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# PROCHLORPERAZINE INCREASES KCC2 FUNCTION AND REDUCES SPASTICITY AFTER SPINAL CORD INJURY

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**ABSTRACT**

In mature neurons, low intracellular chloride level required for inhibition is maintained by the potassium-chloride co-transporter KCC2. Impairment of $Cl^-$ extrusion following KCC2 dysfunction has been involved in many CNS disorders such as seizures, neuropathic pain or spasticity after a spinal cord injury (SCI). This makes KCC2 an appealing drug target for restoring $Cl^-$homeostasis and inhibition in pathological conditions. In the present study, we screen the Prestwick Chemical Library® and identify conventional *antipsychotics phenothiazine derivatives as enhancers of KCC2 activity. Among them,* prochlorperazine hyperpolarizes the $Cl^-$ equilibrium potential in motoneurons of neonatal rats and restores the reciprocal inhibition after SCI. The compound alleviates spasticity in chronic adult SCI rats with an efficacy equivalent to the anti-spastic agent baclofen, and rescues the SCI-induced downregulation of KCC2 in motoneurons below the lesion. These preclinical data support prochlorperazine for a new therapeutic indication in the treatment of spasticity after SCI and neurological disorders involving a KCC2 dysfunction.

## INTRODUCTION

Glycine and GABA are the major inhibitory transmitters in the CNS. The strength, as well as the polarity, of $GABA_A$ and glycine receptor-mediated chloride synaptic inputs depends on the regulation of the intracellular concentration of chloride ions ($[Cl^-]_i$). [1,2]. A low $[Cl^-]_i$ is needed for inhibition to occur and is primarily maintained in healthy mature neurons by the potassium-chloride co-transporters KCC2 in the plasma membrane, which extrude $Cl^-$ [3,4]. In many neurological disorders associated with enhanced excitation, the global expression of KCC2 is downregulated thereby depolarizing the $Cl^-$ equilibrium potential and reducing the strength of postsynaptic inhibition [5-8,8-13].We have previously reported that the disinhibition of motoneurons linked to a dysfunction of KCC2 after spinal cord injury (SCI) predisposes to spasticity [14,15], a motor disorder clinically characterized by spasms, clonus and hyperreflexia [16-18]. Spasticity negatively influences quality of life, but none of the currently available drugs (baclofen, tizanidine, botulinum toxin A…) are uniformly useful in reducing all symptoms associated to spasticity [19]. Therefore, efforts which aim at finding chemical activators of KCC2 to restore inhibition by lowering $[Cl^-]_i$ might provide a new effective therapy for spasticity and, more broadly, for a wide range of neurological disorders.

A recent high-throughput screening assay identified CLP257 as a positive modulator of KCC2 able to restore impaired $Cl^-$ homeostasis in dorsal horn neurons derived from a rat model of neuropathic pain [20]. Likewise, activation of 5-$HT_{2A}$ receptors with TCB-2 repairs alterations of $Cl^-$ homeostasis in motoneurons of spastic rats following SCI [21]. Although promising, transfer of CLP257 or TCB-2 from research discovery to patients is a costly and arduous undertaking. The strategy of repurposing approved drugs for another indication reduces time and effort in bringing a new chemical entity from bench to bedside. To this end, we have screened molecules from the Prestwick Chemical Library® consisting of off-patent approved drugs to identify a new KCC2 enhancer in a bioavailable and safe formulation. We identified piperazine phenothiazine derivatives as pharmacologically active agents for activating KCC2. We focused on prochlorperazine and found that in addition to activate KCC2 function, it reduces spasticity in rats with SCI.

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## MATERIAL AND METHODS

### Animals.

Wistar rats were housed under a 12 h light/dark cycle in a temperature-controlled area with *ad libitum* access to water and food. All animal care and use conformed to the French regulations (Décret 2010-118) and were approved by the local ethics committee CEEA 71 - Comité d'éthique en neurosciences - INT Marseille (authorization Nb A9 01 13).

### Surgery.

The surgery and postoperative care were described previously. [18] Briefly, we anesthetized adult female Wistar Rats (225/250g Charles River, Burlington MA USA) with a mixture of ketamine (Imalgen®, Merial, Duluth, Georgia, USA, 50 mg/kg ip) and medetomidine (Domitor®, Janssen Pharmaceutica, Beerse, Belgium, 0.25 mg/kg ip) and neonates by hypothermia. Amoxicillin was administered after anesthesia (150 mg/kg, s.c.; Duphamox LA® ; Pfizer Inc, NY, USA). After laminectomy, the spinal cord was transected at T8 thoracic level (for neonates at the day of birth). In some adult rats, a catheter was inserted with the distal end in proximity to the lumbar enlargement. Sham-operated animals were subjected to all procedures except the spinal cord transection.

### Drugs

We purchased: perphenazine, prochlorperazine dimaleate (PCPZ), fluphenazine, trifluoperazine dihydrochloride, thioproperazine dimesylate, thiethylperazine from Prestwick Chemical® (Illkirch, France) or Tocris (Bristol, UK); ouabaine, bumetanide, acetophenazine, baclofen, DIOA [(dihydroindenyl)oxy]alkanoic acid] and NEM (N-Ethylmaleimide) from Sigma Aldrich (St Louis, MO, USA) ; perazine from Santa Cruz Biotechnology (Dallas, TX, USA); 2-amino-5-phosphonovaleric acid (AP5) and 6-cyano-7-nitroquin-oxaline-2,3-dione (CNQX) from Abcam (Cambridge, UK). AP5 and CNQX were

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prepared in aCSF, ouabaine in $H_2O$, and bumetanide, phenothiazines piperazine, baclofen and DIOA in DMSO.

**Drug administration and assessment of spasticity in adult spinal rats.**

We used the rate-dependent depression (RDD) of the H-reflex as a measurement of spastic symptoms. [15] A pair of stainless steel stimulating needle electrodes was inserted transcutaneously into the surroundings of the tibial nerve, a recording electrode into the flexor digitorum brevis muscle (FDB, flexor of the lateral four toes) and the reference electrode was placed s.c. into the foot. We stimulated the tibial nerve for 0.2 ms at 0.2 Hz with increasing current intensities and determined the intensity necessary to get a maximal H response. Then, we used this intensity for trains of 12 stimulations at 0.2, 1, 2 and 5 Hz. Normalization of the amplitude of responses (the first three discarded) to the controls evoked at 30-s intervals (mean of five pulses) determined the level of the RDD. Chronic spastic rats were treated with a single i.v. administration of PCPZ (10µg/kg in saline + 0.1% DMSO), baclofen (2mg/kg; in saline + 0.1% DMSO) or vehicle (saline + 0.1% DMSO). DIOA was i.t. delivered (40 µg in DPBS, calcium, magnesium + 10% DMSO). The RDD was evaluated twice before drug injection and at intervals of 20, 40, 60 and 80 min after the drug injection.

