2017

Inhibition of Nkcc1 Promotes Axonal Growth and Motor Recovery in Ischemic Rats

Mu XP

Elsevier BV

info:eu-repo/semantics/article
info:eu-repo/semantics/acceptedVersion
© IBRO
CC BY-NC-ND https://creativecommons.org/licenses/by-nc-nd/4.0/
http://dx.doi.org/10.1016/j.neuroscience.2017.09.036

https://erepo.uef.fi/handle/123456789/4995

Downloaded from University of Eastern Finland's eRepository
Inhibition of Nkcc1 Promotes Axonal Growth and Motor Recovery in Ischemic Rats


PII: S0306-4522(17)30689-9
DOI: https://doi.org/10.1016/j.neuroscience.2017.09.036
Reference: NSC 18044

To appear in: Neuroscience

Received Date: 14 March 2017
Accepted Date: 21 September 2017


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
INHIBITION OF NKCC1 PROMOTES AXONAL GROWTH AND MOTOR RECOVERY IN ISCHEMIC RATS

X. P. Mu, a,b H. B. Wang, a X. Cheng, a L. Yang, c X. Y. Sun, d H. L. Qu, d S. S. Zhao, a Z. K. Zhou, e T. T. Liu, d T. Xiao, f,g B. Song, h J. Jolkkonen, i C. S. Zhao a*

a Department of Neurology, The First Affiliated Hospital, China Medical University, Shenyang, China
b Department of Neurology, The Fourth Affiliated Hospital, China Medical University, Shenyang, China
c Department of Cardiology, The Affiliated Center Hospital, Shenyang Medical College, Shenyang, China
d Department of Neurology, The People’s Hospital of Liaoning Province, Shenyang, China
e Department of Geriatrics, The First Affiliated Hospital, China Medical University, Shenyang, China
f Department of Dermatology, The First Affiliated Hospital, China Medical University, Shenyang, China
g Key Laboratory of Immunodermatology, Ministry of Health, Ministry of Education, Shenyang, China
h Regenerative Medicine, Cardiff Institute of Tissue Engineering and Repair, School of Dentistry, Cardiff University, Cardiff, UK.
i Institute of Clinical Medicine – Neurology, University of Eastern Finland, P. O. Box 1627, 70211 Kuopio, Finland.

Running title: NKCC1 Inhibition and Post-stroke Axonal Growth
*Corresponding author. Address: No. 155, North Nanjing Street, Heping District, Shenyang 110001, Liaoning, PR China. Tel: +86-24-83283026; fax: +86-24-83282315.

Email-address: cszhao@mail.cmu.edu.cn (C. S. Zhao)
**Abbreviations:** BDA, biotinylated dextran amine; CST, corticospinal tract; MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; ROCK, Rho-associated kinase; GABA, Gamma-aminobutyric acid; NKCC1, Na\(^+\)-K\(^+\)-Cl\(^-\)-co-transporter 1; KCC2, K\(^+\)-Cl\(^-\)-co-transporter 2; ET-1, endothelin-1; DAPI, 4,6-diamino-2-phenyl indole; PVDF, polyvinylidene fluoride; LSD, least significant difference; BBB, blood-brain-barrier; MCAO, middle cerebral artery occlusion.
Abstract Bumetanide is a selective inhibitor of the Na⁺-K⁺-Cl⁻ co-transporter 1 (NKCC1). We studied whether bumetanide could affect axonal growth and behavioral outcome in stroke rats. Adult male Wistar rats were randomly assigned to four groups: sham-operated rats treated with vehicle or bumetanide, and ischemic rats treated with vehicle or bumetanide. Endothelin-1 was used to induce focal cerebral ischemia. Bumetanide administration (i.c.v.) started on postoperative day 7 and continued for 3 weeks. Biotinylated dextran amine (BDA) was injected into the right imotor cortex on postoperative day 14 to trace corticospinal tract (CST) fibers sprouting into the denervated cervical spinal cord. Nogo-A, NKCC1, KCC2 and BDNF in the perilesional cortex and BDA, PSD-95 and vGlut1 in the denervated spinal cord were measured by immunohistochemistry and/or Western blot. Behavioral outcome of rats was assessed by the beam walking and cylinder tests. The total length of CST fibers sprouting into the denervated cervical spinal cord significantly increased after stroke and bumetanide further increased this sprouting. Bumetanide treatment also decreased the expressions of NKCC1 and Nogo-A, increased the expressions of KCC2 and BDNF in the perilesional cortex and enhanced the synaptic plasticity in the denervated cervical spinal cord after cerebral ischemia. The behavioral performance of ischemic rats was significantly improved by bumetanide. In conclusion, bumetanide promoted post-stroke axonal sprouting together accompanied by an improved behavioral outcome possibly through restoring and maintaining neuronal chloride homeostasis and creating a recovery-promoting microenvironment by overcoming the axonal growth inhibition encountered after cerebral ischemia in rats.
**Key words**  axonal growth; bumetanide; NKCC1; stroke; functional recovery
INTRODUCTION

