condition.

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In models of neuropathic pain, impaired $GABA_A$ receptor-mediated spinal inhibition has been attributed to reduced spinal activity of the potassium chloride co-transporter (KCC2) [3; 13; 29]. We recently identified both loss of RDD and reduced KCC2 protein levels in diabetic rats that exhibit neuropathic pain without spasticity or rigidity [29]. The absence of RDD in diabetic rats likely reflects inversion of spinal $GABA_A$ receptor function from inhibition to excitation, rather than loss of inhibitory GABAergic interneurons, because spinal GABA release is not diminished and RDD can be restored by $GABA_A$ receptor antagonism [29; 38]. This identifies RDD as a potential tool to determine whether spinal $GABA_A$ receptor-mediated inhibitory systems are disrupted.

To test the hypothesis that loss of RDD is indicative of a contribution by spinal disinhibition to neuropathic pain states, we examined three models of neuropathic pain. Pain induced by spinal delivery of BDNF was chosen because BDNF acutely reduces spinal KCC2 levels and causes inversion of spinal $GABA_A$ receptor function [12; 45], allowing the association between RDD and neuropathic pain due to inversion of spinal inhibitory function to be tested. We used the streptozotocin (STZ)-diabetic rat, which develops RDD deficits and impaired spinal $GABA_A$ receptor-mediated inhibition [29], and examined the effects of a spinal BDNF-sequestering molecule on RDD and indices of neuropathic pain to determine whether restoration of RDD coincides with alleviation of neuropathic pain. Taxol-induced neuropathy was selected because it is thought to arise primarily from peripheral nerve injury [48], providing a model in which to evaluate whether RDD deficits and painful neuropathy disassociate when the underlying causes are not related to spinal disinhibition. These three models were used to investigate whether the presence or absence of RDD can predict the contribution of spinal GABAergic inhibitory systems to associated neuropathic pain.

2. Materials and Methods

2.1 Animals

All studies were performed using adult female Sprague–Dawley rats (250–300 g; Harlan Industries, San Diego CA, USA). Animals were housed 2–3 per cage with free access to food (5001 diet, Harlan) and water 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 in 14 week-old rats following an overnight fast by a single injection of streptozotocin (STZ, Sigma, St. Louis, MO, USA) at 50 mg/kg i.p. that was freshly dissolved in 0.9% sterile saline. Blood glucose concentration was measured in tail-vein blood samples by a strip-operated reflectance meter (OneTouch Ultra, LifeScan, Inc., Milpitas, CA, USA) both 4 days after the STZ injection and at the conclusion of each study. Rats were only considered diabetic if they had non-fasting blood glucose levels of 15 mmol/L or greater at both times. Studies were carried out after an 8-week duration of hyperglycemia (diabetic) or in age-matched non-diabetic rats (normal).

2.3 Induction of taxol neuropathy

Neuropathy was induced using paclitaxel (Taxol®) dissolved in a vehicle composed of 1:1 ethanol and Fluka CremophorEL (all sourced from Sigma, St Louis, MO, USA). Taxol (1 mg/kg) was administered intraperitoneally 4 times at 48 hour intervals. Rats received an intrathecal (IT) catheter 1 week after the development of allodynia, corresponding to 3 weeks after the start of taxol administration.

2.4 Drugs

Bicuculline, a $GABA_A$ receptor antagonist (TCI America, Portland, OR, USA), muscimol, a $GABA_A$ receptor agonist (Sigma, St. Louis, MO, USA), BDNF (Regeneron, Tarrytown, NY, USA) and TrkB/Fc, a BDNF sequestering molecule, (Sigma, St. Louis, MO, USA) were dissolved in 0.9% sterile saline. Denatured TrkB/Fc, which was produced by boiling TrkB/Fc in saline for 15 min, was used as a control for some of the TrkB/Fc experiments. [(dihydroindenyl)oxy] alkanoic acid (DIOA: Alexis Biochemicals, San Diego, CA, USA), a KCC2 inhibitor, was dissolved in saline + 10 % DMSO. Drugs or vehicle were injected directly to the lumbar spinal cord via an indwelling IT catheter implanted 3–7 days prior to drug delivery as described in detail elsewhere [57]. Drugs were injected in a volume of 10 μL, or, in cases where two drugs were administered during the same experiment, a volume of 5 μL per drug was used. Drug delivery was followed by delivery of 10 μL saline to completely flush the catheter. No more than 30 μL total volume was injected per experiment. Drug doses were chosen according to the relevant literature [12; 29; 42].

2.5 Tactile response threshold

Paw 50% withdrawal threshold (PWT) was assessed using a series of calibrated von Frey filaments, (Kom Kare, Middletown, OH, USA) using the up-down method exactly as described elsewhere [6; 10]. For studies in which BDNF was administered to control rats, percent maximum potential effect (% MPE) was calculated as (baseline PWT – PWT) / (0.25 – baseline PWT) × 100. This assumes that the maximal effect of BDNF would be to reduce PWT to its minimum measurable output, 0.25 g. For all other studies, % MPE was calculated as (PWT – baseline PWT) / (15 - baseline PWT) *100. This assumes that the maximal drug effect would be to increase the PWT to its maximum measurable output, 15 g. Area under the curve (AUC) was calculated using the trapezoidal rule.

2.6 Formalin-evoked flinching

Rats were restrained manually, and formalin (50 μl) was injected sub-dermally into the dorsum of one hind paw. Depending on the purpose of the experiment, a concentration of 0.5 or 5 % formalin was used as indicated in the text. Rats were then placed in an observation chamber that was maintained at a constant temperature of 26°C. Flinching was counted in 1 min blocks every 5 min for 1 h. Flinches were grouped to highlight two specific phases of the test corresponding to the initial injury afferent barrage (phase 1) and the subsequent spinal sensitization (phase 2)[7]. BDNF was administered 1 h before formalin, and bicuculline or muscimol were administered 15 min before formalin. TrkB/Fc was administered 10 min before formalin.

2.7 Rate-dependent depression

The H-reflex was recorded as previously described [29]. Under isoflurane anesthesia, one hind limb of the rat was secured and a transcutaneous stimulating needle electrode (Grass Technologies, West Warwick, RI, USA) inserted adjacent to the tibial nerve at the ankle. Two recording needle electrodes were inserted into interosseous muscles of the hind paw. A grounding electrode was placed in the skin at the back of the neck. Stimulus generation and recording of M- and H-waves from the resulting electromyogram were performed using a Powerlab 4/30 connected to a computer running Scope software (AD Instruments, Colorado Springs, CO, USA). The tibial nerve was stimulated using bursts of 5 × 200 μs duration square waves with 40 μs intervals between each square wave, for a total burst duration of 1160 μs. The resulting M-wave and H-reflex in response to each burst were recorded. Bursts were repeated at frequencies ranging from 0.2–5 Hz to elicit RDD. The stimulation intensity was increased by 0.125 V increments until the intensity that produced Hmax, defined as the maximum amplitude of the H-wave, was found. M waves were generally detected at

stimulation intensities giving rise to H-max, but showed a distinct recruitment curve profile (data not shown). Burst stimulation was compared to single stimulation (1 × 50 μs duration square pulse) during pilot studies. Burst stimulation produced larger Hmax values (0.115 ± 0.017 V) compared to single stimulation (0.062 ± 0.008 V). Both stimulation parameters generated the same magnitude of depression in response to 1 Hz stimulation frequency (% depression of burst stimulation = 48.6 ± 8.2 vs. single stimulation = 48.0 ± 5).