***In vitro* electrophysiological recordings.**

The spinal cord below T8 was isolated from neonatal rats [postnatal day (P)4–P6] as previously described (Vinay et al., 1999) and transferred to the recording chamber perfused with an oxygenated (95% $O_2$/5% $CO_2$) aCSF composed of the following (in mM): 130 NaCl, 4 KCl, 3.75 $CaCl_2$, 1.3 $MgSO_4$, 0.58 $NaH_2PO_4$, 25 $NaHCO_3$ and 10 glucose, pH 7.4 (24-26°C). Extracellular recording/stimulation were made from lumbar L3-L5 ventral (VR) and dorsal (DR) roots by contact stainless steel electrodes insulated with Vaseline. A glass suction electrode was also used to stimulate the ipsilateral ventral funiculus at the L2–L3 level. AC recordings from VR were amplified (×2,000) and band-pass filtered from 70 Hz to

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3 kHz. Current clamp recordings from L4–L5 motoneurons were performed in DCC mode with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA). Motoneurons were identified by an antidromic response to VR stimulation. Microelectrodes were filled with 2 M K-acetate (90–150 MΩ). Intracellular signals were sampled at 10 kHz (Digidata 1440a; Clampex 10 software; Molecular Devices).

To estimate the ionic gradient of $Cl^-$ efflux in motoneurons, we measured the reversal potential of the inhibitory postsynaptic potential ($E_{IPSP}$) evoked by ipsilateral ventral funiculus stimuli and pharmacologically isolated with CNQX (10 µM) and AP5 (50-100 µM). The amplitudes of IPSPs measured at various holding potentials (500-ms-long current pulses) were plotted to obtain $E_{IPSP}$ from the regression line.

The reciprocal inhibition was assessed by using a variation of the monosynaptic model technique [22]. We evoked monosynaptic reflexes in VR L5 by a supramaximal stimulation of the DR L5 (0.3 ms duration). When this stimulation was preceded by a stimulation of the DR L3 (2 x times the threshold (T) for the monosynaptic reflex) with a delay ranging from 0 to 40 ms, the amplitude of the L5 monosynaptic reflex was reduced, indicative of the reciprocal inhibition. Normalization of the amplitude of the L5 monosynaptic reflex recorded before and after the conditioning DR L3 stimulation were used to assess the reciprocal inhibition.

**Stable mammalian cell line expressing KCC2**

The HEK-293 cells stably expressing KCC2 were grown to confluence at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 supplemented with foetal bovine serum (10%), penicillin (50 U/m) and streptomycin (50 pg/ml; Life technologies, Carlsbad, CA, USA). Puromycin (20µg/ml, Life technologies) was added to select KCC2-expressing cells.

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**Thallium ($Tl^+$) influx assay**

The FluxOR™ Thallium detection kit from Life Technologie was used. Briefly, HEK-293 cells were loaded with the $Tl^+$-sensitive fluorescent dye FluxOR for 90 min. Once the unloaded dye was removed, cells were supplemented with assay buffer containing ouabain (200 µM) and bumetanide (10 µM). Cells were then plated (100.000 cells/75 µl/well) in clear-bottomed, black-walled 96-well plates (Greiner Bio-One, Monroe, NC, USA). Compounds to be assayed from the Prestwick library collection were added (5 µl/well) by using the Biomek® NX Laboratory automation workstation (Beckman Coulter, Villepinte, France) and incubated for 15 min before measuring an initial baseline fluorescence (490 nm excitation and 520 nm emission) onto the screening system POLARstar omega (BMG Labtech, Ortenberg, Allemagne). The fluorescence signal was initiated by adding 20 µl of a 5×$Tl_2SO_4$ solution (final concentration of 2 mM $Tl_2SO_4$) and recorded 30 minutes after. The test was conducted for each plate using 2 columns on which cells on half were treated with 33 µM NEM, an accepted activator of KCC2, [23] as positive control and the other half with no compounds as negative control. After subtracting each well's fluorescence obtained after compound addition (baseline fluorescence), activity was expressed as percentage change of $Tl^+$ influx in compound-treated cells versus influx in vehicle-treated cells. Hits were selected as compounds that cause an increase >20% without apparent effect on parental untransfected HEK cells. Hits were retested in triplicate. Positive compounds were further evaluated by testing them at varying concentrations. To evaluate the quality of the $Tl^+$ flux assay, the value of Z' factor was calculated using the following equation: Z'=1-[3 SD of sample + 3 SD of control]/[mean of sample-mean of control]. At the end of the fluorescent assay, cell viability was checked by the PrestoBlue® Cell Viability Reagent (Life technologies) protocol. Briefly, cells viability in 96-well plates was expressed as the 570-nm absorbance ratio of drug-treated cells compared with cells incubated in the absence of drug (medium). The result was expressed as percentage of the control (defined as 100%). The initial screen of 880 compounds was performed at 2µg/ml (3 to 10µM), 12µg/ml (18 to 60µm) and 20µg/ml (30 to 100µM).

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**Immunostaining KCC2**

Adult rats were perfused transcardially with 4% paraformaldehyde (PFA, Sigma Aldrich) in PBS (bioMérieux SA, Marcy l'Etoile, France) at 4°C. Tissues were post-fixed overnight (16 hours) in 4% PFA and washed 3 times with PBS. Lumbar spinal cords (L4-L5) were then embedded in 4% low melting agarose and sectioned (30 µm) with a vibratome. Sections from all samples were mounted on the same poly-lysine coated slides and processed for immunohistochemistry. Slices were thus (i) dried overnight, (ii) permeated (45 min) in PBS with 3% Bovin Serum Albumin (BSA, Sigma Aldrich) and 0.2% Triton x-100 (iii) washed in PBS (2 x 5 min), (iv) preincubated (1 h) in PBS with normal goat serum (3% BSA, 1/100 normal Goat serum), (v) washed and incubated overnight at room temperature in the affinity-purified rabbit anti-KCC2 polyclonal antibody (diluted 1:500; Merck-Millipore Billerica, Massachusetts, USA; Cat# 07-432, RRID:AB_310611), (vi) washed in PBS (3 x 5 min) (vii) incubated (1 h) with a goat anti-rabbit IgG conjugated to Alexa-546 (diluted 1:500; Life Technologies), (viii) washed in PBS 3x5 min, and finally (ix) coverslipped with a gelatinous aqueous medium. In control experiments, the primary antiserum was replaced with rabbit immunoglobulin fraction during the staining protocol. Images were taken using the confocal microscope Zeiss, LSM500 at X60 magnification, digitized in stacks of 0.5 µm-thick optical sections and processed with the Zen (Zeiss) software.

We quantified KCC2 immunolabeling of motoneuron (identified as the biggest cells in the ventral horn). Line scans show peaks of intensity at the periphery of the cell body (Fig. 3D), likely plasma membrane. We drew a region of interest within the presumed plasma membrane and measured the mean pixel intensities in this delimited area [15,18,21]. Values from Sham, SCI and PCPZ treated-rats were then normalized to the mean value measured from sections of SCI rats on the same slide.

**Statistical analysis**

Group measurements were expressed as means ± s.e.m. We used Mann-Whitney tests, Kruskal-Wallis tests and one- or two-way ANOVAs with or without repeated

measures as appropriate (Graphpad Prism 5 software). For all statistical analyses, the data met the assumptions of the test and the variance between the statistically compared groups was similar. The significance was set at $P < 0.05$.