Current treatment options for stroke patients are limited. Evidence is emerging to suggest that the brain is able to respond to injury to restore lost functions (Carmichael, 2003; Huie et al., 2017). The repair mechanisms include axonal and dendritic reorganization, altered excitability, neurogenesis and angiogenesis; these processes are activated with a delay, thus opening a wider therapeutic window for treatment.

It is well documented that stroke leads to axonal sprouting in experimental models (Caleo, 2015; Carmichael et al., 2017). Following a unilateral stroke, the axons of corticospinal tract (CST) from the contralateral motor cortex sprout collaterals which cross over to the ipsilateral cervical spinal cord (Zhao et al., 2013; Zai et al., 2009; Qu et al., 2014). These newly formed, long-descending projections to the ipsilateral spinal cord may contribute to behavioral recovery (Zai et al., 2009; Lee et al., 2004). However, the extent of axonal sprouting is constrained by the unfavorable local microenvironment; this contains either intrinsic growth-inhibitory molecules or insufficient support from appropriate neurotrophic agents (Carmichael et al., 2017; Benowitz and Carmichael, 2010). The effects of several intrinsic myelin-associated neurite growth inhibitors including NogoA, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) are crucial in preventing the injured axons from regenerating (Pernet and Schwab, 2012; Baldwin and Giger, 2015). After interacting with the common Nogo receptor complex, Nogo-A can activate intracellular components of Nogo signaling-RhoA and its downstream target Rho-associated kinase (ROCK), which ultimately results in the inhibition of axon regeneration and growth cone collapse (Pernet and Schwab, 2012). Blockade or inhibition of
Nogo-A/NgR or RhoA/ROCK can counteract these detrimental effects on intrinsic myelin-associated neurite outgrowth and have been associated with an improved behavioral recovery (Schwab, 2004; Domeniconi and Filbin, 2005). Therefore, strategies that reverse the hostile microenvironment by overcoming the intrinsic inhibition would be predicted to enhance rewiring and improve the functional outcome after stroke.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult mammalian brain. Under normal physiological conditions, the Na$^+\cdot$K$^+$-Cl$^-$-co-transporter 1 (NKCC1) transports Cl$^-$ from the extracellular space into neurons, whereas the K$^+$-Cl$^-$-co-transporter 2 (KCC2) has an opposite effect. NKCC1 is predominately expressed in immature neurons (Young et al., 2011; Ge et al., 2006). Interestingly, focal cerebral ischemia leads to increased expression of NKCC1 and decreased expression of KCC2 (Jaenisch et al., 2010; Wang et al., 2014; Martin-Aragon Baudel et al., 2017). The disruption of neuronal chloride homeostasis results in a shift of the chloride equilibrium potential, which triggers GABA-mediated depolarization. More importantly, there is evidence that GABA-mediated depolarization evokes axonal inhibition by activating Rho/Rock signaling (Shulga et al., 2012).