For measurement of RDD curves, bursts were repeated across the 0.2–5.0 Hz range. For drug studies, bursts were repeated at a 1 Hz stimulation frequency. This frequency was chosen because preliminary studies indicated that it is associated with an approximately 40 % decrease in the amplitude of the H-wave between the first and second bursts in normal rats (see Fig. 1). This allows for a subsequent increase or attenuation in response to drug administration to be detected. RDD was calculated as the percent change in the amplitude of the H-wave (% depression) evoked by the second stimulation burst (H2) compared to the H-wave amplitude evoked by the first burst (H1). The magnitude of RDD did not depend upon the amplitude of H1 (data not shown, R^2=0.01), as has been suggested elsewhere [20]. Drugs were administered either at a fixed interval before RDD measurements were taken or immediately after a baseline measurement, as indicated in the text.

2.8 Western blotting

Spinal cords were obtained by hydraulic extrusion after decapitation of anaesthetized rats. Dorsal and ventral portions of the lumbar enlargement were collected into ice-cold homogenization buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % TritonX, protease inhibitor cocktail) and homogenized with sonication before centrifugation (14,000 g). Aliquots of the supernatant were incubated for 30 min at 37 °C in Laemmli LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Fifteen μg of total protein was separated on 4–12 % SDS-PAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and immunoblotted onto nitrocellulose (Amersham, Pittsburg, PA, USA). For blotting of diabetic tissues, membranes were incubated with anti-KCC2 (1:1000; Upstate, Temecula, CA, USA) or anti-actin (loading control; 1:5000, Sigma-Aldritch, St. Louis, MO, USA), followed by incubation with horseradish peroxidase-linked anti-rabbit or anti-mouse secondary antibody (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed on film using WestPico chemiluminescent substrate (Thermo Scientific). Quantification was performed with QuantityOne (BioRad, San Diego, CA, USA). For blotting of BDNF- and taxol-treated tissues, membranes were incubated with anti-KCC2 (1.5:1000) or anti-actin (1:1000), followed by horseradish peroxidase-linked anti-rabbit or anti-mouse (1:2500) using SNAP i.d. (Millipore, Billerica, MA, USA). Blots were incubated with WestPico chemiluminescent substrate and developed using Odyssey Fc imaging device (Li-Cor, Lincoln, NE, USA) and quantified using Image Studio version 2.1 (Li-Cor). Western blots were repeated three times and results were pooled.

2.9 Statistical analysis

Statistical analyses were performed with Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA) using unpaired, two-tailed t-test or one-way ANOVA followed by Tukey or Dunnett's *post-hoc* test for multiple comparisons, as indicated. Paw 50% withdrawal threshold is reported as group median and interquartile range (IQR) for non-parametric data or as group mean % MPE ± SEM. RDD and formalin test data are reported as group mean ± SEM.

3. Results

3.1 RDD in BDNF-treated rats

RDD in normal rats is dependent on functional $GABA_A$ receptor-mediated spinal inhibitory systems [29]. As $GABA_A$ receptor-mediated inhibition can be regulated by local BDNF levels [45], we first investigated the effect of manipulating spinal BDNF levels on RDD in normal rats. Representative traces of the H-reflex from saline and BDNF-treated rats are shown (Fig. 1A). RDD was assessed in normal rats 15 min after the IT administration of saline, BDNF or TrkB/Fc, a chimeric molecule comprised of the extracellular domain of the TrkB receptor fused to the C-terminus Fc domain of human IgG that sequesters BDNF by competing with the endogenous TrkB receptor [56]. BDNF-treated rats had similar H1 values compared to saline-treated rats (Fig. 1B), indicating that BDNF did not alter the intrinsic excitability of motor neurons. TrkB/Fc (5 μg) did not alter RDD compared to saline-treated rats, whereas RDD showed a dose-dependent attenuation in response to BDNF-treatment, with 20 μg BDNF completely abolishing RDD (Fig. 1C).

To determine whether impairment of RDD in normal rats by BDNF could be attributed to failure or reversal of $GABA_A$ receptor-mediated activity, we tested the effect of 0.6 μg bicuculline on RDD in rats pre-treated with IT saline or 20 μg BDNF 15 min before measuring baseline RDD. Bicuculline significantly ($p<0.05$) attenuated RDD within 5 min of administration in rats that had been pre-treated with saline (Fig. 1D). Conversely, in rats lacking RDD due to pre-treatment with BDNF, bicuculline significantly ($p<0.05$) restored RDD within 5 min of delivery, whereas saline did not (Fig. 1E). H1 was not significantly different between any of the experimental groups (baseline: 0.26 ± 0.03 V; BDNF + saline: 0.17 ± 0.04 V; BDNF + bicuculline: 0.24 ± 0.4 V).

3.2 Tactile withdrawal thresholds in BDNF-treated rats

Studies of the impact of spinal BDNF were extended to include behavioral indices of allodynia. After the IT injection of 20 μg BDNF, PWT declined from baseline (median: 15.00 g, IQR: 15.00–15.00) with a maximal effect at 60 min (median: 2.86 g, IQR: 1.81–9.11) and duration of at least 6 h (Fig. 2A). To determine whether BDNF causes allodynia in normal rats by causing a failure of $GABA_A$ receptor-mediated inhibition or a switch in $GABA_A$ receptor function, we injected 0.6 μg bicuculline or saline 30 min after the BDNF injection. Both groups of rats treated with BDNF showed a decline in PWT that was apparent by 15 min. In BDNF-treated rats that were subsequently given saline, PWT continued to decline, reaching a maximal effect 60 min after BDNF injection that persisted for at least 6 h. However, in BDNF-treated rats that were subsequently given bicuculline, PWT returned towards normal, indicating the bicuculline reversed BDNF-induced effects on PWT. Analysis of the AUC of PWT prior to the second injection (0–30 min) shows that both BDNF-treated groups had significantly reduced PWT ($p<0.05$) compared to the group that received saline only and were not significantly different from each other (Fig. 2B). In contrast, analysis of AUC following the second injection (60–345 min) shows that the BDNF + saline group was significantly reduced relative to both the saline + saline ($p<0.001$) and the BDNF + bicuculline ($p<0.05$) groups. There was no significant difference between the BDNF + bicuculline group and the saline + saline group, indicating the bicuculline reversed BDNF-induced effects on PWT.

3.3 Formalin-evoked flinching in BDNF-treated rats

Rats were pre-treated with IT saline or 5 μg of TrkB/Fc 10 min prior to injection of 50 μl 5 % formalin into the hind paw to determine whether endogenous BDNF release plays a role in either phase of formalin-evoked flinching behavior during maximal stimulation. Saline-

injected rats showed robust phase 1 and phase 2 flinching in response to 5 % formalin that was not altered by pre-treatment with TrkB/Fc (Fig. 2C).

To determine whether excess spinal BDNF can alter formalin-evoked flinching, we measured paw flinching in rats pre-treated with 20 μg IT BDNF or saline 1 h before injection of 0.5 % formalin into the paw. This is a dose of formalin that produces a sub-maximal flinching response in normal rats [8]. The interval between administration of BDNF and paw formalin injection was chosen to target the peak efficacy of BDNF according to measurements of PWT (Fig. 2A). Formalin-evoked flinching was increased during both phase 1 and phase 2 in BDNF-treated rats relative to saline-treated rats (Fig. 2D). To determine whether BDNF caused increased flinching via altered $GABA_A$ receptor activity, BDNF pre-treated rats were given saline or 0.6 μg bicuculline 15 min before formalin (45 min after BDNF). Bicuculline, but not saline, completely prevented the increased flinching during both phases of the formalin test in BDNF pre-treated rats (Fig. 2D).