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## RESULTS

### Phenothiazine piperazine derivatives upregulate KCC2 function

To identify KCC2 activators from the Prestwick Chemical Library®, we used a standard thallium ($Tl^+$) flux assay reported as suitable for high throughput screening in HEK-293 cell line expressing KCC2 (Delpire et al., 2009;Zhang et al., 2010; Medina et al., 2013). In principle, upon the activation of KCC2 in reverse mode, the uptake of $Tl^+$ into cells releases a fluorescent signal from the pre-loaded FluxOR™ dye which reports the activity of KCC2. The initial screen of 880 compounds performed at the concentration of 12 µg/ml (18 to 60 µM), yielded 121 hits with more than 20% increase in the fluorescent signal (**Fig.1a**). The Z factor with an average of 0.83 ± 0.004 (range 0.75 to 0.92) conformed to the assay quality requirement (**Fig. 1b**). We then selected 27 compounds among these 121 hits and that were ineffective on naïve HEK293-cells. A secondary screen tested each compound in triplicate and confirmed the activity of approximately 60% of them (17/27). A classification of positive hits revealed 4 molecules (perphenazine, prochlorperazine dimaleate, trifluoperazine dihydrochloride and thioproperazine dimesylate) that belong to antipsychotic piperazine phenothiazine derivatives characterized by a three-ring structure and a piperazine substituent on nitrogen (**Fig. 1c**). Based on their commercial availability, 8 piperazine phenothiazines were purchased for further evaluation (chemical structure of each compound is illustrated in **Fig. 1c**). To determine their potency, dose-response curves (range: 0.1 to 50 µM; example for prochlorperazine in **Fig. 1d**) were performed in parallel to a counter screen to evaluate their cytotoxicity. All compounds were effective with the minimal effective concentration ranging from 3 to 12 µM (see **Fig. 1c**). Prochlorperazine, thiethylperazine, fluphenazine and perazine were the most potent activators (~140% of basal activity). Among these molecules, prochlorperazine dimaleate (termed henceforth as PCPZ) exhibited the most potent effect with the lowest minimal effective concentration (3 µM) without apparent cytotoxic effect at concentration below 200 µM (see **Fig. 1c**). Therefore, from a drug repurposing point of view, PCPZ was selected for further experimental investigations of the molecule.

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**PCPZ enhances $Cl^-$ extrusion capacity in motoneurons**

By means of intracellular recordings, we investigated whether PCPZ could enhance $Cl^-$ extrusion capacity of lumbar motoneurons recorded in *in vitro isolated whole spinal cord preparation* from intact neonatal rats [postnatal day (P)4–P6]. The effect of PCPZ was examined by determining the reversal potential of inhibitory post-synaptic potentials ($E_{IPSP}$) as a measure of the KCC2 function [24]. We found that PCPZ (10 µM) hyperpolarized $E_{IPSP}$ within 20–25 min by -3.3 ± 0.66 mV (**Fig. 2a**; n=6; *p<0.05 Wilcoxon test). Given that there was no change of the resting membrane potential ($V_{rest}$; n=6; **Fig. 2a***;* p>0.05, Wilcoxon test), the net driving force for $Cl^-$ ($E_{IPSP}$-$V_{rest}$) increased significantly (n=6; **Fig. 2a***;* *p<0.05, Wilcoxon test), potentially strengthening the inhibitory synaptic transmission.

**PCPZ strengthens inhibition and decreases spasticity in rats with SCI.**

To test the ability of PCPZ to enhance the post-synaptic inhibition, we took advantage of the recent *in vitro* demonstration that the reciprocal inhibition in the spinal cord is markedly reduced after SCI [25]. Typically, flexor-related Ia interneurons [recruited by stimulation of the dorsal root (DR) L3] inhibits L5 extensor-related motoneurons [26] such as when the DR L5 stimulation was preceded by DR L3 stimulation, there was a reduction of the DR L5-evoked monosynaptic response (**Fig. 2b**). This reduction almost disappeared in spinal cord isolated from neonatal rats that underwent SCI at birth (**Fig. 2b**). In those rats, the bath application of PCPZ at a concentration as low as 10 µM significantly reduced the amplitude of the L5 monosynaptic response to values seen in intact animals (**Fig. 2b**, n=6; ###, p < 0.001; two-way repeated measures ANOVA). Altogether, these results indicate that PCPZ is able to restore the reciprocal inhibition after neonatal SCI.

The Hoffmann reflex (H-reflex), resulting from the monosynaptic activation of motoneurons by Ia afferents, declines in amplitude over repetitive stimulation and becomes more depressed as the frequency of stimulation increases [27,28]. The lower rate-dependent depression (RDD) of the H-reflex in spastic *patients* and *animals* identifies RDD as a reliable assessment of spasticity [15,29,30]. As the low RDD is indicative of a spinal disinhibition and a KCC2 dysfunction (Boulenguez et al., 2010), we tested whether the

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hyperpolarizing shift of $E_{IPSP}$ in motoneurons by PCPZ would restore the RDD in adult rats with chronic SCI (21 d post-SCI). The i.v administration of PCPZ (10 µg/kg) or vehicle (DMSO) did not affect the maximal amplitude of H-reflex and M-wave (**Table 1**) but significantly increased the RDD to values seen in SCI animals (**Fig. 3a**). At frequencies of 1 Hz and higher, the amplitude of the H wave over repetitive stimulation was smaller than after vehicle injection, with a maximal effect 80 min after PCPZ administration (*n* = 9 vehicle, n=8 PCPZ; **Fig. 3a**; *2-way ANOVA test with bonferroni post-test; ns p > 0,5; *p < 0.5; ** p < 0.01; ***p < 0.001*). To identify the mechanisms underlying this effect, we i.t. administered DIOA (40 µg), a highly specific KCC2 blocker [31] 50 minutes after PCPZ injection. KCC2 blockade reversed PCPZ effect (**Fig. 3b**, n=6 in each group), which is consistent with the hypothesis of KCC2 mediating the $E_{IPSP}$ shift induced by PCPZ. Taken together these results indicate that PCPZ reduces spasticity essentially through a modulation of KCC2.

**PCPZ increases the expression of KCC2 in motoneurons below the SCI.**

We used immunohistochemistry to quantify the effect of PCPZ on the expression of KCC2 on lumbar motoneurons after SCI. Peaks of KCC2 labeling were detected in the periphery of cell bodies of sham, SCI and PCPZ treated rats showing a membrane labeling (**Fig. 3c**). Levels of KCC2 labeling surrounding motoneurons were higher in PCPZ-treated compared to vehicle-treated animals (10 µg/kg; i.v. 80 min), but did not return to the level found in sham-operated rats (**Fig. 3c**, Sham vs SCI + prochlorperazine * p < 0,05; SCI vs SCI + prochlorperazine *** p < 0.001; Kruskal–Wallis test, Dunn's post-tests; n = 231 motoneurons in each group).

**PCPZ is as effective in reducing spasticity as baclofen.**

From a translational perspective, the therapeutic profile of a new antispastic drug cannot be defined solely on the basis a comparison to vehicle-treated rats. We thereby assessed PCPZ potential advantages with respect to existing antispastic drug. The $GABA_B$ receptor agonist, baclofen, is the most effective and widely used drug for the treatment of

spasticity after SCI [32-37]. We compared the relative efficacy of PCPZ to that of the "gold standard" baclofen (2mg/kg, i.v) in restoring the RDD. The reduction of the RDD was more pronounced in PCPZ-treated animals at 1 Hz and remained comparable to that of baclofen-treated rats at higher frequencies of stimulation (**Fig. 3d**, 2-way ANOVA test with bonferroni post test; ns $p > 0,5$; *$p < 0.5$, $n = 6$ in each group). These results suggest that antispastic effects of PCPZ is at least similar to that of baclofen.