The diuretic drug, bumetanide, is a selective inhibitor of NKCC1 (Blaesse et al., 2009). Animal experiments have revealed that in the acute phase of cerebral ischemia, administration of bumetanide can reduce brain edema and the severity of damage to neurons and oligodendrocytes (Wang et al., 2014; Yan et al., 2003; Lu et al., 2006). One recent study also found that blocking the activity of WNK3-SPAK / OSR1 signaling which lies upstream of NKCC1 in vivo could reduce the expression of NKCC1 in the brain and prevent the death
of oligodendrocyte precursor cells after cerebral ischemia (Begum et al., 2015). In addition, bumetanide administration has been shown to reverse the GABA-mediated depolarizing effect, which protects and repairs the damaged neurons (Shulga et al., 2012). The aim of this study was to investigate the effect of bumetanide on axonal sprouting and its possible association with a behavioral recovery in rats subjected to experimental stroke.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (200-250g) in the study were obtained from the Experimental Animal Center of China Medical University (Shenyang, China) [No. SYXK (Liao)-2013-0007]. These rats were randomly assigned to four groups: sham-operated rats treated with vehicle (SHAM, n=10) or bumetanide (SHAM+BUM, n=10) and rats subjected to cerebral ischemia and treated with vehicle (ISC, n=12) or bumetanide (ISC+BUM, n=12). The animals were housed in standardized cages (4-5 rats per cage) in a 12-h light/dark cycle with free access to food and water. The protocol of the study was approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC-20150407). All efforts were made to ensure the animals’ welfare and to lessen suffering.

Endothelin-1 stroke model

Rats were anesthetized with 3% isoflurane and maintained under 1.5% isoflurane in 30% O₂ and 70% N₂O. Focal cerebral ischemia was induced by injecting the vasoconstrictive peptide endothelin-1 (ET-1) (Sigma, USA) at the following three stereotaxic coordinates: (1) AP + 0.7 mm, ML +2.2 mm, DV 2.0 mm; (2) AP + 2.3 mm, ML +2.5 mm, DV 2.3 mm; (3) AP + 0.7 mm, ML +3.8 mm, DV 5.8 mm according to the rat brain atlas by Paxinos and Watson.
(Soleman et al., 2010; Biernaskie and Corbett, 2001). ET-1 was injected at a speed of 0.5 µl/min by an infusion pump with the needle left in situ for three minutes before carefully removing it to avoid aspiration. The volume of each injection was 2µl (0.5 µg/µl). Sham-operated rats were injected with saline instead of ET-1.

**Bumetanide infusion**

Rats in the SHAM+BUM and ISC+BUM groups were treated with bumetanide (Sigma, USA, 0.2mg/kg/day) (Mazarati et al., 2009; Xu et al., 2016) via a micro-injection system (RWD Life Science Co., Ltd, China) once a day for 3 weeks; the drug was dissolved in alkaline solution. The micro-injection system was implanted into the lateral ventricle at the appropriate stereotaxic coordinates (AP -0.9mm, ML +1.9mm, DV 4.0mm) on postoperative day 7.

**Biotinylated dextran amine (BDA) tracing**

The corticospinal tract (CST) fibers were traced with BDA (Molecular Probes, 10 000 MW, 10% wt/vol solution in 0.01mol/L PBS) which was injected into the right (contralateral to the lesion side) motor cortex using the following coordinates (Zhao et al., 2013): AP +1.0 mm, ML –2.0 mm, DV 2.0 mm, DV 2.1.5 mm; AP 0 mm, ML –1.5 mm, DV 2.0 mm, DV 2.1.5 mm; AP –1.0 mm, ML –1.4, DV 2.0 mm, DV 2.1.5 mm; and AP +2.0 mm, ML –1.4 mm, DV 2.0 mm, DV 2.1.5 mm. Each injection volume was 1µl and the syringe was left in place for 3 min after each injection.

**Tapered/ledged beam walking test**

The tapered/ledged beam-walking test was used to evaluate hindlimb and forelimb functions (Zhao et al., 2013; Zhao et al., 2005). The rats were pre-trained to complete the task for three
days before the surgical procedure and then tested on postoperative day 31. The test was videotaped and later analyzed by calculating the slip ratio of the impaired (contralateral to lesion) forelimb and hindlimb (number of slips/number of total steps) (Zhao et al., 2013). A step onto the ledge was scored as a full slip; if the limb touched the side of the beam, then a half-slip was scored.