3.4 Effect of BDNF-sequestration on RDD in diabetic rats

Diabetic rats exhibit tactile allodynia, hyperalgesia in response to formalin and RDD deficits that are attributable to inversion of $GABA_A$ receptor function associated with reduced lumbar spinal KCC2 expression [29]. We therefore determined whether normalizing RDD function by manipulation of spinal BDNF levels predicts efficacy in alleviating tactile allodynia and formalin-evoked hyperalgesia. Diabetic rats had significantly ($p<0.05$) attenuated RDD relative to age-matched normal rats (Fig. 3A). Diabetic rats received IT injections of either 5 μg denatured TrkB/Fc, 20 μg BDNF or 5 μg TrkB/Fc and RDD was measured 15 min later. Both denatured TrkB/Fc and BDNF-treated diabetic rats had attenuated RDD (Fig. 3B), while TrkB/Fc restored RDD to values similar to those seen in normal rats (Figs. 3A, B). In order to determine whether TrkB/Fc restored RDD by restoring $GABA_A$ receptor-mediated inhibition, 0.6 μg bicuculline was administered to rats that had been pre-treated with TrkB/Fc 15 min prior to taking baseline RDD measurements. RDD in TrkB/Fc-treated diabetic rats was significantly ($p<0.001$) impaired within 5 min of bicuculline administration (Fig 3C). Changes to the magnitude of RDD were not associated with changes in the amplitude of H1, which was not different between baseline TrkB/Fc (0.62 ± 0.04 V), TrkB/Fc + saline (0.65 ± 0.13 V) and TrkB/Fc + bicuculline (0.52 ± 0.08 V) groups.

3.5 Effect of BDNF-sequestration on behavioral indices of painful neuropathy in diabetic rats

We investigated whether TrkB/Fc could alleviate behavioral indices of painful neuropathy in diabetic rats. After confirming robust tactile allodynia (median PWT: 3.19, IQR: 2.32–4.91), 5 μg TrkB/Fc was delivered to the spinal cord by IT injection. Allodynia was alleviated within 15 min (median PWT: 10.20, IQR 6.68–12.79), and the effect disappeared by 60–90 min (Fig. 4A). Analysis of AUC from 0–90 min showed a significant ($p<0.05$) difference between denatured TrkB/Fc- and TrkB/Fc-treated diabetic rats (Fig. 4B). IT delivery of 5 μg TrkB/Fc 10 min before paw injection of 0.5 % formalin did not significantly reduce formalin-evoked flinching (Figs. 4C, D).

3.6 RDD in taxol-induced painful neuropathy

To further test the association between RDD and neuropathic pain, RDD was assessed in rats with taxol-induced neuropathy. All taxol-treated rats developed robust allodynia within 12 days of administration of the first dose of taxol (Day 12 median PWT: 1.62 g, IQR: 1.34–2.51).

Despite the continued presence of robust allodynia, rats with taxol-neuropathy exhibited normal RDD across a range of stimulation frequencies when measured on day 20 after starting taxol treatment (Fig. 5A). H1 values in taxol-treated rats (0.66 ± 0.09 V) were not different from control rats (0.69 ± 0.09 V). Because normal RDD is dependent upon intact $GABA_A$ receptor-mediated inhibitory systems [29], we verified that the $GABA_A$ receptor system was operational in taxol-treated rats by measuring the effects of 0.3 μg of the $GABA_A$ receptor inhibitor bicuculline or 3 μg of the KCC2 blocker DIOA, both of which impair RDD in normal rats [29]. Bicuculline and DIOA, but not saline vehicle, significantly (both $p<0.001$) impaired RDD within 5 min of administration (Fig. 5B). H1 values were significantly ($p<0.05$) reduced in the bicuculline-treated group (0.33 ± 0.04 V) compared to baseline (0.62 ± 0.08 V), but not ($p= 0.051$) in saline-treated (0.37 ± 0.05 V) compared to baseline (0.67 ± 0.9 V) or DIOA-treated (0.57 ± 0.09 V) compared to baseline (0.68 ± 0.08 V).

3.7 Effect of manipulations of $GABA_A$ergic system on behavioral indices of painful neuropathy in taxol-treated rats

Because RDD was normal in taxol-treated rats, we tested the efficacy of bicuculline and muscimol on indices of neuropathic pain to determine whether $GABA_A$ receptor-mediated inhibition is also intact. After confirming the presence of allodynia (median baseline PWT all rats: 3.67, IQR: 1.84–4.76), taxol-treated rats were given either IT saline, 0.3 μg bicuculline or 0.3 μg muscimol. Muscimol induced a rapid reversal of allodynia with a peak efficacy at 30 min (median PWT: 12.99, IQR:4.95–15.00) and a duration of 120 min (Fig. 6A) that was not accompanied by evidence of motor impairment. In contrast, bicuculline and saline had no effect on PWT. Analysis of AUC revealed that that muscimol treatment significantly ($p<0.01$) increased PWT compared to both saline- and bicuculline-treated animals (Fig. 6B).

Taxol-treated rats exhibited significantly more flinching behavior in response to 0.5 % formalin during both phase 1 ($p<0.05$) and phase 2 ($p<0.001$) compared to normal rats. Muscimol significantly ($p<0.05$) prevented flinching behavior during both phases of the formalin test, whereas bicuculline had no effect (Figs. 6C, D).

3.8 Spinal KCC2 expression in BDNF-treated, diabetic and taxol-treated rats

Spinal KCC2 expression was significantly reduced in the dorsal, but not ventral, spinal cord within 1 hr of BDNF administration, when compared to saline-treated rats ($p<0.05$; Figs. 7A, B). KCC2 was also reduced in the dorsal, but not ventral spinal cord of 8 week diabetic rats compared to control rats ($p<0.05$; Figs. 7C, D). In taxol-treated rats, KCC2 expression was unchanged in the dorsal lumbar spinal cord, but reduced in the ventral lumbar spinal cord (Figs. 7E, F).

Discussion

RDD is an electrophysiological phenomenon reflecting activation of spinal inhibitory circuits. We previously found that RDD is impaired in rats with painful diabetic neuropathy [29]. To test the potential of RDD to identify underlying causes of neuropathic pain, we investigated RDD after pharmacological manipulation of three different models. In normal rats, spinal injection of BDNF produced rapid impairment of RDD accompanied by allodynia and increased formalin-evoked flinching. This agrees with prior reports that BDNF produces allodynia and hyperalgesia in rodents [12; 23; 56]. There are many potential mechanisms by which exogenous BDNF could enhance behavioral sensitivity to peripheral stimuli, including increased synaptic drive to excitatory neurons, decreased synaptic drive to inhibitory interneurons [33; 52], enhanced NMDA receptor-dependent

responses [22; 31] and induction of spinal LTP of C-fiber-evoked potentials [59]. BDNF also caused a reduction in dorsal horn KCC2 protein levels, which would be expected to affect the chloride reversal potential and invert $GABA_A$ receptor function [13; 34]. As all BDNF-induced effects were reversed by the $GABA_A$ receptor antagonist bicuculline, BDNF likely acts via inversion of $GABA_A$ receptor function. This parallels our prior findings that bicuculline reversed RDD deficits and enhanced formalin-evoked flinching in rats treated with the KCC2 inhibitor DIOA [29]. If allodynia and increased formalin-evoked flinching had been caused by mechanisms other than altering excitatory $GABA_A$ receptor function, bicuculline should not alter, or even exacerbate, these indices of neuropathic pain. This suggests that RDD can be used to identify a contribution of impaired $GABA_A$ receptor-mediated function to neuropathic pain.