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## DISCUSSION

We identified PCPZ as a positive modulator of KCC2 capable to restore endogenous inhibition of motoneurons after SCI. PCPZ leads to a hyperpolarizing shift of $E_{IPSP}$ and reduces SCI-induced spasticity. The observed effect of PCPZ on spasticity likely results from an enhancement of KCC2 function. In line with this assumption, PCPZ increases cell surface expression of KCC2 in motoneurons after SCI and blocking KCC2 prevents the effect of PCPZ to alleviate spasticity. Likewise, the reciprocal inhibition, downregulated after SCI and determined by the level of KCC2 function, [25] is restored by PCPZ.

Deficits in KCC2 function have been documented in brain and spinal cord injuries, [15,38] in temporal lobe epilepsy [39,40] and in neuropathic pain. [41-44] Accordingly, KCC2 appears to be an attractive target to restore endogenous inhibition in pathological conditions. Previous investigations showed KCC2 as a druggable target in modulating $[Cl^-]_i$ for the development of therapeutics. The 5-$HT_{2A}$ high-affinity agonist, TCB-2, reduces spasticity after SCI by boosting the KCC2 function [21] while CLP257, a new KCC2 enhancer, alleviates hypersensitivity in a rat model of neuropathic pain. [20] Unfortunately, these two compounds are not approved human drugs. Conversely, PCPZ is currently licensed for its neuroleptic actions with an adult daily dosage ranging from 15 to 150 mg.[45] Typical antipsychotic drugs such as PCPZ may produce extrapyramidal side-effects at clinically effective doses against schizophrenia. [45] However, the antispastic effect of PCPZ, equivalent to that of baclofen, was achievable with a low human equivalent dose estimated at 1.6µg/kg for a 70 kg human [HED = Animal Dose (mg/kg) x (animal wt/human wt in kg) $^{0.33}$; see Nair and Jacob 2016)]. The phenothiazine ring possesses a high degree of lipophilicity and these drugs easily cross the blood-brain barrier [46-48]. Chlorine substitution of C-2 atom of prochlorperazine confers less anti-psychotic activity than other substituents (X = -SO2NR2 > -CF3 > -CO-CH3 > -Cl). [48] Collectively, the use of PCPZ at lower doses represents an effective and tolerable therapeutic strategy for reducing spasticity, likely better than the common *anti-spastic agent* baclofen which often causes sedation and dizziness. [35,36]

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The mechanism by which PCPZ modulates KCC2 function in motoneurons has not been investigated in the present study. The therapeutic potential of PCPZ in reducing psychosis has been mainly attributed to its anti-dopaminergic action. [49,50] However, the main source of dopamine in the mammalian spinal cord arises from the diencephalon and disappears after SCI. [51,52] Furthermore, dopamine appears to be anti-spastic by restoring the RDD in chronic SCI rats, [53] thereby suggesting that inhibition of dopaminergic receptors unlikely accounts for the upregulation of KCC2. Antipsychotic drugs have been also found to display anti-serotoninergic actions most notably through an inverse agonist activity of 5-$HT_{2C}$ receptors. [54]. Interestingly, 5-$HT_{2C}$ receptors in motoneurons become constitutively active after SCI and contribute to muscle spasms. [55] We recently showed that blocking 5-$HT_{2B}$Rs and 5-$HT_{2C}$Rs hyperpolarized $E_{IPSP}$ in rats with SCI. [21] However, PCPZ is one of the typical antipsychotic devoided of inverse agonist activity [54] and thus unlikely modify KCC2 function via 5-$HT_{2C}$ receptor-mediated mechanisms.

PCPZ may also interact indirectly via some intermediate signaling molecules, as suggested by the potent inhibitory action of phenothiazines on $Ca^{2+}$-dependent PKC [56]. The phosphorylation of KCC2 by PKC constitutes a powerful and dynamic mechanism to influence KCC2 function. [57] Following SCI, a tonic activation of a $Ca^{2+}$-dependent PKC signaling pathway alters motoneuronal $Cl^-$ homeostasis by depolarizing shift $E_{IPSP.}$ [21] A similar relationship between $Ca^{2+}$-dependent PKC isozymes and the positive shift of $E_{Cl}$ has been pointed out in hippocampal neurons. [58] Therefore, the potency of PCPZ in restoring KCC2 function may be connected with its inhibitory action on $Ca^{2+}$-dependent PKC. [56]

In sum, we provide strong preclinical evidence for translation to chronic SCI subjects, a process that will be facilitated as PCPZ is already an approved compound and has been recently patented for treating spasticity (WO 2015135947 A1). [59]

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**Author Disclosure Statement**

No competing financial interests exist.

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References

1. Doyon, N., Vinay, L., Prescott, S.A., and De, K.Y. (2016). Chloride Regulation: A Dynamic Equilibrium Crucial for Synaptic Inhibition. Neuron 89, 1157-1172.

2. Ben-Ari, Y., Khalilov, I., Kahle, K.T., and Cherubini, E. (2012). The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist. 18, 467-486.

3. Payne, J.A., Rivera, C., Voipio, J., and Kaila, K. (2003). Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. 26, 199-206.

4. Vinay, L. and Jean-Xavier, C. (2008). Plasticity of spinal cord locomotor networks and contribution of cation-chloride cotransporters. Brain Res. Rev. 57, 103-110.

5. Coull, J.A., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A., De, K.P., and De, K.Y. (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424, 938-942.

6. Price, T.J., Cervero, F., and De, K.Y. (2005). Role of cation-chloride-cotransporters (CCC) in pain and hyperalgesia. Curr. Top. Med. Chem. 5, 547-555.

7. Huberfeld, G., Wittner, L., Clemenceau, S., Baulac, M., Kaila, K., Miles, R., and Rivera, C. (2007). Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. J. Neurosci. 27, 9866-9873.

8. Cramer, S.W., Baggott, C., Cain, J., Tilghman, J., Allcock, B., Miranpuri, G., Rajpal, S., Sun, D., and Resnick, D. (2008). The role of cation-dependent chloride transporters in neuropathic pain following spinal cord injury. Mol. Pain 4, 36.

9. Hewitt, S.A., Wamsteeker, J.I., Kurz, E.U., and Bains, J.S. (2009). Altered chloride homeostasis removes synaptic inhibitory constraint of the stress axis. Nat. Neurosci. 12, 438-443.

Journal of Neurotrauma
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10. Tao, R., Li, C., Newburn, E.N., Ye, T., Lipska, B.K., Herman, M.M., Weinberger, D.R., Kleinman, J.E., and Hyde, T.M. (2012). Transcript-specific associations of SLC12A5 (KCC2) in human prefrontal cortex with development, schizophrenia, and affective disorders. J. Neurosci. 32, 5216-5222.

11. He, Y., Xu, S., Huang, J., and Gong, Q. (2014). Analgesic effect of intrathecal bumetanide is accompanied by changes in spinal sodium-potassium-chloride co-transporter 1 and potassium-chloride co-transporter 2 expression in a rat model of incisional pain. Neural Regen. Res. 9, 1055-1062.