**Cylinder test**

The cylinder test was used to assess spontaneous forelimb use (Windle and Corbett, 2005; Sun et al., 2016). The rat was placed in a transparent plexiglass cylinder (20cm in diameter, 45cm high) and videotaped for 3 minutes through a mirror placed below the cylinder. A person blinded to the tested groups analyzed video recordings by counting left forelimb, right forelimb and both forelimb contacts with the cylinder wall. The impaired forelimb use percentage was calculated as follows: impaired forelimb contacts/(impaired+unimpaired+both forelimb contacts)×100% (Sun et al., 2016).

**Tissue preparation**

On postoperative day 33, half of the animals were anesthetized and then transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde. The brains and spinal cords were dissected (Figure 1B, 2C), post-fixed overnight and cryoprotected in 30% sucrose solution for 5-7 days at 4 °C. OCT embedded brain tissue was cut into 30 μm sections and C6-C8 segment of spinal cord was cut into 30-50 μm sections on a cryotome (Leica, CM1900, Munich, Germany). In both immunostaining and western blot experiments, the perilesional cortex was defined as the region of the cerebral cortex immediately adjacent to the stroke core (Cheatwood et al., 2008; Hinmanet al., 2013; Carmichael et al., 2005)(Figure
1B,1C); this was the location from where samples were taken in our study.

Infarct volume measurements

For evaluating the infarct volume, coronal sections (30 μm) were collected at 1 mm intervals and stained with cresyl violet (Sigma, USA). The infarcted area was calculated by subtracting the non-injured area of the injured hemisphere from the area of the normal hemisphere (contralateral) for each section (NIH ImageJ). Total volumes were obtained by multiplying infarcted areas by the distance between sections (Popp et al., 2009).

Immunohistochemistry and quantification

Immunofluorescence staining was performed on free-floating sections as previously described (Jessberger et al., 2007). Spinal sections were stained with the following primary antibodies: rabbit anti-PSD-95 (1:800, Abcam) and rabbit anti-vGlut1 (1:100, Abcam) and brain sections were stained with rabbit anti-Nogo-A (1:400, Abcam) and rabbit anti-BDNF (1:400, Abcam) overnight at 4°C. After rinsing, the sections were incubated with the secondary antibodies: Alexa Fluor 488 goat anti-rabbit (1:200, Invitrogen, USA) for PSD-95, vGlut1, Nogo-A (with 4,6-diamino-2-phenyl indole, DAPI) and BDNF (with DAPI); and for double staining of synaptic markers and BDA, streptavidin Alexa Fluor 594 conjugate (1:200, Invitrogen, USA). The sections were rinsed, mounted, and cover-slipped.

Axonal sprouting was assessed in a confocal microscope at 20× magnification (Olympus FV-1000, Japan) with caudal cervical enlargement (C6-C8) from 6 coronal sections per rat. Then, three-dimensional reconstruction images of the BDA-positive fibers were obtained. The total length of the crossing CST fibers were traced and analyzed by the NIH ImageJ software. When studying vGlut1 and PSD-95 stainings, immunostaining pictures of every
tenth section from C6 to C8 of the spinal cord were captured at 20× magnification. The pixels of integrated density were measured by NIH ImageJ.

To measure the expression of Nogo-A and BDNF in the perilesional cortex, every tenth section (30 μm) was selected. Pictures were captured with a confocal microscope (Olympus FV-1000, Japan). The number of immunopositive cells was measured with NIH ImageJ software.

**Western blot analysis**

Western blot analysis was performed from the perilesional cortex from half of the animals on postoperative day 33. Total protein was extracted in lysis buffer and quantified using the Bradford method, separated by SDS–PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with primary antibodies against NKCC1, KCC2, NogoA and BDNF (1:800, Abcam). After being incubated with peroxidase-coupled anti-rabbit IgG (Cell Signaling Technology) at 37°C for 2 hours, proteins were visualized with using ECL (Pierce, Rockford, IL USA) and then the images were captured by DNR Bio-Imaging Systems (Jerusalem, Israel).

