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Theophylline Regulates Inflammatory and Neurotrophic factor Signals in Functional Recovery after C2-Hemisection in Adult Rats

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Abstract

Recovery of respiratory activity in an upper cervical hemisection model (C2H) of spinal cord injury (SCI) can be induced by systemic theophylline administration 24–48 h after injury. The objectives in the present study are (1) to identify pro-inflammatory and neurotrophic factors expressed after C2H and (2) molecular signals involved in functional recovery. Four groups of adult female rats classified as (i) sham (SH) controls, (ii) subjected to a left C2 hemisection (C2H) only, (iii) C2H rats administered theophylline for 3 consecutive days 2 days after C2H (C2H-T Day 5) and (iv) C2H rats treated with theophylline for 3 consecutive days 2 days after C2H and then weaned for 12 days (C2H-T Day 17) prior to assessment of respiratory function and molecular analysis were employed. Corresponding Sham controls, C2H untreated (vehicle only controls) and C2H treated (theophylline) rats were sacrificed, C3-C6 spinal cord segments quickly dissected and left (ipsilateral) hemi spinal cord and right (contralateral) hemi spinal cord were separately harvested 2 days post surgery. SHAM operated and C2H untreated-controls corresponding to C2H-T Day 5 and C2H-T Day 17 rats, respectively, were prepared similarly. Messenger RNA levels for pro-inflammatory genes (TXNIP, IL-1 β , TNF- α and iNOS) and neurotrophic and survival factors (BDNF, GDNF, and Bcl2) were analyzed by real time quantitative PCR. Gene expression pattern was unaltered in SH rats. TXNIP, iNOS, BDNF, GDNF and Bcl2 mRNA levels were significantly increased in the ipsilateral hemi spinal cord in C2H rats. BDNF, GDNF and Bcl2 levels remained elevated in the ipsilateral hemi spinal cord in C2H-T Day 5 rats. In this same group, there was further enhancement in TXNIP and IL-1 β while iNOS returned to basal levels. Theophylline increased DNA binding activity of transcription factors - cyclic AMP responsive element (CRE) binding protein (CREB) and pro-inflammatory NF- κ B. Messenger RNA levels for all genes returned to basal levels in C2H-T Day 17 rats. However, BDNF mRNA levels remained significantly elevated after weaning from the drug. Our results suggest that enhanced resolution of early inflammatory processes and expression of pro-survival factors may underlie theophylline-induced respiratory recovery. The results identify potential targets for gene and drug therapies.

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Keywords

Spinal cord injury; Theophylline; Respiratory Recovery; Neurotrophic factor; Transcription factor; Inflammation Resolution

1. INTRODUCTION

The National Spinal Cord Database indicates that the major cause of mortality and morbidity following SCI is respiratory failure. The mainstay of current therapy for patients with severe respiratory deficits following upper cervical SCI is mechanical respiratory support (National Spinal Cord Injury Statistical Center, 2010). Patients tethered to mechanical ventilators feel a loss of independence and long term use of mechanical support can often lead to infections and pneumonia (Winslow and Rozovsky, 2003). There is therefore a need for alternative approaches to alleviate respiratory deficits. In order to understand the respiratory system and improve impaired respiratory function after injury, several animal models have been employed in basic research. One of the most extensively studied is the C2 hemisection (C2H) model. In this model, interruption of the descending bulbospinal respiratory pathways paralyzes the hemidiaphragm ipsilateral to the hemisection. However, respiratory function can be induced chemically or pharmacologically by activation of a latent respiratory pathway that is referred to as the crossed phrenic pathway or “CPP” (Goshgarian, 2003; 2009).