12. Hubner, C.A. (2014). The KCl-cotransporter KCC2 linked to epilepsy. EMBO Rep. 15, 732-733.

13. Tyzio, R., Nardou, R., Ferrari, D.C., Tsintsadze, T., Shahrokhi, A., Eftekhari, S., Khalilov, I., Tsintsadze, V., Brouchoud, C., Chazal, G., Lemonnier, E., Lozovaya, N., Burnashev, N., and Ben-Ari, Y. (2014). Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. Science 343, 675-679.

14. Boulenguez, P. and Vinay, L. (2009). Strategies to restore motor functions after spinal cord injury. Curr. Opin. Neurobiol. 19, 587-600.

15. Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., Stil, A., Darbon, P., Cattaert, D., Delpire, E., Marsala, M., and Vinay, L. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. Nat. Med. 16, 302-307.

16. Nielsen, J.B., Crone, C., and Hultborn, H. (2007). The spinal pathophysiology of spasticity--from a basic science point of view. Acta Physiol (Oxf) 189, 171-180.

17. Nair, K.P. and Marsden, J. (2014). The management of spasticity in adults. BMJ 349, g4737.

18. Brocard, C., Plantier, V., Boulenguez, P., Liabeuf, S., Bouhadfane, M., Viallat-Lieutaud, A., Vinay, L., and Brocard, F. (2016). Cleavage of Na(+) channels by calpain increases

persistent Na(+) current and promotes spasticity after spinal cord injury. Nat. Med. 22, 404-411.

19. Graham, L.A. (2013). Management of spasticity revisited. Age Ageing 42, 435-441.

20. Gagnon, M., Bergeron, M.J., Lavertu, G., Castonguay, A., Tripathy, S., Bonin, R.P., Perez-Sanchez, J., Boudreau, D., Wang, B., Dumas, L., Valade, I., Bachand, K., Jacob-Wagner, M., Tardif, C., Kianicka, I., Isenring, P., Attardo, G., Coull, J.A., and De, K.Y. (2013). Chloride extrusion enhancers as novel therapeutics for neurological diseases. Nat. Med. 19, 1524-1528.

21. Bos, R., Sadlaoud, K., Boulenguez, P., Buttigieg, D., Liabeuf, S., Brocard, C., Haase, G., Bras, H., and Vinay, L. (2013). Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. Proc. Natl. Acad. Sci. U. S. A 110, 348-353.

22. Wang, Z., Li, L., Goulding, M., and Frank, E. (2008). Early postnatal development of reciprocal Ia inhibition in the murine spinal cord. J. Neurophysiol. 100, 185-196.

23. Payne, J.A. (1997). Functional characterization of the neuronal-specific K-Cl cotransporter: implications for [K+]o regulation. Am. J. Physiol 273, C1516-C1525.

24. Jean-Xavier, C., Pflieger, J.F., Liabeuf, S., and Vinay, L. (2006). Inhibitory postsynaptic potentials in lumbar motoneurons remain depolarizing after neonatal spinal cord transection in the rat. J. Neurophysiol. 96, 2274-2281.

25. Gackiere, F. and Vinay, L. (2015). Contribution of the potassium-chloride cotransporter KCC2 to the strength of inhibition in the neonatal rodent spinal cord in vitro. J. Neurosci. 35, 5307-5316.

26. Talpalar, A.E., Endo, T., Low, P., Borgius, L., Hagglund, M., Dougherty, K.J., Ryge, J., Hnasko, T.S., and Kiehn, O. (2011). Identification of minimal neuronal networks involved in flexor-extensor alternation in the mammalian spinal cord. Neuron 71, 1071-1084.

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27. Ishikawa, K., Ott, K., Porter, R.W., and Stuart, D. (1966). Low frequency depression of the H wave in normal and spinal man. Exp. Neurol. 15, 140-156.

28. Kiser, T.S., Reese, N.B., Maresh, T., Hearn, S., Yates, C., Skinner, R.D., Pait, T.G., and Garcia-Rill, E. (2005). Use of a motorized bicycle exercise trainer to normalize frequency-dependent habituation of the H-reflex in spinal cord injury. J. Spinal Cord. Med. 28, 241-245.

29. Thompson, F.J., Reier, P.J., Lucas, C.C., and Parmer, R. (1992). Altered patterns of reflex excitability subsequent to contusion injury of the rat spinal cord. J. Neurophysiol. 68, 1473-1486.

30. Grey, M.J., Klinge, K., Crone, C., Lorentzen, J., Biering-Sorensen, F., Ravnborg, M., and Nielsen, J.B. (2008). Post-activation depression of soleus stretch reflexes in healthy and spastic humans. Exp. Brain Res. 185, 189-197.

31. Delpire, E., Days, E., Lewis, L.M., Mi, D., Kim, K., Lindsley, C.W., and Weaver, C.D. (2009). Small-molecule screen identifies inhibitors of the neuronal K-Cl cotransporter KCC2. Proc. Natl. Acad. Sci. U. S. A 106, 5383-5388.

32. Burns, A.S. and Meythaler, J.M. (2001). Intrathecal baclofen in tetraplegia of spinal origin: efficacy for upper extremity hypertonia. Spinal Cord. 39, 413-419.

33. Soni, B.M., Mani, R.M., Oo, T., and Vaidyanathan, S. (2003). Treatment of spasticity in a spinal cord-injured patient with intrathecal morphine due to intrathecal baclofen tolerance--a case report and review of literature. Spinal Cord. 41, 586-589.

34. Plassat, R., Perrouin, V.B., Menei, P., Menegalli, D., Mathe, J.F., and Richard, I. (2004). Treatment of spasticity with intrathecal Baclofen administration: long-term follow-up, review of 40 patients. Spinal Cord. 42, 686-693.

35. Dario, A. and Tomei, G. (2004). A benefit-risk assessment of baclofen in severe spinal spasticity. Drug Saf 27, 799-818.

Journal of Neurotrauma
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36. Dario, A., Calandrella, D., Bono, G., Scamoni, C., Picano, M., and Tomei, G. (2004). Chronic Intrathecal Baclofen Infusion for Spasticity: Relationship between Pump and Host. Neuromodulation. 7, 201-204.

37. Hornby, T.G., Kahn, J.H., Wu, M., and Schmit, B.D. (2006). Temporal facilitation of spastic stretch reflexes following human spinal cord injury. J. Physiol 571, 593-604.

38. Jin, X., Huguenard, J.R., and Prince, D.A. (2005). Impaired Cl- extrusion in layer V pyramidal neurons of chronically injured epileptogenic neocortex. J. Neurophysiol. 93, 2117-2126.

39. Miles, R., Blaesse, P., Huberfeld, G., Wittner, L., and Kaila, K. (2012). Chloride homeostasis and GABA signaling in temporal lobe epilepsy.

40. Loscher, W., Puskarjov, M., and Kaila, K. (2013). Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. Neuropharmacology 69, 62-74.

41. Price, T.J., Cervero, F., Gold, M.S., Hammond, D.L., and Prescott, S.A. (2009). Chloride regulation in the pain pathway. Brain Res. Rev. 60, 149-170.

42. Doyon, N., Ferrini, F., Gagnon, M., and De, K.Y. (2013). Treating pathological pain: is KCC2 the key to the gate? Expert. Rev. Neurother. 13, 469-471.