**Statistics**

All the statistical analyses were performed with SPSS software version 22. The data were analyzed using one-way ANOVA. Statistical differences between groups were analyzed using the least significant difference (LSD) post hoc test. Data were presented as the mean ± SD and significance was set at P<0.05. Pearson’s correlation coefficients were used to analyze the correlation between the CST axonal density and behavioral outcomes.
RESULTS

Infarct volume measurements

There was no significant difference in infarct volumes between the ISC group (126.9±10.1mm$^3$) and ISC+BUM group (130.8±12.2mm$^3$). A typical infarct evoked damage in the cortex and striatum is shown in Figure 1C. Two rats died after the ET-1 injection. Three rats did not display any signs of behavioral impairment and were excluded from the study.

Bumetanide enhances CST fibers to cross the midline in the spinal cord after stroke

Consistent with previous studies, the CST fibers from the intact side innervated the denervated lesion side after cerebral ischemia (Zai et al., 2009; Lee et al., 2004). A Z-stack reconstructed photograph of BDA-labeled fibers revealed a significant overall group effect in the total length of crossing CST fibers (F$_{(3,20)}$=86.468; P<0.001). There was an increase in the total length of the CST fibers crossing into the denervated side in the ISC group (1113.2±166.9µm) as compared to the SHAM group (433.8±201.1µm; P<0.01) and the SHAM+BUM group (466.3±89.2µm; P<0.01). Interestingly, bumetanide treatment further increased the total length of crossing fibers in the ISC+BUM group (2133.2±315.0µm; P<0.01, Figure 2A, B) compared with the ISC group. In the SHAM group, only a few BDA-labeled axons crossed the midline of the spinal cord. There were no differences observed between the SHAM and SHAM+BUM groups.

Recovery of synaptic markers by bumetanide after stroke

Immunostaining of two synaptic markers, PSD-95 and vGlut1, was reduced in the denervated gray matter of spinal cords in the ISC group in comparison to either SHAM or SHAM+BUM groups (P<0.01). There were no differences noted between the SHAM and SHAM+BUM
groups. The expression of these synaptic markers was restored after bumetanide infusion in the ISC+BUM group (P<0.01, Figure 3A, 3C). Moreover, PSD-95 and vGlut1 with BDA double staining showed a close-proximity relationship (Figure 3B), which indicated that the collaterals sprouting into the denervated side of the spinal cord were possibly making synaptic contacts.

**Bumetanide downregulates expression of NKCC1 but upregulates that of KCC2 after cerebral ischemia**

Next, we measured NKCC1 and KCC2 in the perilesional cortex by Western blot (Figure 4). There were significant overall group effects in NKCC1 expression (F(3,12)=128.571, P<0.001) as well as in KCC2 (F(3,12)=17.780, P<0.001). The expression of NKCC1 increased whereas that of KCC2 decreased after cerebral ischemia compared to the SHAM and SHAM+BUM groups (P<0.01). Interestingly, bumetanide almost completely reversed both the increase in NKCC1 expression as well as the decrease in KCC2 expression in the perilesional cortex (P <0.05).

**Bumetanide decreases Nogo-A levels but increases BDNF levels in perilesional cortex**

We also examined the expressions of axonal growth inhibitor Nogo-A as well as BDNF expression by immunostaining and Western blot in the peri-infarct cortex. There was a significant overall group effect in both Nogo-A expression (F(3,20)=9.447, P<0.001, Figure 5A; F(3,12)=12.342, P<0.005, Figure 6A) and the BDNF expression (F(3,20)=4.494, P<0.05, Figure 5A; F(3,12)=96.329, P<0.00, Figure 6A). The number of Nogo-A+ cells with DAPI was significantly increased after cerebral ischemia compared with the corresponding situation in the SHAM group (P<0.01). Bumetanide decreased the number of Nogo-A+ cells with DAPI
in the perilesional cortex in the ISC+BUM group (P<0.01, Figure 5A) compared with the ISC group. The same results with respect to NogoA were observed when assessed by western blot. Bumetanide significantly decreased BDNF expression in the sham-operated rats (P<0.01, as assessed by western blot) and increased BDNF expression in the ischemic animals (P<0.01) (Figure 5A, 6A).