We previously demonstrated that theophylline, a methylxanthine clinically used in the treatment of asthma, chronic pulmonary obstructive disease (COPD), and premature infants with respiratory deficits can activate the CPP and restore function to the hemidiaphragm paralyzed by C2 hemisection (Nantwi et al., 1996; Nantwi and Goshgarian, 1998a, 1998b, Nantwi, et al., 2003a, 2003b) via adenosine receptor antagonism (Nantwi and Goshgarian, 1998b). While adenosine receptor antagonism has been demonstrated as a mechanism of theophylline-induced functional restoration in the C2H model of SCI, the molecular and cellular pathways that may mediate the recovery are yet to be elucidated. The pharmacologic profile of theophylline includes phosphodiesterase (PDE) inhibition and recent studies suggest that PDE inhibition can lead to increases in cellular cAMP levels and cAMP-dependent signaling and gene expression that may enhance synaptic connections (Walteriet and Weller, 2003; Feldman et al., 2003; Kajana and Goshgarian, 2009, 2008).

In addition, cAMP signaling has been suggested as a possible stimulator of respiratory activity (Brock et al., 2010). A convergent point in the cAMP pathway is the activation of the transcription factor, cAMP responsive element (CRE) binding protein (CREB) and expression of neurotrophic factor (BDNF, GDNF) and survival factor (Bcl2) (Chen et al., 2006; Hoh et al., 2010; Suzuki et al., 2011). In addition, acute spinal cord injury evokes pro-inflammatory gene expression and neural inflammation (Gonzales et al., 2003, 2007). Inflammation is required for the removal of the cellular debris by infiltrating monocytes/macrophages and resident glial cells in the spinal cord, which is essential for wound healing and establishment of tissue homeostasis (Speyer and Ward, 2011). Similarly, neurotrophic factors provide support neuronal survival and axonal and dendritic regeneration after the injury (Ying et al., 2008; Deng et al., 2011). In this report, we asked: (i) whether differences in gene expression pattern for neurotrophic factors and pro-inflammatory genes between the left (ipsilateral) hemi spinal cord and right (contralateral) hemi spinal cord in C2 hemisected and theophylline-treated animals (C2H-T) exist; and (ii) how gene expression pattern changes in the (ipsilateral hemi spinal cord or contralateral hemi spinal cord) in SHAM operated (uninjured) control, C2H operated but treated with vehicle only (injured and untreated control) and C2H theophylline-treated rats. We hypothesize that theophylline-

induced respiratory recovery in C2H rats is mediated at the cellular/molecular level by activation of both neurotrophic factors and pro-inflammatory genes. We also hypothesize that theophylline activates transcription factors CREB and NF- κ B that are responsible for neurotrophic factor and inflammatory gene expression in the injured spinal cord during respiratory recovery. In support of our hypotheses, our results demonstrate that theophylline enhances neurotrophic and survival factor expression (BDNF, GDNF and Bcl2) and induces pro-inflammatory TXNIP and IL-1 β expression while suppressing iNOS. We show that BDNF expression is sustained even after drug withdrawal while pro-inflammatory TNF- α expression remains high suggesting the presence of persistent inflammation in spinal cord injury.

2. MATERIALS AND METHODS

2.1. Materials

Antibodies for BDNF and Bcl2, and Actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CREB and phosphor-CREB antibodies were from Cell Signaling Technologies. The Electrophoresis Mobility Shift Assay (EMSA) kit for Transcription factors CREB and NF- κ B to consensus DNA were purchased from Signosis Inc. (Sunnyvale, CA). TRIZOL for RNA isolation was from Invitrogen and PCR primers were also synthesized by Invitrogen or Applied Biosystems. PVDF and ECL reagents were purchased from GE Amersham. First strand cDNA synthesis kit and SYBR green reagents were purchased from Biorad and Applied Biosystems (now Invitrogen).

2.2. Experimental animals

Adult female Sprague Dawley rats (250–320 g) were used in this study. Animals were divided into four experimental groups (n=6/group): (1) sham-operated controls (SHAM Day 2), (2) rats subjected to a left C2 hemisection only (C2H Day 2), (3) C2H rats administered theophylline for three days starting at day 2 of C2 hemisection (C2H-T Day 5) and (4) C2H rats administered theophylline for 3 days as in (3) but weaned from the drug for 12 days (C2H-T Day 17). Appropriate controls were: (1) SHAM Day 5 and C2H Day 5 untreated (vehicle only), which are compared with C2H-T Day 5. (2) SHAM Day 17 and C2H Day 17 untreated (vehicle only), which are compared with C2H-T Day 17 (n=5 animals/group).