The pain phenotype of BDNF-treated rats is similar to that of STZ-diabetic rats, with both exhibiting RDD deficits, tactile allodynia and hyperalgesia in the formalin test secondary to $GABA_A$ receptor-mediated excitatory function [29]. STZ-diabetic rats also have reduced dorsal, but not ventral, spinal KCC2 protein expression. We therefore investigated the involvement of BDNF in diabetes-induced RDD impairment using the BDNF-sequestering molecule, TrkB/Fc, which reverses KCC2 deficits and behavioral indices of painful neuropathy after nerve injury [12; 42; 43]. Spinal delivery of TrkB/Fc produced a rapid, $GABA_A$ receptor inhibition-dependent restoration of RDD in diabetic rats, implying that continuous spinal BDNF signaling may be required to maintain aberrant $GABA_A$ receptor function. Likewise, TrkB/Fc acutely reversed tactile allodynia, indicating that BDNF signaling contributes to the pain phenotype in diabetic rats, as reported in other experimental models of pain [39; 42; 55; 56]. BDNF mRNA is increased in the dorsal root ganglia of diabetic rats [18], though whether this promotes increased release of BDNF from central projections of primary afferents remains to be established. Spinal BDNF could also be derived from activated microglia [12], which have been reported in diabetic rats [14; 43; 51; 53]. Spinal BDNF tissue levels are unchanged by diabetes [43], but this does not provide information on BDNF release. Our findings suggest that BDNF may be involved in a pathogenic cascade that leads to altered $GABA_A$ receptor function, impaired RDD and neuropathic pain in diabetic rats, and that RDD evaluation can identify treatments that reverse neuropathic pain mediated by central disinhibition.

In contrast to diabetic neuropathy, which results from a systemic disease that disrupts all levels of the nervous system, taxol-induced neuropathy has been described as a peripherally-mediated neuropathy [48]. Taxol is found in sensory cell bodies after systemic administration and does not cross efficiently into the central nervous system [9]. Taxol causes damage to peripheral sensory terminals [1; 19] and spontaneous afferent activity that may occur via peripheral sensitization or ectopic and/or ephaptic signals [54]. Our finding of increased flinching during phase 1 of the formalin test supports the occurrence of exaggerated primary afferent activity in taxol-treated rats. RDD was not impaired by taxol, despite concurrent tactile allodynia and exaggerated responses in the formalin test. RDD defects are therefore not an indicator of neuropathic pain *per se*. However, the presence of normal RDD in taxol-treated rats allowed us to test our hypothesis that RDD deficits only accompany neuropathic pain when spinal disinhibition is a contributory mechanism. The impairment of RDD when the $GABA_A$ receptor system was disrupted by bicuculline or DIOA excludes the possibility that RDD appeared normal due to the involvement of other inhibitory systems and confirms that normal RDD is indicative of normal $GABA_A$ receptor function in taxol-treated rats. Likewise, muscimol retained its antinociceptive effects against tactile allodynia and formalin-evoked flinching, demonstrating that the spinal $GABA_A$ receptor system retains its inhibitory function. RDD is therefore retained in a painful neuropathy that is not associated with spinal disinhibition.

The H-reflex is frequently considered to be monosynaptic, with RDD (sometimes called low-frequency depression) resulting from reduced primary afferent neurotransmitter release onto motor neurons. Our prior [29] and present data show that RDD of the H-reflex in rats depends upon normal inhibitory $GABA_A$ receptor function, as RDD in both normal and taxol-treated rats is lost after administration of bicuculline or DIOA. Impairment of RDD has recently been attributed to KCC2 depletion and consequent changes to post-synaptic $GABA_A$ receptor function in motor neuron cell bodies of the ventral horn [3]. However, our data appear in conflict with this view, as taxol did not alter RDD despite reduced KCC2 protein in the ventral horn. Furthermore, as ventral motor neurons are not hyperpolarized at stimulation frequencies that produce RDD [25], inhibitory inputs acting directly on motor neurons likely do not participate in RDD. The loss of RDD in both diabetic and BDNF-treated rats, accompanied by a selective reduction of KCC2 protein in the dorsal horn, may provide some guidance as to the circuits involved. KCC2 is not expressed by DRG neurons [44], so reduced KCC2 protein is unlikely to directly affect neurotransmitter release from primary afferents. Primary afferent neurotransmitter release onto motor neurons could plausibly be modulated by as yet unknown signals from the dorsal horn that are disrupted when dorsal horn KCC2 levels are reduced. Another potential explanation is that the H-reflex measured in our studies involves oligosynaptic excitatory interneurons located in the dorsal horn, and that reduced KCC2 expression by these neurons renders them impervious to GABAergic inhibition, leading to impaired RDD in diabetic and BDNF-treated rats. This is supported by evidence in cats [27; 28] and humans [4; 21] indicating that group Ia afferents make both monosynaptic and oligosynaptic excitatory connections onto motor neurons, and that the EPSP rise time measured in ventral motor neurons is longer than would be expected from a purely monosynaptic H-reflex [4; 41]. While we cannot confirm a specific wiring of the spinal cord that drives the H-reflex and RDD, our spinal pharmacology and location-specific western blotting data are consistent with an oligosynaptic component to H-reflex modulation during RDD. Future studies investigating why diabetes and BDNF selectively deplete KCC2 in the dorsal horn and identifying the circuitry of cells that express KCC2 and contribute to H-reflex modulation in that region may clarify this issue.

Our study highlights loss of RDD as an indicator of conditions in which impaired GABAergic inhibitory function contributes to behavioral indices of neuropathic pain. Impaired RDD has also been identified in spinal cord injury models characterized by hind limb spasticity and rigidity that are thought to be a consequence of impaired inhibitory function in the ventral spinal cord [3; 30]. However, this need not contradict use of RDD as an indicator of neuropathic pain arising from spinal disinhibition, as spinal cord injury has also been associated with the presence of pain. For example, RDD deficits have been observed in a model of ischemia-induced spinal cord injury that exhibits trunk allodynia and specific loss of inhibitory interneurons [24; 30; 58] and a shift in chloride reversal potential has been reported in dorsal horn neurons of spinal cord injured rats with tactile allodynia [35]. RDD deficits also occur in patients with spinal cord injury [26; 47], but whether impaired RDD is associated with a greater incidence of neuropathic pain has not been reported. In all cases, RDD deficits appear to be associated with impaired $GABA_A$ receptor function, suggesting that RDD is a useful electrophysiological indicator by which to evaluate the underlying cause of neuropathic pain.

The present data demonstrate that RDD can be used to assess spinal inhibitory function and identify spinal disinhibition as a mechanism contributing to neuropathic pain (summarized in Table 1). Our data also suggest that BDNF contributes to the mechanism underlying spinal cord disinhibition caused by altered $GABA_A$ receptor function in diabetic rats. A notable aspect of RDD is that the relationship between inter-stimulus interval and H-reflex depression remains consistent across different species and experimental designs [5; 17; 26; 37]. This suggests that our findings in rodents may be relevant to the human condition. The

measurement of RDD in both upper and lower limbs of humans may also allow segmental discrimination of etiological mechanisms of neuropathic pain [46]. The measurement of RDD may therefore be a useful indicator of the underlying mechanisms of neuropathic pain states and could provide a tool for characterizing experimental models of neuropathic pain and defining clinical treatment options.

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The unpredictable efficacy of current therapies for neuropathic pain may reflect diverse etiological mechanisms operating between, and within, diseases. As descriptions of pain rarely establish specific mechanisms, a tool that can identify underlying causes of neuropathic pain would be useful in developing patient-specific treatments. Rate-dependent depression (RDD), a measure of the change in amplitude of the Hoffman reflex over consecutive stimulations, is attenuated in diabetic rats that also exhibit impaired spinal $GABA_A$ receptor function, reduced spinal KCC2 expression and indices of painful neuropathy. To investigate whether loss of RDD is a reliable indicator of the contribution of spinal GABAergic dysfunction to neuropathic pain, we assessed RDD, tactile allodynia and formalin-evoked hyperalgesia in three models: rats treated acutely with brain-derived neurotrophic factor (BDNF), diabetic rats treated with the BDNF-sequestering molecule TrkB/Fc and rats with taxol-induced neuropathy. Delivery of BDNF to the spinal cord of normal rats produced RDD deficits and features of painful neuropathy associated with disrupted $GABA_A$ receptor-mediated inhibitory function and reduced dorsal spinal KCC2 expression. Treating diabetic rats with TrkB/Fc restored RDD and alleviated indices of painful neuropathy. In taxol-treated rats, RDD was not impaired and behavioral indices of neuropathic pain were not associated with spinal GABAergic dysfunction or reduced dorsal spinal KCC2 expression. Our data reveal BDNF as part of the mechanism underlying spinal cord disinhibition caused by altered $GABA_A$ receptor function in diabetic rats and suggest that RDD deficits may be a useful indicator of neuropathic pain states associated with spinal disinhibition, thereby revealing specific therapeutic targets.