**Bumetanide promotes behavioral performance in ischemic rats**

The beam walking and cylinder tests were used to evaluate sensorimotor impairment and recovery on post-operative day 31-32. There were significant overall group effects in slip ratios of the impaired forelimb (F(3,36)=20.007, P<0.001), slip ratio of the hindlimb (F(3,36)=19.335, P<0.001) and in the impaired limb use ratio (F(3,36)=28.845, P<0.001). The SHAM and SHAM+BUM groups displayed no significant differences. The slip ratios increased after ischemia compared to the SHAM and SHAM+BUM groups (P<0.01), and this was partially reversed by bumetanide administration (ISC+BUM group) (P<0.05; Figure 7A, B). Furthermore, the spontaneous use of the impaired limb in the cylinder test decreased significantly after ischemia in comparison to the SHAM and SHAM+BUM groups (P<0.01). Again, bumetanide partially restored the asymmetry in limb use (P<0.05, Figure 7C). Correlation analysis revealed significant negative correlations between slip ratios of forelimb and hindlimb in beam walking test and the CST axonal density (r = −0.77, P<0.05; r= −0.79, P<0.05) in ISC and ISC+BUM groups (Figure 7D). A positive correlation was found between impaired limb use ratio in the cylinder test and axonal density (r=0.68, P<0.05, Figure 7D). However, there was no significant correlation between behavioral outcome and axonal density in the SHAM and SHAM+BUM groups, which suggested that bumetanide-induced
CST axonal density contributed to neurological recovery only under ischemic conditions. Furthermore, no significant correlation existed between axonal density and behavior when data from all four groups were analyzed together (P>0.05, Figure 8).

**DISCUSSION**

The present study is the first to reveal that bumetanide can enhance axonal sprouting and possibly promote synapse formation of CST fibers from the intact side of the brain reaching into the denervated cervical spinal cord after focal cerebral ischemia in rats. In addition, bumetanide decreased the expression of NKCC1 and Nogo-A and increased the levels of KCC2 and BDNF in the perilesional cortex after an experimental stroke in these animals. More importantly, the structural and molecular remodeling achieved by chronic bumetanide treatment was associated with a significant behavioral improvement.

The neurotransmitter GABA activates chloride-permeable channels by binding to GABA\(_A\) receptors; this effect has different consequences in mature and immature neurons depending on the gradient of intracellular and extracellular chloride ions (Ge et al., 2006; Tozuka et al., 2005). In immature neurons or under ischemic conditions, GABA\(_A\) receptor-mediated responses are depolarizing and excitatory because of the downregulation of KCC2 and the upregulation of NKCC1, leading to an extracellular flow of Cl\(^-\) (Blaesse et al., 2009; Nabekura et al., 2002). Consistent with previous reports (Jaenisch et al., 2010; Wang et al., 2014), our study further demonstrated evidence of a long-term change of NKCC1 and KCC2 in the perilesional cortex after stroke. Consequently, the disruption of neuronal chloride homeostasis might lead to a reduction in the inhibitory actions mediated by the GABA\(_A\) receptor. At least to some extent, this reduced GABAergic inhibition may be
helpful in permitting plasticity remodeling and behavioral recovery after stroke (Rossini et al., 2003; Filippo et al., 2008). However, the enhanced, prolonged GABA-mediated depolarization has been claimed to trigger secondary post-stroke damage in the situation of sustained ischemia due to deranged chloride homeostasis, which eventually leads to a detrimental cytosolic calcium overload in the injured neurons (Jaenisch et al., 2010; Nabekura et al., 2002).

In the present study, however, there was no difference in infarct size between vehicle and bumetanide treated rats after a 3-week-infusion of the drug. This might indicate that bumetanide can decrease the infarct size in the acute phase but not during the recovery period. Importantly, there is evidence demonstrating that the intracellular Ca\(^{2+}\) overload induced by GABA\(_A\) mediated depolarization after axonal injury might activate the Rho/Rock signaling, which is known to ultimately exert inhibitory effects on axonal growth (Shulga et al., 2012). Therefore, blockade of GABA\(_A\)-mediated depolarization in neurons and maintenance of chloride homeostasis following brain injury could help to rescue the injured neurons. In support of this proposal, there is evidence showing that bumetanide may be able to reverse the depolarizing shift in GABA\(_A\)-mediated responses induced by the axotomy of mature neurons; one consequence would be that it could prevent neuronal death (Shulga et al., 2012).