Prior to surgery, rats were anesthetized (IP) (ketamine, 70 mg/kg; xylazine 7 mg/kg) and then subjected to a left upper cervical (C2) hemisection as described previously (Liou and Goshgarian, 1994). Theophylline was administered by oral gavage (20 mg/kg, 3 $\times$ daily). The drug dose and route of administration were based on a previous study that the drug induces and maintains recovery after C2 hemisection (Nantwi, et al., 2003). All surgical procedures employed in the present study were conducted strictly in accordance with the guidelines set out by the National Institutes of Health and approved by the IACUC and the Division of Laboratory Animal Research (DLAR) at Wayne State University.

2.3. Electrophysiology

Based on our experience with the C2H model and our prior publications (Nantwi et al., 1996; Nantwi and Goshgarian, 1998, 2001) we did not subject all rats to a complete electrophysiologic assessment of respiratory activity prior to assessing molecular/cellular signals. Instead, we used separate rats (2 per group) to demonstrate functional completeness of the C2 hemisection and efficacy of theophylline in restoring function (C2H-T Day 5) and maintaining function even after 12 days cessation of drug administration (C2H-T Day 17). Since rats in the latter groups were used to confirm and extend our previous study, only a qualitative record of diaphragmatic activity under spontaneous breathing conditions was conducted. In this representative group, assessment of the functional completeness of the

hemisection was conducted 24 hr after C2 hemisection by monitoring respiratory activity in the left and right hemidiaphragm as described previously (Nantwi et al., 1996). After recording stable levels of activity the recording was stopped, the abdominal incisions closed with fine sutures, and all skin incisions closed with stainless steel wound clips. Representative C2H-T Day 5 and C2H-T Day 17 rats were similarly assessed at the conclusion of the respective experimental periods.

2.4. Tissue harvesting experimental groups

Rats were anesthetized with chloral hydrate (400 mg/kg, IP) and decapitated with a guillotine. Tissue harvesting was done at 2 days post-surgery for SHAM Day 2 and C2H Day 2 rats. For C2H-T Day 5 and C2H-T Day 17 rats, tissue harvesting was done at day 5 and day 17 after C2 hemisection respectively.

For molecular and biochemical studies, spinal cords at levels C3–C6 were dissected at the region containing the phrenic motoneurons that modulate activation of the CPP (Goshgarain, 2003, 2009). Left (Ipsilateral) hemi spinal cord and right (Contralateral) hemi spinal cord segments were separated in the mid-sagittal plane using a sharp steel blade while the dissected tissue was kept frozen on a block of dry ice. The separated segments were immediately stored according to experimental groups at -80°C until needed.

For immunohistochemical (IHC) analysis, separate representative groups of rats ($n=4$) from all groups were perfused with 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cords (C3–6) were dissected, and cryoprotected in a sucrose gradient (10%, 20%, and 30% w/v in PB, respectively) and tissues were embedded in Tissue Tek (OCT mounting medium). Twenty μm thick sections through C3–C5 spinal cord levels were collected on poly-L-lysine-slides and stored at -80°C until used.

Immunohistochemistry: IHC was performed similar to what has been described recently (Perrone et al., 2010a). The ipsilateral hemi spinal cord as well as the contralateral hemi spinal cord sections were blocked for 1 h in a PBS solution that contained 5% Chemiblocker (Chemicon, Temecula, CA) or 5% BSA, 0.5% Triton X-100, and 0.05% sodium azide. The primary antibodies for BDNF (rabbit) were diluted in the same solution (1:200) and applied overnight followed by appropriate secondary antibodies (1:600 dilutions) conjugated with Alexa Fluor 594 for 2 hours. The images were captured by an OLYMPUS BX 51 fluorescence microscope, which is fitted with a triple DAPI/FITC/TRITC cube, a DP70 digital camera and image acquisition software. Similar magnification (400 $\times$) and exposure time were maintained throughout for comparing images unless otherwise mentioned.