43. Janssen, S.P., Truin, M., Van, K.M., and Joosten, E.A. (2011). Differential GABAergic disinhibition during the development of painful peripheral neuropathy. Neuroscience 184, 183-194.

44. Modol, L., Cobianchi, S., and Navarro, X. (2014). Prevention of NKCC1 phosphorylation avoids downregulation of KCC2 in central sensory pathways and reduces neuropathic pain after peripheral nerve injury. Pain 155, 1577-1590.

45. Jack DeRuiter (2001). Dopamine antagonists: Phenothiazine/Thioxanthene sar. Principles of Drug Action 2, Fall

46. Seelig, A., Gottschlich, R., and Devant, R.M. (1994). A method to determine the ability of drugs to diffuse through the blood-brain barrier. Proc. Natl. Acad. Sci. U. S. A 91, 68-72.

47. Gerebtzoff, G., Li-Blatter, X., Fischer, H., Frentzel, A., and Seelig, A. (2004). Halogenation of drugs enhances membrane binding and permeation. Chembiochem. 5, 676-684.

48. Jaszczyszyn, A., Gasiorowski, K., Swiatek, P., Malinka, W., Cieslik-Boczula, K., Petrus, J., and Czarnik-Matusewicz, B. (2012). Chemical structure of phenothiazines and their biological activity. Pharmacol. Rep. 64, 16-23.

49. Smith, H.S., Cox, L.R., and Smith, B.R. (2012). Dopamine receptor antagonists. Ann. Palliat. Med. 1, 137-142.

50. Tsuchihashi, H., Sasaki, T., Kojima, S., and Nagatomo, T. (1992). Binding of [3H]haloperidol to dopamine D2 receptors in the rat striatum. J. Pharm. Pharmacol. 44, 911-914.

51. Skagerberg, G., Bjorklund, A., Lindvall, O., and Schmidt, R.H. (1982). Origin and termination of the diencephalo-spinal dopamine system in the rat. Brain Res. Bull. 9, 237-244.

52. Sharples, S.A., Koblinger, K., Humphreys, J.M., and Whelan, P.J. (2014). Dopamine: a parallel pathway for the modulation of spinal locomotor networks. Front Neural Circuits. 8, 55.

53. Liu, H., Skinner, R.D., Arfaj, A., Yates, C., Reese, N.B., Williams, K., and Garcia-Rill, E. (2010). L-Dopa effect on frequency-dependent depression of the H-reflex in adult rats with complete spinal cord transection. Brain Res. Bull. 83, 262-265.

54. Herrick-Davis, K., Grinde, E., and Teitler, M. (2000). Inverse agonist activity of atypical antipsychotic drugs at human 5-hydroxytryptamine2C receptors. J. Pharmacol. Exp. Ther. 295, 226-232.

55. Murray, K.C., Nakae, A., Stephens, M.J., Rank, M., D'Amico, J., Harvey, P.J., Li, X., Harris, R.L., Ballou, E.W., Anelli, R., Heckman, C.J., Mashimo, T., Vavrek, R., Sanelli, L., Gorassini, M.A., Bennett, D.J., and Fouad, K. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat. Med. 16, 694-700.

56. Aftab, D.T., Ballas, L.M., Loomis, C.R., and Hait, W.N. (1991). Structure-activity relationships of phenothiazines and related drugs for inhibition of protein kinase C. Mol. Pharmacol. 40, 798-805.

57. Kahle, K.T., Deeb, T.Z., Puskarjov, M., Silayeva, L., Liang, B., Kaila, K., and Moss, S.J. (2013). Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. Trends Neurosci. 36, 726-737.

58. Fiumelli, H., Cancedda, L., and Poo, M.M. (2005). Modulation of GABAergic transmission by activity via postsynaptic Ca2+-dependent regulation of KCC2 function. Neuron 48, 773-786.

59. Boulenguez, P., Liabeuf, S., Gourmand, L., Viallat-Lieutaud.A., and Vinay.L. (2015). Piperazine Phenothiazine derivatives for treating spasticity Patent. WO 2015135947 A1



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**TABLE 1**

Maximal amplitudes of M and H waves (mV)

| | | M-Max | | | | | | H-max | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Before inject.** | **T0** | **T20** | **T40** | **T60** | **T80** | **Before inject.** | **T0** | **T20** | **T40** | **T60** | **T80** |
| **PCPZ** | Mean | 5.5 | 5.93 | 5.50 | 5.44 | 5.38 | 5.33 | 1.28 | 1.49 | 1.38 | 1.36 | 1.31 | 1.54 |
| | SEM | 0.37 | 0.44 | 0.43 | 0.43 | 0.39 | 0.41 | 0.13 | 0.11 | 0.11 | 0.12 | 0.08 | 0.14 |
| **DMSO** | Mean | 6.48 | 6.60 | 6.06 | 5.89 | 6.30 | 6.22 | 1.81 | 1.74 | 1.49 | 1.61 | 1.74 | 1.55 |
| | SEM | 0.73 | 0.55 | 0.63 | 0.58 | 0.49 | 0.50 | 0,31 | 0.20 | 0.22 | 0.29 | 0.32 | 0.21 |
| **PCPZ + DIOA** | Mean | 8.79 | 8.20 | 8.62 | 8.46 | 8.22 | 7.98 | 2.70 | 2.61 | 2.51 | 2.40 | 2.39 | 2.43 |
| | SEM | 1.63 | 1.43 | 1.21 | 1.26 | 1.47 | 1.48 | 0.33 | 0.26 | 0.23 | 0.25 | 0.33 | 0.30 |
| **PCPZ** | Mean | 5,76 | 5.46 | 4.91 | 5.03 | 4.95 | 4.63 | 1.52 | 1.50 | 1.58 | 1.40 | 1.38 | 1.35 |
| | SEM | 0.68 | 0.58 | 0.47 | 0.60 | 0.61 | 0.81 | 0.16 | 0.17 | 0.27 | 0.17 | 0.16 | 0.17 |
| **Baclofen** | Mean | 6,10 | 5.84 | 5.46 | 5.15 | 5.28 | 5.20 | 1,80 | 1.67 | 1.37 | 1.26 | 1.213 | 1.29 |
| | SEM | 0,73 | 0.55 | 0.80 | 0.53 | 0.64 | 0.60 | 0.30 | 0.21 | 0.26 | 0.13 | 0.11 | 0.11 |