In the present study, we demonstrated that chronic bumetanide infusion significantly decreased the level of NKCC1, as well as downregulating the expression of NogoA in the perilesional areas, with more CST fibers crossing over the midline and sprouting into the denervated gray matter in the cervical cord in ischemic rats after the experimentally-induced focal stroke. In addition, bumetanide significantly increased the expressions of PSD-95 and
vGlut-1, which were used as the markers for axonal growth and reactive synaptogenesis. Importantly, the double staining of BDA positive fibers and the above synaptic markers revealed their close-proximity, suggesting that the sprouting fibers may have formed functional synapses. Thus, bumetanide might exert a positive effect on axonal sprouting after a focal cerebral ischemia. One way to test this hypothesis would be to conduct an electrophysiological study to investigate the chloride equilibrium potential with the perforated patch-clamp technique in a slice taken from an ischemic brain.

Recently, it was reported that a delayed, sustained, low-dose GABA_A receptor antagonism could promote the sensorimotor recovery in a skilled-reaching task during the chronic stage in an ET-1 rat model of focal ischemia and this behavioral improvement was accompanied by a smaller infarct volume (Lake et al., 2015). In the present study, chronic treatment with bumetanide starting 7 days after stroke resulted in a significant improvement in terms of the behavioral recovery as assessed in the beam-walking and cylinder tests but the drug did not affect the infarct size.

The levels of BDNF have been demonstrated to be upregulated and to promote survival and sprouting of corticospinal tract (CST) neurons after neuronal injury (Blesch and Tuszynski, 2007; Jain A et al., 2011; Tuinstra et al., 2012; Ollivier-Lanvin et al., 2014; Lynskey et al., 2006; Sasaki et al., 2009; Vavrek et al., 2006; He et al., 2013; Brock et al., 2010; Cook et al., 2017). Our data showed that the expression of BDNF was significantly increased in the ISC+BUM group but decreased in SHAM+BUM group, indicating that chronic bumetanide treatment could induce endogenous BDNF levels only during ischemia. Interestingly, there is an interplay between Nogo-A and BDNF (Seiler et al., 2016).
injury-induced upregulation of Nogo-A can be counterbalanced by exercise following a traumatic brain injury, with this effect being mediated through a BDNF-dependent mechanism (Gabriela et al., 2008). Therefore, we cannot completely exclude the possibility that the downregulated expression of NogoA in the perilesional cortex by bumetanide observed here might be also mediated via a BDNF-dependent mechanism. Under normal conditions, BDNF might decrease the expression of KCC2, whereas after a neuronal injury, BDNF would increase KCC2 expression and this would help to restore GABAergic inhibition (Boulenguez et al., 2010; Garraway and Huie, 2016). Consistently, a decreased level of KCC2 was observed after a focal stroke in the present study, and this deficit was reversed by bumetanide. Thus one could speculate that chronic bumetanide treatment would not only restore the chloride homeostasis but also combat the unfavorable microenvironment and thus promote axonal sprouting. Electrophysiological studies will be required for the future studies.

CONCLUSION

Our experimental data reveals the therapeutic efficacy of bumetanide treatment started during recovery period after a stroke on axonal sprouting. Thus enhanced axonal rewiring after cerebral ischemia represents a potential novel approach to support a functional recovery after a stroke. In rats subjected to experimental stroke, these beneficial effects are possibly mediated by reconstructing neuronal chloride homeostasis and creating a helpful microenvironment by overcoming the detrimental effects of axonal growth inhibitors.

Acknowledgments- This study was supported by the National Natural Science Foundation of China (No. 81372104, No. 30872736, No. 81401002), the Shenyang Population and Health
Technical Critical Special Project (No. F16-206-9-01); the Program of the Distinguished Professor of Liaoning Province, Neurology. National Science Foundation of Liaoning Province of China (No. 2014021074).

REFERENCES


Domeniconi M, Filbin MT (2005) Overcoming inhibitors in myelin to promote axonal


Ollivier-Lanvin K, Fischer I, Tom V, Houle JD, Lemay MA (2014) Either brain-derived neurotrophic factor or neurotrophin-3 only neurotrophin-producing grafts promote
locomotor recovery in untrained spinalized cats. Neurorehabil Neural Repair 29:90-100.