2.5. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNAs were isolated by the TRIZOL method and the first-strand cDNAs were synthesized in 20 μl volume using 1 μg RNA of the Bio-Rad iScript cDNA synthesis kit (Perrone et al 2009, Cheng et al., 2006). Messenger RNA expression was analyzed by real-time quantitative PCR using the Bio-Rad Chromo 4 detection system and SYBR Green PCR Master Mix from Bio-Rad or Applied Biosystems (Foster City, CA). Primers were designed using Primer Express v 2.0 [Applied Biosystems] and synthesized by Invitrogen (Carlsbad, CA). The real time PCR reaction mixture contained 1X SYBR Green PCR Master Mix, 400 nM forward and reverse primers, and 2.5 μl cDNA in a final volume of 25 μl . The PCR cycling was programmed as 95°C for 15s, 55°C for 30s and 72°C for 30s 40 cycles followed by the construction of a melting curve through increasing the temperature from 60°C to 95°C at a ramp rate of 2% for 20 min. The real time PCR samples were evaluated using a single predominant peak as a quality control. Ct values were used to calculate the

relative expression level of mRNAs that were normalized to actin. Rat primers for the qPCR are as follows:

- a. BDNF (Brain derived neurotrophic factor):
 FORWARD: 5'-CTTTACTGGCTTAGGGGATGG-3'
 REVERSE: 5'-CGCTTTATCAACCAGAAATGGA-3'
- b. GFNF (Glia derived neurotrophic factor):
 FORWARD: 5'-CAGAGAATTCCAGAGGGAAAG-3'
 REVERSE: 5'-GAAAGATCAGTTCCTCCTTGG-3'
- c. Bcl2:
 FORWARD: 5'-GGGATGCTTTTGTGGAACTAT-3'
 REVERSE: 5'-AGGTATGCACCCAGAGTGATG-3'
- d. beta-actin:
 FORWARD: 5'-AAGCTGTGCTATGTTGCCCTA-3'
 REVERSE: 5'-ATTGCCGATAGTGATGACCTG-3'
- e. IL-1 β (Interleukin 1beta):
 FORWARD: 5'-GGACAGAACATAAGCCAACAA-3'
 REVERSE: 5'-CTTTCATCACACAGGACAGGT-3'
- f. TNF- α (Tumor necrotic factor alpha):
 FORWARD: 5'-AGCAGATGGGCTGTACCTTAT-3'
 REVERSE: 5'-GCTGACTTTCTCCTGGTATGA-3'
- g. TXNIP (Thioredoxin interacting protein):
 FORWARD: 5'-CTGAAGTTACCCGAGTCAAAGC-3'
 REVERSE: 5'-CTCACCTGTAGCCTGGTCTTCT-3'
- h. iNOS (Inducible nitric oxide synthase):
 FORWARD: 5'-ATGGTTCCTGGGCGTGTTGGAATA-3'
 REVERSE: 5'-TGTAAGCTGTGGCCCTGACAGTA-3'

In addition, pre-designed rat PCR primer sets for iba-1 (also known as Aif1, microglial activation marker, cat# QT00407057) and glial fibrillary acidic protein (GFAP, an astroglial activation marker, cat# QT00195517), purchased from Qiagen (Valencia, CA), were used in order to determine astroglia activation in spinal cord injury.