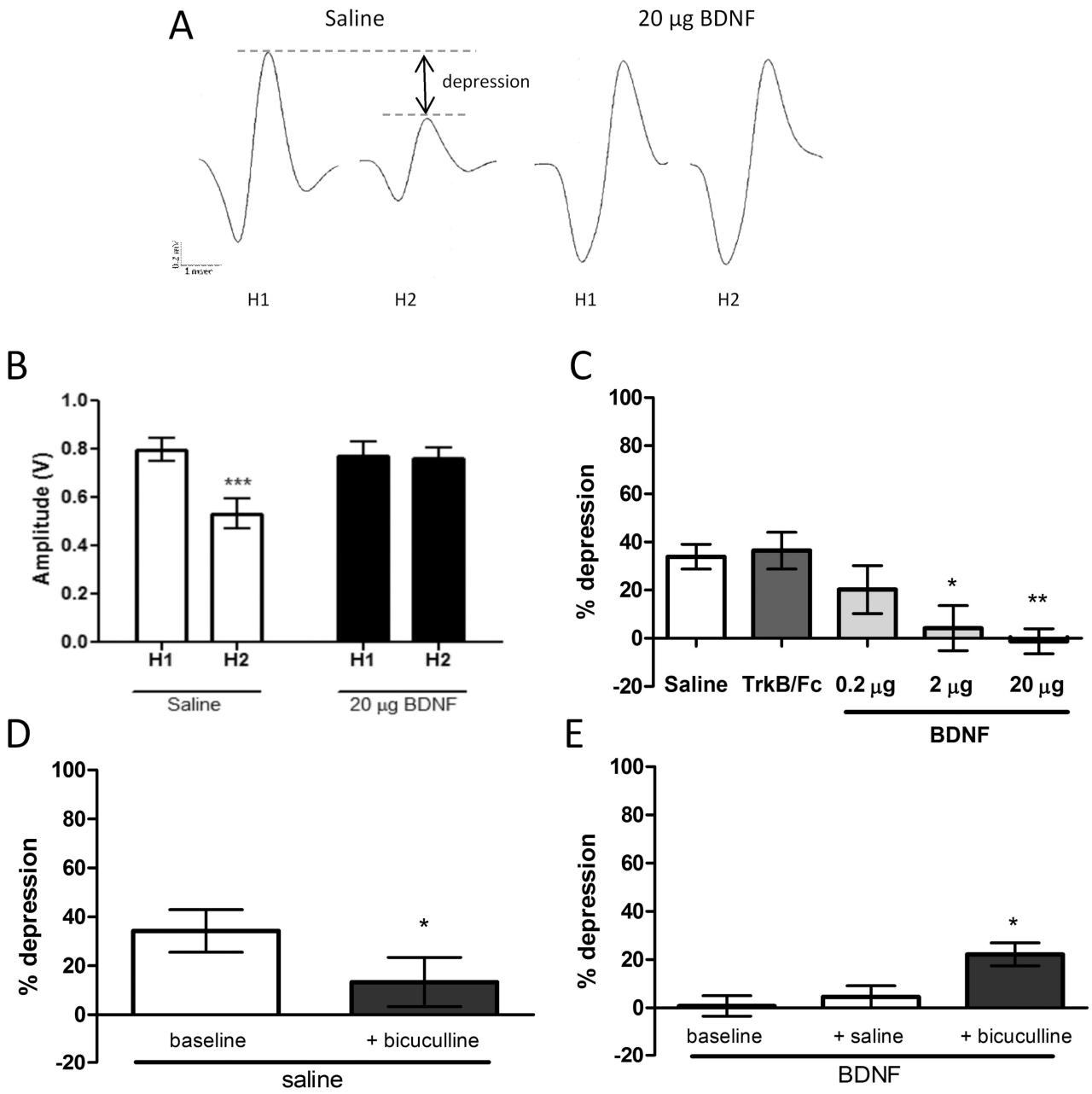

Figure 1. Effect of spinal BDNF on RDD in normal rats

A) Representative first (H1) and second (H2) H-reflex traces taken 15 min after administration of either saline or BDNF (20 µg) illustrating RDD in saline-treated rats. B) Corresponding group mean H1 and H2 amplitudes taken 15 min after administration of saline of 20 µg BDNF. C–E) % depression of the H-wave (RDD) in: C) rats treated with saline (IT), TrkB/Fc (5 µg; IT) or BDNF (0.2–20 µg; IT) 15 min before measuring RDD, D) rats pre-treated with saline (IT) 15 min before measuring RDD (baseline), followed by RDD measurement 5 min after delivery of bicuculline (0.6 µg; IT), E) rats pre-treated with BDNF (20 µg; IT) 15 min before measuring RDD (baseline), followed by RDD measurement 5 min after delivery of saline or bicuculline (0.6 µg; IT). Data are group mean ± SEM. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ compared to H1 by paired t-test (B), to saline or baseline by

one-way ANOVA followed by Dunnett's *post-hoc* test (C, E) or to baseline by unpaired t-test (D). N=5–8 per group.