Figure Legends

Fig 1. Study design of the experiment and overview of the cerebral ischemia. A, Study design, the arrows indicate the timing of pre-training, stroke, bumetanide treatment, biotinylated dextran amine (BDA) injection, beam-walking test, cylinder test and sacrifice. B, Gross evaluation of the ischemic area as indicated by the arrow. C, A schematic picture revealing the typical infarct region (blue color). The black-bordered area designates the perilesional cortex area in B and C.

Fig 2. Sprouting of the cortical spinal tract (CST) fibers which cross the midline of spinal cord to the gray matter on the denervated side. A, Representative photographs of BDA-immunolabeling of CST collaterals (the arrows indicated) which had sprouted across the spinal midline into the denervated side from the BDA labelled side of spinal cord. CC indicated central canal of spinal cord. B, The total length of the CST axons collaterals which had sprouted and crossed the midline. Statistical significance: *P<0.01 compared to the SHAM and SHAM+BUM groups, **P<0.01 compared to the ISC group. Data are mean±SD. n=6 per group. Scale bar=100µm. C, The gross image of the spinal cord and the segment between the two arrows indicates C6-C8 of the spinal cord (on left side); on right side of C: a schematic diagram of the corticospinal projections and the lesion site (blue) and the injection site of BDA (red points). BDA was injected into the contralesional cortex to trace sprouting CST fibers (sprouting fibers, indicated by blue arrows) after the experimental stroke.

Fig 3. Expressions of synaptic markers in denervated gray matter of spinal cord. A,
Representative micrographs of immunostaining of PSD-95 and vGlut-1. B, Double staining of BDA with PSD-95 and vGlut-1 revealed their close proximity relationship (arrows). C, Statistical significance: **P<0.01 compared to the SHAM and SHAM+BUM groups, #P<0.01 compared to the ISC group. Data are mean±SD. n=6 per group. Scale bar: A= 100µm; B= 50µm.

Fig 4. Expression levels of NKCC1 and KCC2 in the perilesional cortex. A, Western blots analysis of NKCC1 and KCC2. B, Statistical significance: **P<0.01 compared to the SHAM and SHAM+BUM groups, #P<0.05 compared to the ISC group. Data are mean±SD. n=4 per group for Western blot.

Fig 5. Expression levels of Nogo-A and BDNF in the perilesional cortex as assessed by immunostaining. A, Immunostaining of Nogo-A and BDNF with DAPI. B, Statistical significance: For Nogo-A, *P<0.01 compared to the SHAM and SHAM+BUM groups, **P<0.01 compared to ISC group. For BDNF, *P<0.01 compared to the SHAM group, **P<0.01 compared to ISC group. Data are mean±SD. n=6 per group. Scale bar=50µm.

Fig 6. Expression levels of Nogo-A and BDNF in the perilesional cortex as assessed by western blot. A, Western blot analysis of NKCC1 and KCC2. B, Statistical significance: For Nogo-A, *P<0.01 compared to the SHAM and SHAM+BUM groups, **P<0.05 compared to ISC group. For BDNF, *P<0.01 compared to the SHAM+BUM group, **P<0.01 compared to ISC group, and #P<0.05 compared to SHAM group. Data are mean±SD. n=4 per group.
Fig 7. Behavioral tests of rat and correlation analysis. A, Beam walking test for impaired forelimb (slip ratio). B, Beam walking test for impaired hindlimb (slip ratio). C, Cylinder test (impaired limb use ratio). Statistical significance: *P<0.01 compared to the SHAM and SHAM+BUM groups, **P<0.05 compared to the ISC group. Data are mean±SD. n=10 per group. D, Correlation analysis between behavioral outcomes and CST axonal density. Statistical significance: ISC and ISC+BUM groups (P<0.05); and SHAM and SHAM+BUM groups (P>0.05).

Fig 8. Correlation analysis between behavioral outcomes and CST axonal density. Statistical significance: SHAM, SHAM+BUM, ISC and ISC+BUM groups analyzed together (P>0.05).
1. Bumetanide promoted axonal growth after cerebral ischemia in rats.
2. Bumetanide upregulated KCC2, BDNF, downregulated NKCC1, NogoA after stroke in rats.