2.6. SDS-PAGE and Western blotting

Spinal cord proteins were extracted in RIPA buffer containing protease inhibitors for cytosolic extract and a nuclear extraction kit from Active Motif (Carlsbad, CA) for nuclear proteins. Protein concentrations were determined using a Coomassie-based reagent from Bio-Rad with BSA as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis of proteins were performed as described previously (Devi et al 2011). Membranes were blocked with 5% BSA for 1 h. Then, rabbit anti-CREB and anti-phospho-CREB primary antibodies were diluted 1:1000 (v/v) in 5% BSA and incubated overnight at 4°C with shaking in cold room. Secondary antibodies were used

at 1:3000 dilutions. ECL used to detect the immunoreactive bands and the images were captured using a Cell Biosciences FluorChem E System (Santa Clara, CA).

2.7. Electrophoretic Mobility Shift Assay

The gel-shift or electrophoretic mobility-shift assay (EMSA) provides a simple and rapid method for detecting DNA-binding activity of proteins. We investigated the consensus DNA binding activity of nuclear CREB and NF- κ B in spinal cord nuclear extracts by using a commercially available gel-shift assay kit from Signosis (Sunnyvale, CA) according to manufacturer's instruction and as described before (Singh et al 2007). After incubation at 16 °C for 30 min with biotin-labeled DNA probes with nuclear extracts (5.0 μ g protein) in a PCR machine, the protein-DNA complexes were subjected to 6.5% non-denaturing polyacrylamide gels prepared with TBE gel formulation. The gels were run at 100 V for 1 h in 0.5x TBE running buffer until the Bromophenol blue dye front reaches ~one inch from the bottom of gel. For competitive inhibition, cold probes were pre-incubated in ice for 20 min before adding the biotin-label probes. Probe alone controls were run without the nuclear extract. Streptavidin-HRP and ECL were used to detect and capture the reactive bands using the FluorChem E System.

2.8. Statistical analysis

Data are expressed as mean $\pm$ se of the indicated number of animals (5–6 rats/group) in each experiment. One-way ANOVA was used to determine differences among group means (SHAM operated control, C2H-untreated (vehicle treated control) and C2H-theophylline treated (experimental animals) followed by Bonferroni post hoc test. In case of comparison between the ipsilateral hemi spinal cord and right contralateral hemi spinal cord in the same animal within an experimental group, Student's t-test was used. A preset value of ($p < 0.05$) was considered statistically significant both for ANOVA and t-test.

3. RESULTS

3.1. C2 hemisection abolishes respiratory-related activity in the ipsilateral hemidiaphragm and theophylline administration partially restores respiratory function

Respiratory-related activity was assessed by electromyographic (EMG) recording in the diaphragm as previously described (Nantwi et al., 1996). The EMG activity is not affected by SHAM operation in both the left and right diaphragms (Fig 1A). Functional completeness of the C2 hemisection resulted in an absence of EMG activity in the left hemidiaphragm (LHD, ipsilateral diaphragm). In marked contrast, activity on the intact or right hemidiaphragm (RHD, contralateral) is evident (Fig 1B). Administration of theophylline (C2H-T Day 5) partially restored function to the paralyzed left diaphragm (compare LHD in Fig 1B and Fig 1C). Recovery persisted after cessation of theophylline for 12 days (C2H-T Day 17, Fig 1D). All figures (1B–D) illustrate qualitative aspects of drug-induced functional improvement that support and extend our previous findings (Nantwi, et al., 1996). Here we would like to emphasize that recordings were conducted in rats under spontaneously-breathing conditions.

3.2. C2 hemisection increases neurotrophic and pro-inflammatory gene expression in the C3-C6 segments of the ipsilateral hemi spinal cord

C2 hemisection abolishes the respiratory activity in the injured spinal cord and theophylline partially restores the activity (Fig 1). The levels of neurotrophic and survival factors (BDNF, GDNF, and Bcl2) and pro-inflammatory mRNA expression (TXNIP, IL-1 β and iNOS) in the left (injured) ipsilateral hemi spinal cord and the right (uninjured) contralateral hemi spinal cord were not altered in sham operated rats (SHAM Day 2, Fig 2), a pattern that

correlated with the electrophysiologic assessment of respiratory activity in SHAM Day 2, (Fig 1A and (Fig 2).