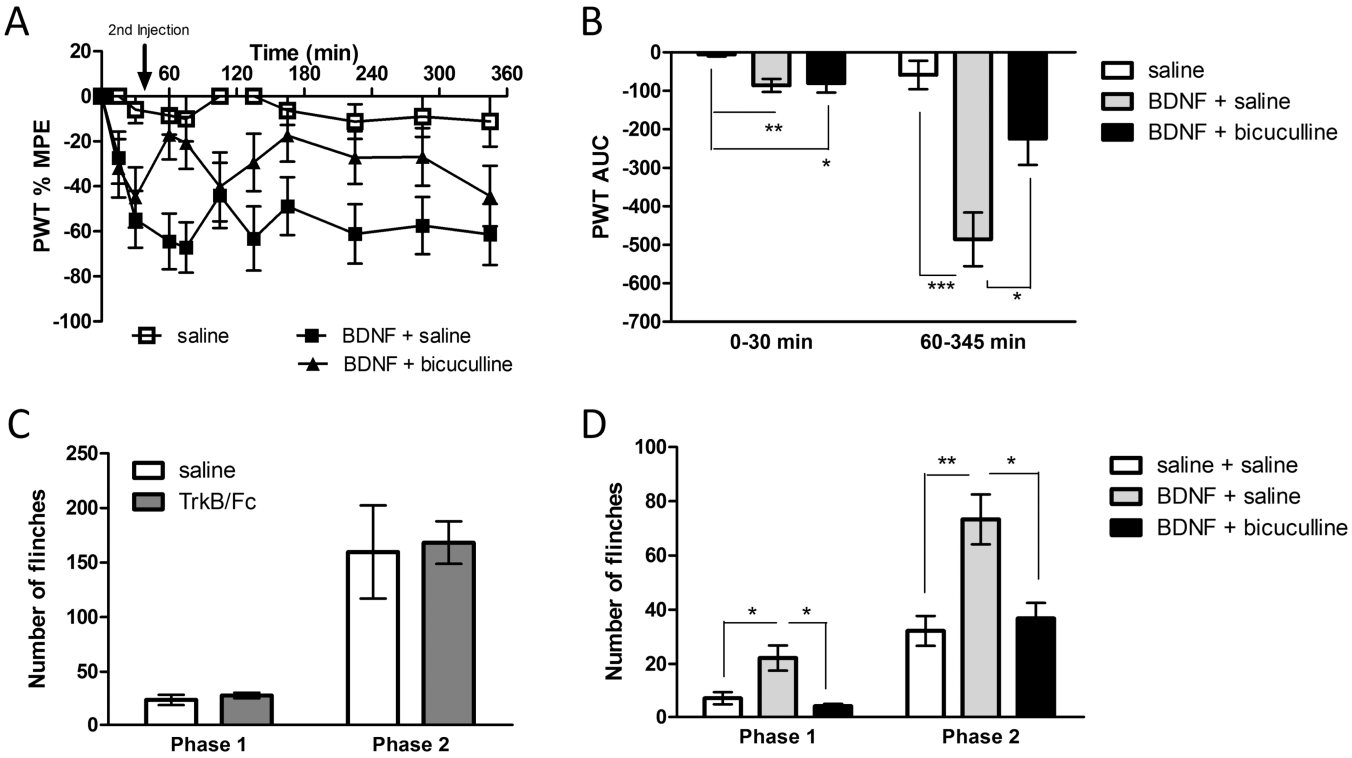

Figure 2. BDNF-induced allodynia and formalin-evoked flinching are reversed by bicuculline

PWT was assessed in normal rats before and after the administration of saline (IT) followed by saline (saline), BDNF (20 μg; IT) followed by saline (BDNF + saline), or BDNF followed by bicucuclline (0.6 μg; IT: BDNF + bicuculline). The second injection was administered 30 min after the initial saline or BDNF injection. A) PWT expressed as percent maximum potential effect (% MPE) over time. B) Area under the curve (AUC) of PWT data separated into the periods before (0–30 min) and after (60–345 min) the second injection. C) Saline or TrkB/Fc (5 μg; IT) was administered 5 min before 5 % formalin injection to the paw and sum of flinches were quantified during phase 1 (0–10 min) and phase 2 (15–60 min). D) Saline or BDNF (20 μg; IT) were administered 1 h before, and saline or bicuculline (0.6 μg; IT) administered 15 min before 0.5 % formalin injection to the paw. Sum of flinches were quantified during phase 1 (0–10 min) and phase 2 (15–60 min). Data are group mean ± SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ by one-way ANOVA followed by Tukey's multiple comparisons *post-hoc* test. N=5–9 per group.

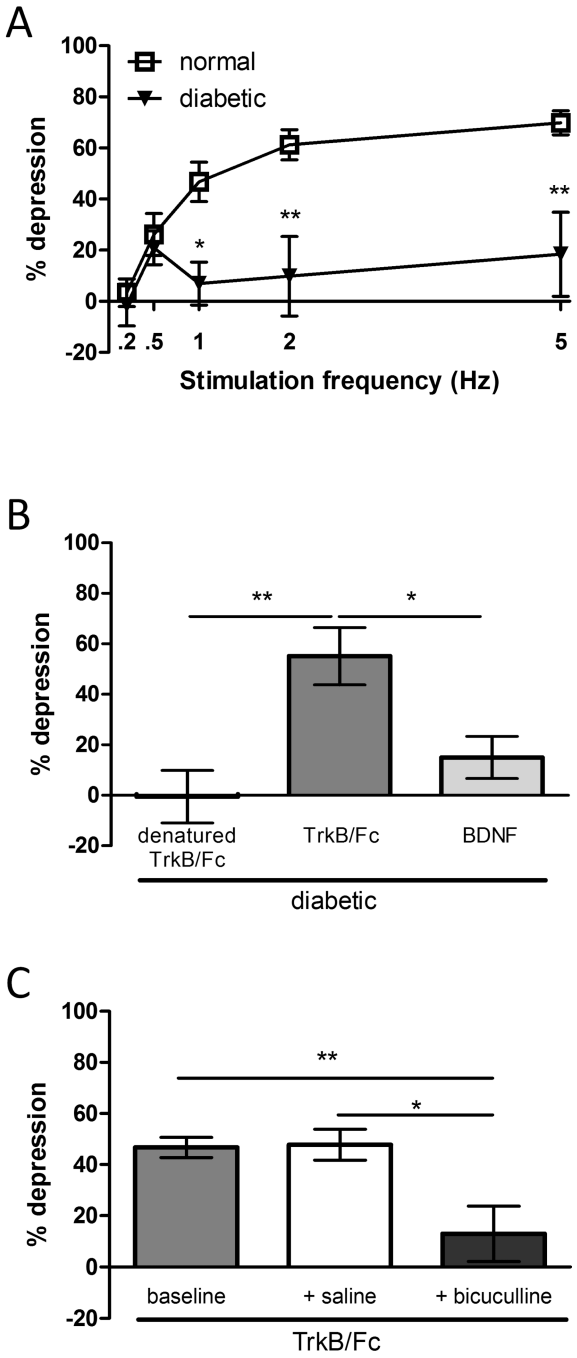

Figure 3. Effect of modulating spinal BDNF on RDD in diabetic rats

A) Depression of the H-wave over consecutive stimulations (RDD) at 0.2–5 Hz frequencies in normal and diabetic rats. RDD in response to 1 Hz stimulation frequency in: B) diabetic rats treated with denatured TrkB/Fc (5 μg; IT), TrkB/Fc (5 μg; IT) or BDNF (20 μg; IT) 15 min prior to taking measurements, and C) diabetic rats treated with TrkB/Fc (5 μg; IT) 15 min before taking measurements (baseline), followed by RDD measurements 5 min after administration of saline or bicuculline (0.6 μg; IT). Data are group mean ± SEM. ** = $p<0.01$ and * = $p<0.05$ by one-way ANOVA followed by Tukey's *post-hoc* test (B) or unpaired two-tailed t-test (A, C). N=6–8 per group.