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**FIGURE LEGENDS**

Fig.1

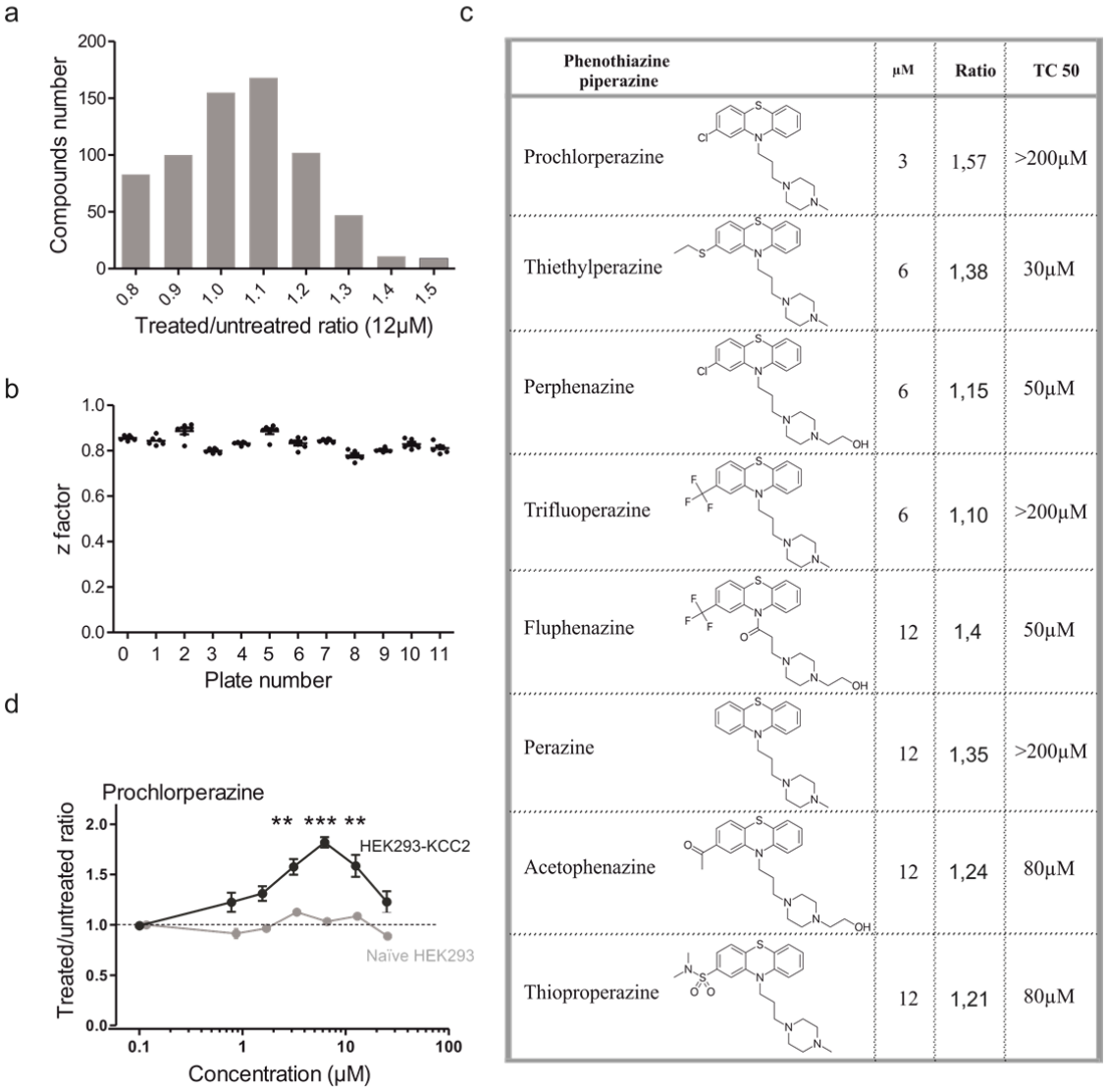


Figure 1: Prestwick library screening on KCC2 using the thallium flux assay in HEK-293 cells. (a): Distribution of identified hits in a total of 880 compounds. The x-axis represents the potentiation activity of the hits on the KCC2-mediated fluorescence signal. Molecules with a KCC2 activity below 80% of the control level are not shown. (b): Z factors of the thallium

flux assay. (c): Dose-response curve of prochlorperazine on KCC2 function, in KCC2 expressing (black) and in naïve (grey) HEK293 cells. K+ uptakes were measured in triplicate at different concentrations of drug ranging from 0.1 to 25 µM. Flux was expressed as a percentage of control (flux without drug). (d): Chemical structures of selected compounds with their minimal effective concentration (µM) on the activity of KCC2 (expressed in % of control) and their toxic concentration for half of the sample population (TC50).