However, after C2 hemisection (C2H Day 2), there were significant increases in BDNF, GDNF and Bcl2 mRNA levels in the ipsilateral hemi spinal cord ($p < 0.05$ vs. the contralateral hemi spinal cord (Fig 3A). Similarly, C2 hemisection enhanced expression of pro-inflammatory TXNIP ($p < 0.05$) and iNOS ($p = 0.02$) in the ipsilateral hemi spinal cord compared to the contralateral hemi spinal cord (Fig 3B). However, no significant changes in expression of IL-1 β were evident, although there was a trend towards increased levels.

Theophylline treatment (C2H-T Day 5) further increased GDNF and Bcl2 expressions while the level of BDNF mRNA remained at levels similar to that observed in C2H Day 2 rats (Fig 3C). Theophylline treatment significantly increased TXNIP and IL-1 β levels ($p < 0.05$, $n = 6$) however; iNOS levels were reduced (Fig 3D). In summary, neurotrophic factor expression especially BDNF (and also GDNF and Bcl2) and pro-inflammatory gene expressions were enhanced by injury and theophylline in the ipsilateral hemi spinal cord. Levels of iNOS however, appeared to decrease.

In order to more thoroughly characterize changes in expression of the various molecular factors, we analyzed expression of BDNF, Bcl2 and TNF- α in the ipsilateral hemi spinal cord and contralateral hemi spinal cord separately in SHAM operated controls, C2H vehicle only treated controls (C2H-UNT), and C2H theophylline treated (C2H-T) animals at day 5. In this series, we compared changes in the homolateral spinal cord in all groups of rats at identical periods. As shown in Fig 4A, BDNF expression in the ipsilateral hemi spinal cord is significantly increased ($p < 0.05$) both in C2H-UNT and C2H-T 5 (theophylline treated 3 days and weaned for 2 days) when compared with SHAM. In the contralateral hemi spinal cord, BDNF expression is reduced in both C2H-UNT control and C2H-T compared with SHAM Day 5 (Fig 4B). Expression of Bcl2 is increased in ipsilateral hemi spinal cord (Fig 4C) but down-regulated in the contralateral hemi spinal cord (Fig 4D).

Pro-inflammatory TNF- α expression is not significantly changed in the ipsilateral hemi spinal cord among SHAM, C2H-UNT and C2H-T cord; however it is down-regulated in C2H-T contralateral hemi spinal cord (Supplementary Fig S1A and S1B). In addition, we also measured the mRNA levels of iba-1 and GFAP, as markers of microglial and astrocyte activation in C2 hemisected cervical spinal cords. Microglia marker, iba-1 mRNA level is not significantly changed both in ipsilateral and contralateral cords although there is an increasing trend in both sides upon C2 hemisection (Fig S1C and S1D). GFAP an astroglial activation marker, level shows a decrease in C2H-UNT compared with SHAM but is not different in C2H-T (Fig S2A and S2B). Our results show that following C2 hemisection, responses for gene expression for neurotrophic factors and pro-inflammatory genes in both the ipsilateral hemi spinal cord and contralateral hemi spinal cord are different.

3.3. Theophylline enhances the DNA binding activity of transcription factors CREB and NF- κ B in the ipsilateral hemi spinal cord

We examined expression of CREB and its phosphorylation activation site Ser 133 on Western blots and the DNA binding activity with Electrophoretic Mobility Shift Assay (EMSA). As shown in Fig 5A, the DNA binding activity of CREB is enhanced (~2-fold) in the ipsilateral hemi spinal cord (C2H-T Day 5) compared with the contralateral hemi spinal cord. This is indicated by the presence of a shift in the electrophoretic mobility of the free biotinylated CRE (lane P). In addition, the protein level of CREB (Fig 5B) and the phosphorylation at Ser 133 of CREB (Fig 5C) are also enhanced in the ipsilateral hemi spinal cord vs. the contralateral hemi spinal cord. The specificity of the reaction is further demonstrated by the absence of the biotin-labeled CRE shift in the presence of competitive

cold CRE probes (lane C). The activation of the CREB DNA binding activity in the ipsilateral hemi spinal cord correlates positively with its potential gene target expression for BDNF, GDNF and Bcl2 (Fig 5C and Fig 3C).