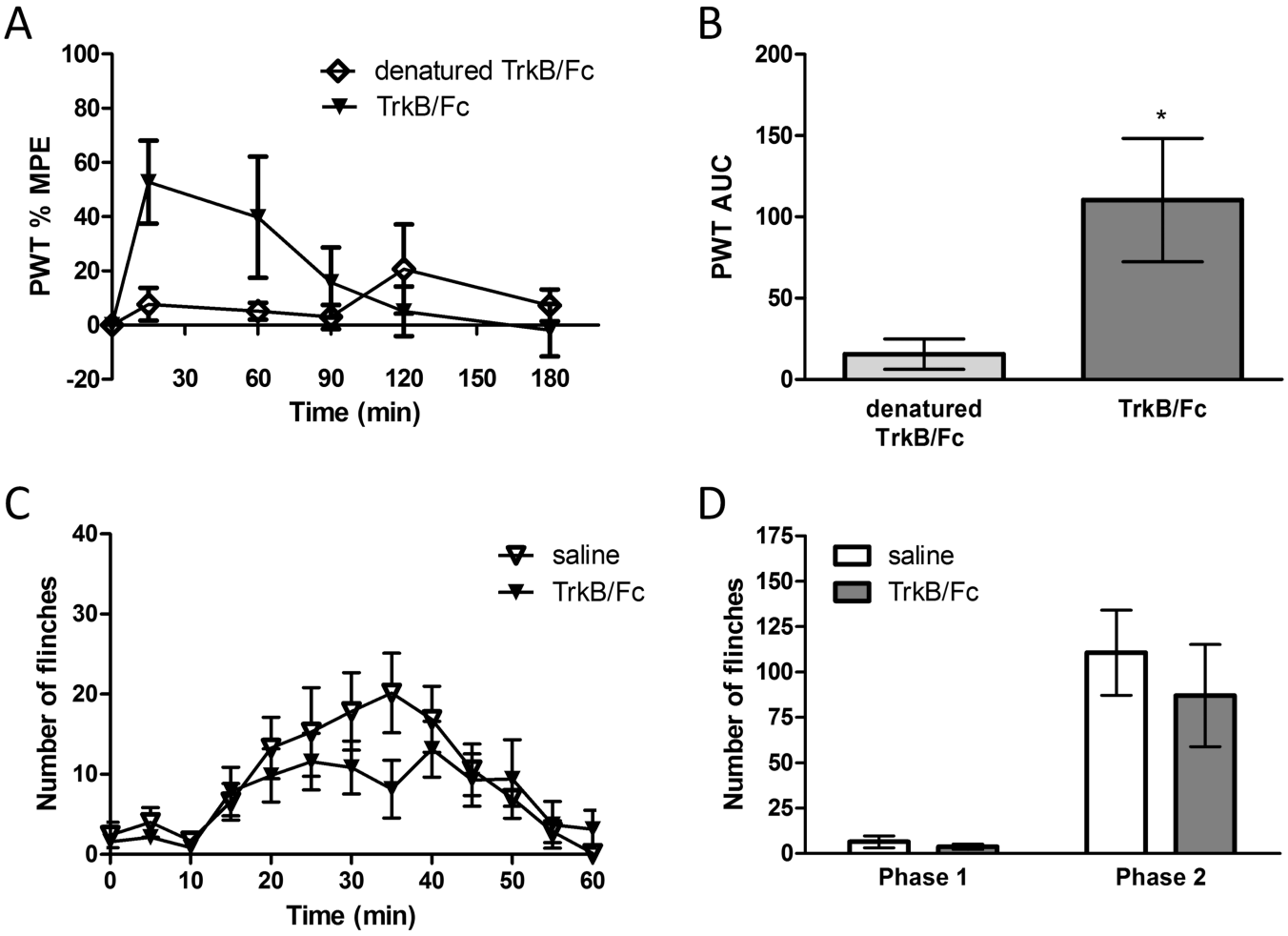

Figure 4. Effect of TrkB/Fc on PWT and formalin-evoked flinching in diabetic rats

A) PWT expressed as percent maximum potential effect (% MPE) as a function of time assessed in diabetic rats before and after administration of TrkB/Fc or denatured TrkB/Fc (both 5 μg; IT). B) AUC of the effect of TrkB/Fc or denatured TrkB/Fc on PWT from 0–90 min. C) Time course of paw flinching in response to paw injection of 0.5 % formalin 10 min after IT delivery of saline or TrkB/Fc (5 μg; IT). D) Sum of flinches during phase 1 (0–10 min) and phase 2 (15–60 min) of the formalin test. Data are group mean ± SEM.* = $p<0.05$ by unpaired t-test. N=5–8 per group.

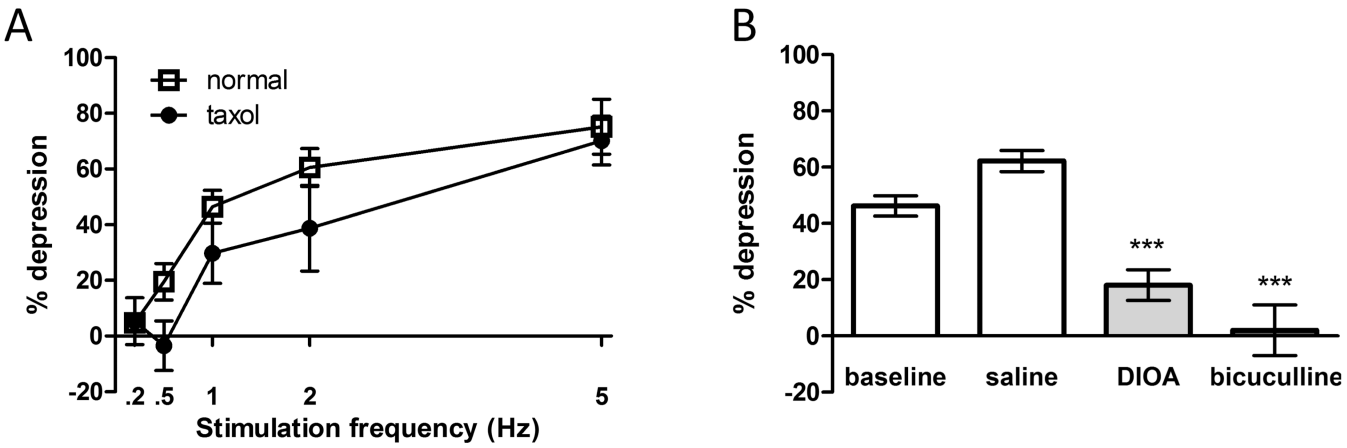

Figure 5. RDD in taxol-treated rats

A) Depression of the H-wave (RDD) over consecutive stimulations at 0.2–5 Hz frequencies in normal and taxol-treated rats. B) RDD in response to 1 Hz stimulation frequency in taxol-treated rats at baseline and 5 min after saline, DIOA (3 μg; IT) or bicuculline (0.3 μg; IT). Data are group mean ± SEM. *** = $p<0.01$ compared to baseline by one-way ANOVA followed by Dunnett's *post-hoc* test. N=6–7 per group.