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Fig.2

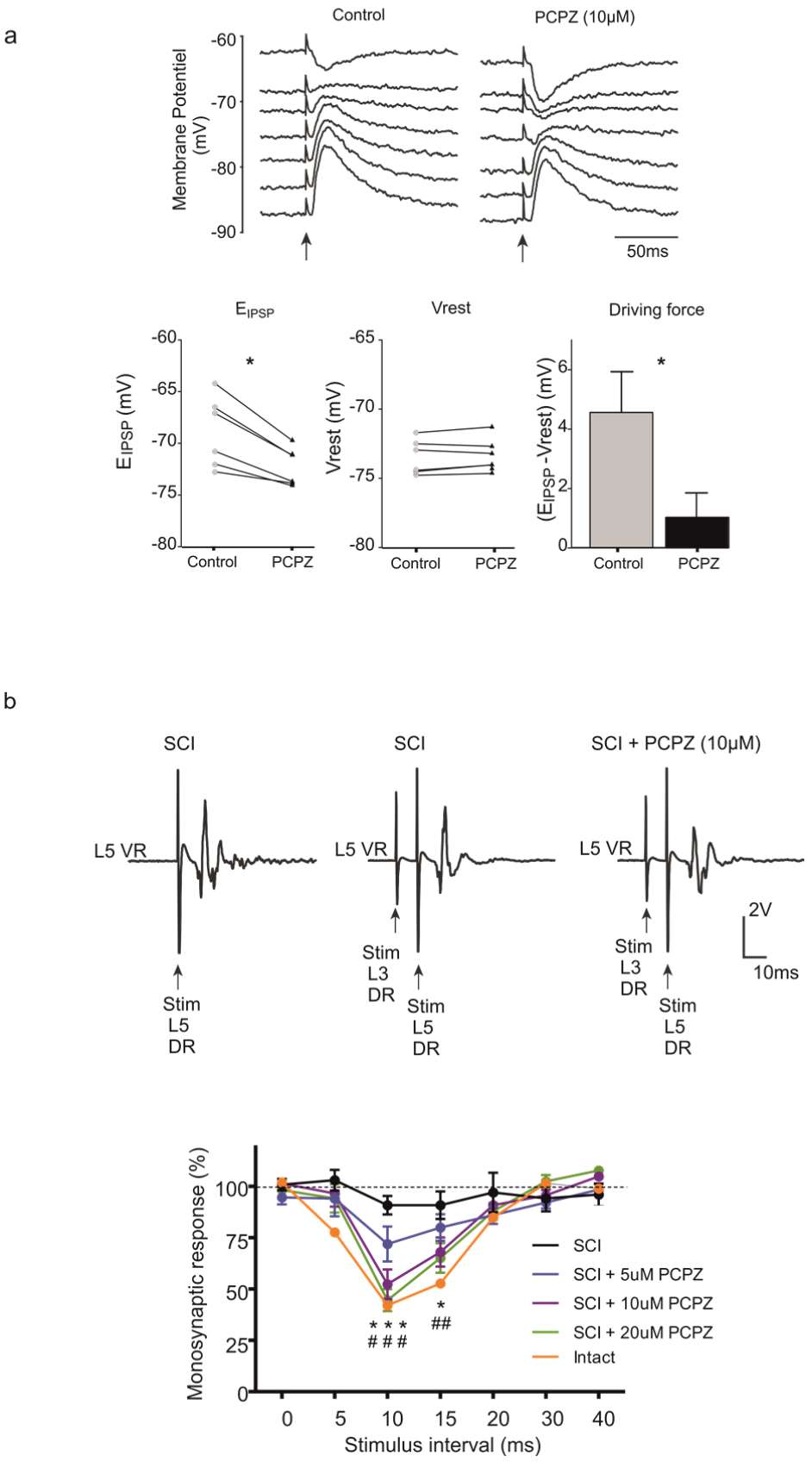

Figure 2: Prochlorperazine hyperpolarizes EIPSP and increases the strength reciprocal inhibition. (a) Top: Inhibitory post-synaptic potentials (IPSPs) evoked by stimulation of the ventral funiculus of the spinal cord (arrow) at different holding potentials in a motoneuron from a P6 intact rat before and after adding PCPZ (10 μM). Bottom: EIPSP, Vrest and driving force (EIPSP-Vrest) measured in six motoneurons before and after PCPZ. Measurements were done 20-25 minutes after adding PCPZ. *P < 0.05 (Wilcoxon paired test). (b) Top: Representative traces of extracellular recordings from a L5 ventral root (VR) of a P6 rat with SCI. Traces show the responses to the L5 dorsal root (DR) stimulation without (left) or with a conditioning stimulation of the L3 DR before (middle) or after (right) adding PCPZ (10 μM). Bottom: Amplitude of the response plotted against the interstimulus interval. Statistical differences between SCI and PCPZ conditions are noted above delays (Wilcoxon test;

* p<0.05, 10uM vs. SCI; *** p<0.001, 10uM vs. SCI; ## p<0.05, 20uM vs. SCI; # # # p<0.001, 20uM vs. SCI).



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Fig.3

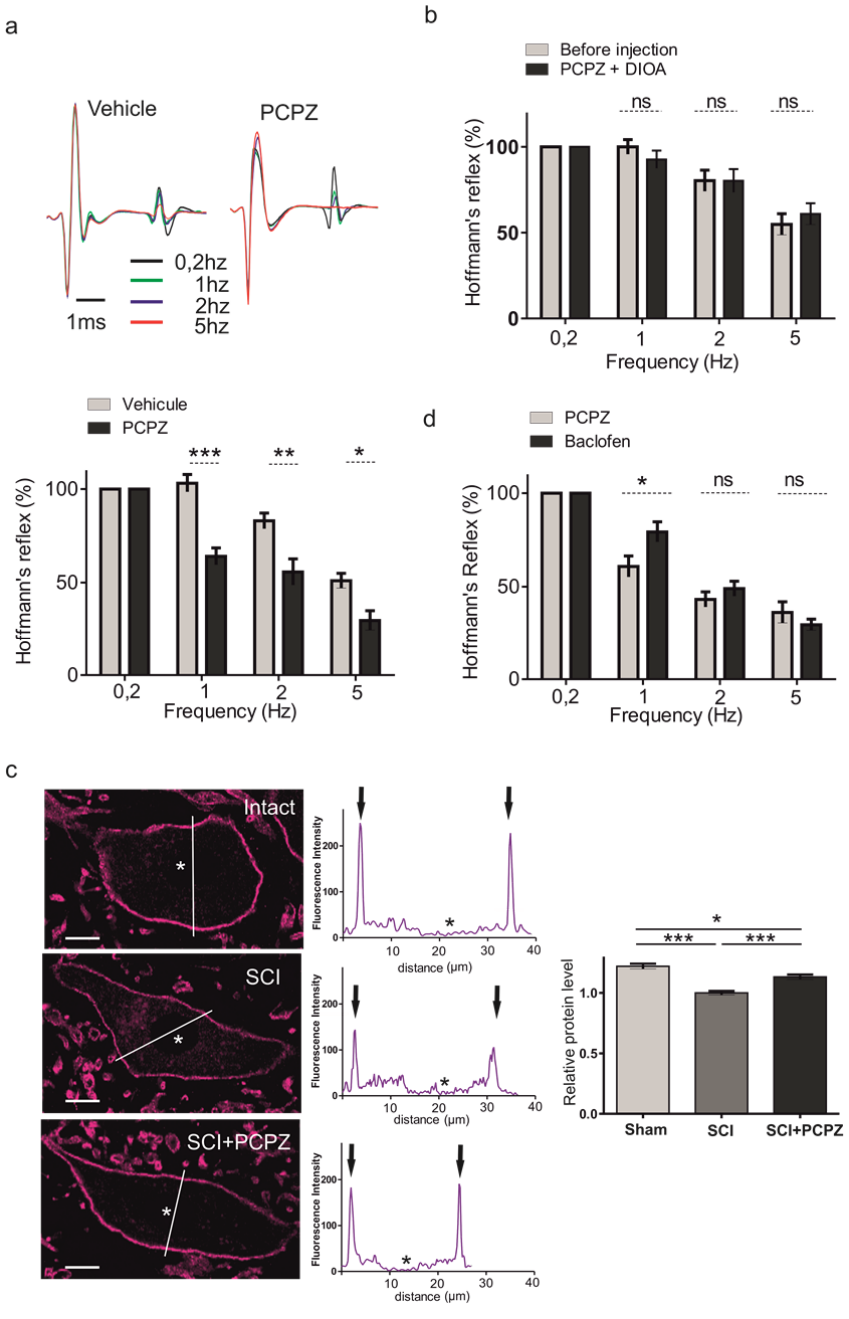


Figure 3: PCPZ decreases spasticity in rats with SCI via KCC2. (a) Prochlorperazine increases the RDD of the H reflex in chronic SCI adult rats. Top: M an H waves evoked in control (vehicle) and 80 min after PCPZ i.v injection (10 µg / kg). Each trace is the mean response to 3 last consecutive stimulations at 0.2, 1, 2, or 5 Hz. Bottom: Mean relative amplitudes of the H reflex after (80 min) the injection of vehicle (grey) or PCPZ (black, n= 9 rats with vehicle; n= 8 rats with PCPZ). (b): Pharmacological blockade of KCC2 by DIOA, (i.t. injection)

50 minutes after i.v. injection of PCPZ, inhibits the PCPZ-induced increase of the RDD (n=6 rats in each group). Mean relative amplitudes of the H reflex before injection (grey) or after (80 min) the injection of PCPZ + DIOA. (c): Left: Example single optical sections showing immunostaining of KCC2 of lumbar motoneurons (L4–L5) in sham-operated (intact), in vehicle-treated SCI rats (SCI) and PCPZ-treated SCI rats (SCI+PCPZ). Scale bars, 10 µm. Line scans illustrate the KCC2 distribution profile. Arrows indicate peaks of KCC2 immunofluorescence, stars indicate nuclei position. Right: Relative immunostaining intensities of the plasma membrane obtained with KCC2 antibody in sham-operated rats (white) and SCI rats vehicle-treated (grey) or PCPZ-treated (black) and normalized to vehicle-treated SCI rats. (n = 231: 77 cells per rat, 3 rats per group). Data are mean ± s.e.m. *, $p < 0,05$; ***$p < 0.001$ (Kruskal–Wallis test, Dunn's post tests). (d): PCPZ is as effective in reducing spasticity as baclofen. Mean relative amplitudes of the H reflex after (80 min) of PCPZ (grey) or baclofen injection (black, n= 6 ). 2-way ANOVA test with bonferroni post test; ns $p > 0,5$; *$p < 0.5$; ** $p < 0.01$; ***$p < 0.001$).