We examined the DNA binding activity of the transcription factor NF- κ B in the C2H model after theophylline treatment for 3 days (C2H-T Day 5). As shown in Fig 6, theophylline increased the mobility of the biotin-labeled NF- κ B probe in the ipsilateral hemi spinal cord when with the contralateral hemi spinal cord. The specificity of NF- κ B binding is further demonstrated by the absence of biotin-labeled probe in the presence of competitive cold probes.

3.4. Theophylline increases BDNF protein levels in the ipsilateral hemi spinal cord

Since BDNF is important for neuronal survival and synaptic regeneration in development and disease, we sought to determine whether BDNF and survival factor expression are sustained during respiratory activity after weaning from theophylline treatment (C2H-T Day 17). As shown in Fig 7A, BDNF mRNA levels remained significantly enhanced (2.2 ± 0.43 ; $p < 0.05$; $n = 5$) in the ipsilateral hemi spinal cord compared with the contralateral hemi spinal cord. Note that all other genes are similar in levels ($p = 0.5$).

We further assessed expression of BDNF message over time (Day 17) in SHAM controls, C2H-UnT and C2H-D17-T. As shown in Fig 7B, BDNF expression is significantly increased in the ipsilateral hemi spinal cord in C2H-UnT and C2H-T compared to SHAM ($P < 0.05$). In the contralateral hemi spinal cord, BDNF expression is also elevated in C2H-UnT (although not significant) compared with SHAM controls, but reduced in C2H-D17-T compared with C2H-UnT controls (Fig 7C). The decrease in BDNF expression in C2H-D17-T in the contralateral hemi spinal cord may be reflected in a significant increase in BDNF levels in the ipsilateral hemi spinal cord (Fig 7A).

With IHC, we observed that BDNF staining was of greater intensity in the SHAM Day 2 control spinal cord neurons (Fig 8A) and was reduced following hemisection (C2H Day 2) (Fig 8B). After theophylline treatment for 3 days (C2H-T Day 5), BDNF staining appeared to increase in the neurons (Fig 8C) and the staining appeared to be sustained after cessation of theophylline (C2H-T Day 17, (Fig 8D). However, it should be noted that the level of BDNF (in C2H Day 2) was lower than in SHAM Day 2 (compare Fig 8A vs. Fig 8B). We observed hypercellularity of satellite cells in the injured cord, i.e., higher number cell nuclei in the C2 hemisected cord (~ 1.43 fold) than in sham-operated controls. To further investigate the effect of hypercellularity, we examined expression of pro-inflammatory cytokine TNF- α which is important in inflammation and neurodegeneration. As shown in Fig 9A, TNF- α mRNA levels were unchanged both in SHAM Day 2 and C2H Day 2 spinal cord segments. However, theophylline treatment significantly enhanced TNF- α mRNA in C2H-T Day 5 (4.7-fold, $p < 0.05$, $n = 6$) and in C2H-T Day 17 its expression was still high (1.5 fold, $p < 0.05$, $n = 6$).

TNF- α expression was analyzed in SHAM control, C2H-UNT and C2H-T at Day 17 (similar to Fig S1 for Day 5). As shown in Fig. 9B, TNF- α expression remains significantly elevated at day 17 in the ipsilateral hemi spinal cord in C2H-UnT and C2H-T compared with SHAM control ($p < 0.05$), however; no difference is observed between C2H-UnT and C2H-T ($p = 0.35$). In the contralateral hemi spinal cord, C2H-D17-T shows a significant reduction in TNF- α ($p < 0.01$) compared with SHAM control. There is no significant difference between C2H-UnT and C2H D17-T ($p = 0.11$). A similar pattern is also observed for iba-1 (Fig S3A-B).