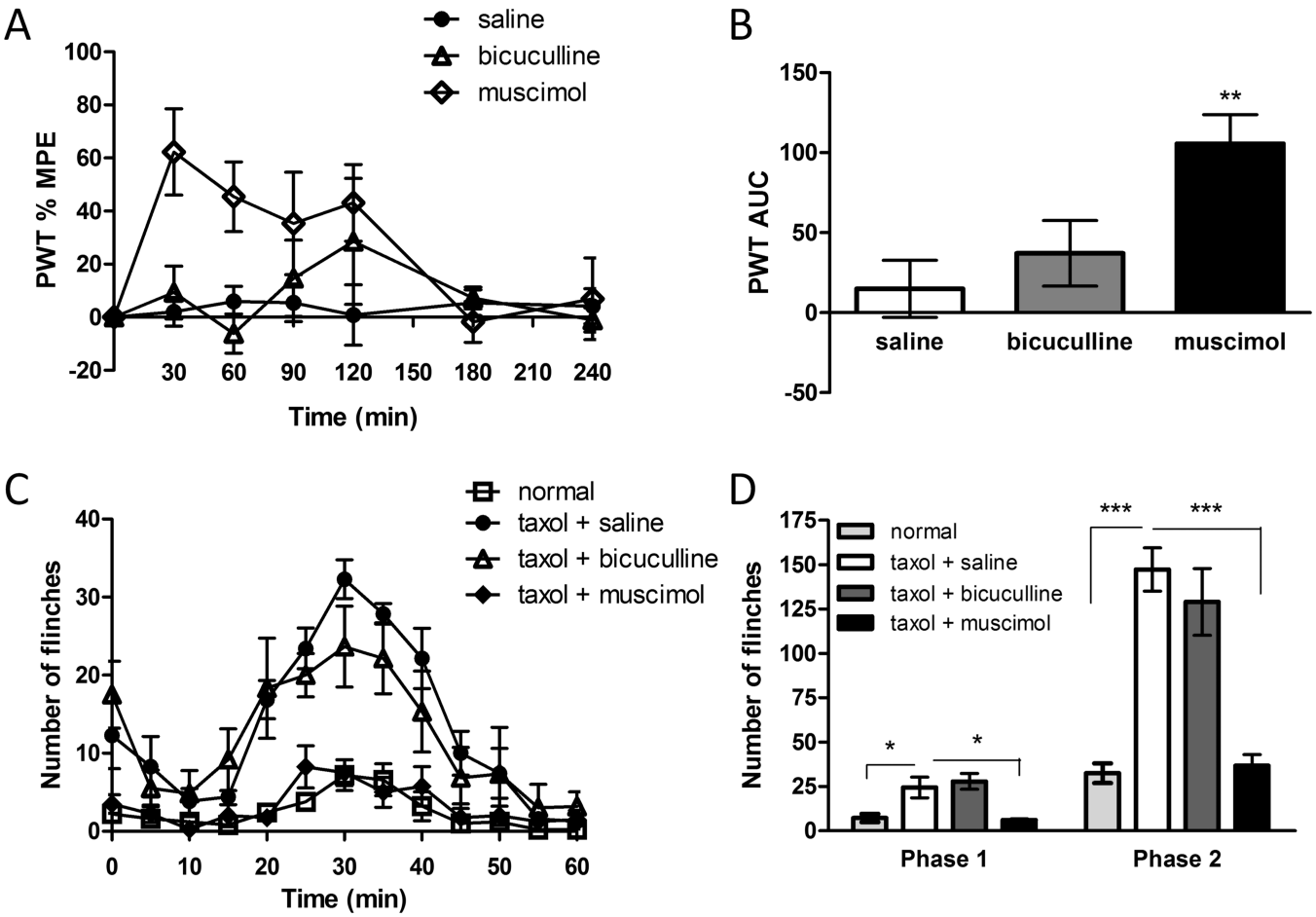

Figure 6. Effects of bicuculline and muscimol on behavioral indices of neuropathic pain in taxol-treated rats

A) PWT assessed in taxol-treated rats before and after administration of saline, bicuculline (0.3 μg; IT), or muscimol (0.3 μg; IT). B) AUC of the effect of saline, bicuculline or muscimol on PWT. C) Time course of paw flinching in response to paw injection of 0.5 % formalin in saline-treated normal rats (normal) and 10 min after administration of saline, bicuculline (0.3 μg; IT) or muscimol (0.3 μg; IT) to taxol-treated rats. D) Sum of flinches during phase 1 (0–10 min) and phase 2 (15–60 min). Data are group mean ± SEM.* = $p<0.05$ compared to saline (B) or taxol + saline (D) by one-way ANOVA followed by Dunnett's *post-hoc* test. N = 5–7 per group.

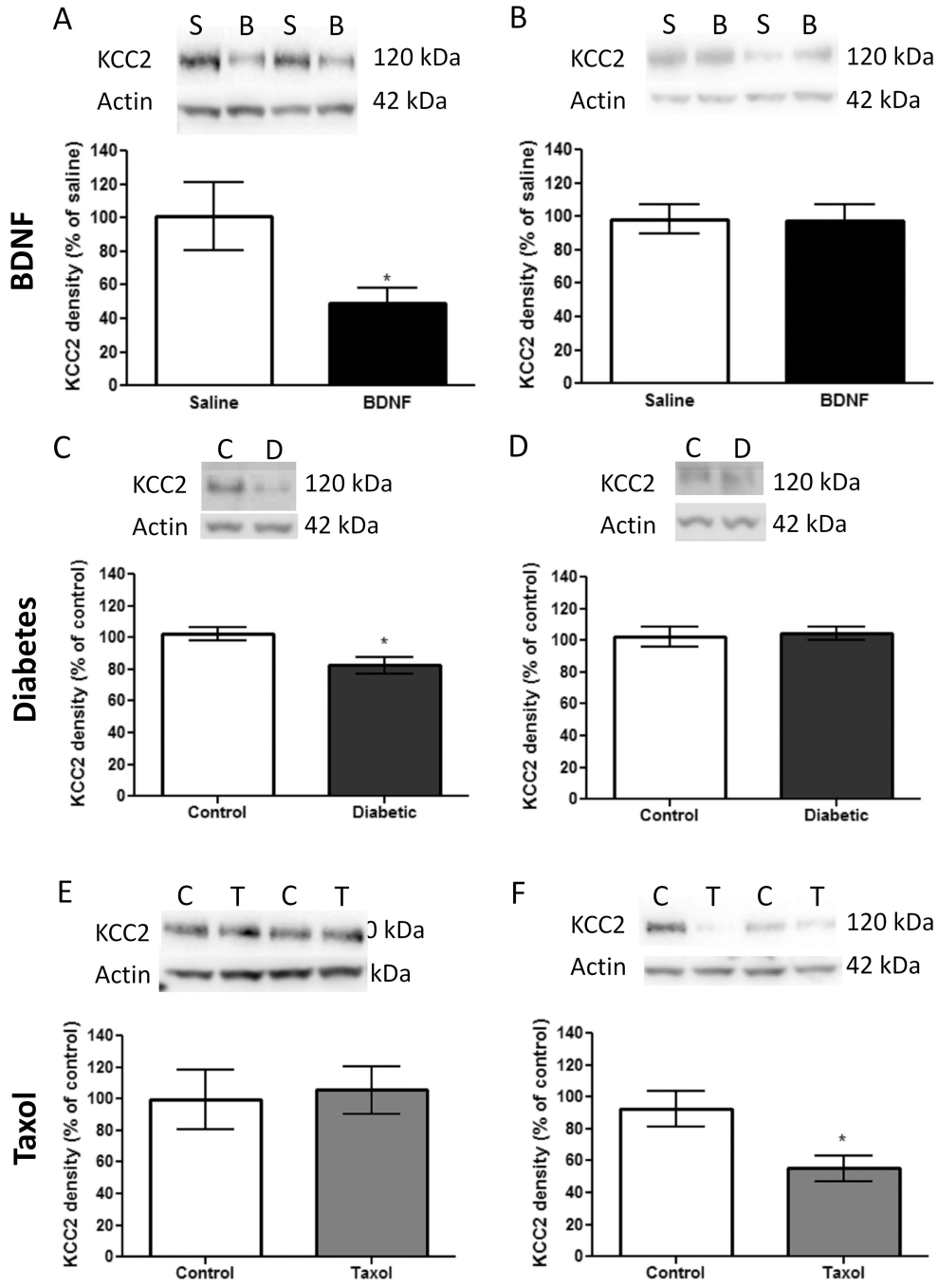

Figure 7. Spinal KCC2 expression in BDNF-treated, diabetic and taxol-treated rats

KCC2 expression in A) lumbar dorsal and B) lumbar ventral spinal cord from rats 1 hr after administration of saline or BDNF (20 μg; IT). KCC2 expression in C) lumbar dorsal and D) lumbar ventral spinal cord from 8 week diabetic rats and age-matched control rats. KCC2 expression in E) lumbar dorsal and F) lumbar ventral spinal cord from taxol-treated rats and age-matched control rats, 30 days after first injection of 1 mg/kg taxol. Data are normalized to actin loading control and expressed as mean ± SEM. *$p<0.05$ compared to relevant control by unpaired two-tailed test. n=7 per group.

Table 1

Summary table demonstrating the relationship between inverted $GABA_A$ receptor function associated with reduced dorsal lumbar spinal KCC2 expression and RDD deficits.

	Normal inhibition	**Spinal disinhibition**
Condition	Normal rats Taxol-treated rats	Diabetic rats BDNF-Treated rats DIOA-treated rats[25]
RDD	Present	Impaired
Dorsal lumbar spinal KCC2	Normal	Decreased[a]
Dorsal spinal $GABA_A$ function during stimulation to evoke RDD	GABA ↓ $GABA_A$ receptors ↓ chloride influx ↓ INHIBITION	GABA ↓ $GABA_A$ receptors ↓ chloride efflux ↓ EXCITATION
Effect of bicuculline	No effect on pain/impairs RDD	Alleviates pain[b]/restores RDD

[a] KCC2 is blocked rather than decreased in DIOA-treated rats

[b] Alleviation of pain inferred from a significant increase in 50% PWT and a significant reduction in formalin-evoked flinching