On the other hand, GFAP level (astroglial activation marker) is unchanged in the ipsilateral hemi spinal cord in C2H-UNT but has a significant decrease in the C2H -D17-T (Fig S3C). GFAP mRNA level is significantly reduced in the contralateral hemi spinal cord both in C2H-UNT and C2H-T compared with SHAM (Fig S3D).

4. DISCUSSION

The results from the present study confirm and extend previous observations that theophylline induces and maintains functional recovery even in the absence of detectable levels of serum of the drug (Nantwi et al., 1996; Nantwi et al., 2003, Fig 1). The present study provides evidence of putative molecular signals that may be important in functional recovery and suggests novel therapeutic strategies to improve recovery after upper cervical SCI. Specifically; our results demonstrate that in the C2H model, neurotrophic and pro-inflammatory gene expression are induced in the ipsilateral side of the spinal segments C3-C6 compared to the contralateral side. We now extend findings from our previous studies of theophylline-induced recovery. The findings described in this investigation are in agreement with previous reports that injury to the spinal cord leads to inflammation and neurotrophic factor expression (Kroner, 2011; Côté et al., 2011; Deng et al., 2011). Specifically, we identify neurotrophic and survival gene expression for BDNF, GDNF, and Bcl2 as factors that may mediate and sustain recovery (Figs 2–4). Furthermore, pro-inflammatory gene expression for TXNIP, IL-1 β and TNF- α are activated while iNOS is suppressed (Figs 2–4). More importantly, we demonstrate that gene activation is associated with enhanced DNA activity of transcription factors CREB and NF- κ B that are known to be involved in neuronal survival and inflammation (Figs 5–6).

Theophylline is a non-selective PDE inhibitor and a nonspecific adenosine receptor antagonist that blocks adenosine A₁ and A_{2a} receptors. Blockade of the A₁ receptor leads to enhanced intracellular cAMP levels (Feldman et al., 2003; Walteriet and Weller, 2003). cAMP-activated protein kinase PKA phosphorylates downstream target cyclic AMP response element binding transcription factor CREB at Ser 133 and enhances its DNA binding activity and gene transcription (Singh et al., 2004; Perrone et al., 2010). This is also evident from our results that theophylline treatment of C2 hemisected rats for 3 days increases total CREB, phosphorylation of CREB at Ser 133 and its CRE binding activity (See Fig 5). Theophylline-induced increased expression of total CREB, phosphorylation of CREB at Ser 133 and its CRE-binding activity reported in this study, may be associated with increases in neurotrophic and survival factors such as BDNF, GDNF and Bcl2 that are known targets of CREB as suggested in other studies (Chen et al., 2006; Hoh et al., 2010; Suzuki et al., 2011). Specifically, our findings indicate that theophylline may be acting through the CREB signaling pathway to target BDNF, GDNF, and Bcl2 expression. In addition our results also suggest that theophylline enhances TXNIP and IL-1 β , in the ipsilateral segments of C3-C6 of the C2 hemisected spinal cord. Expression of these factors correlates with pro-inflammatory and survival transcription NF- κ B activation. The results from the present investigation suggest that theophylline may activate both CREB and the transcription factor NF- κ B which lead to downstream target gene expression for neurotrophic factors and pro-inflammatory genes, both of which are necessary for damaged tissue removal, survival and regeneration of injured neuronal cells. In addition, we demonstrate that the contralateral hemi spinal cord also undergoes changes in gene expression for neurotrophic factor and pro-inflammatory genes. While an early resolution of inflammation might occur in the contralateral hemi spinal cord compared to the ipsilateral hemi spinal cord, the influence of injury on the ipsilateral hemi spinal cord may still limit neurotrophic factor expression in the contralateral cord (e.g., BDNF in Fig 7C). In addition, we also observe a reduction in BDNF protein staining in the contralateral cord (unpublished data), which is more or less similar to that observed with the ipsilateral cord in Fig 8.

Interestingly, we observed that iNOS level is down regulated after theophylline treatment after C2 hemisection (See Fig 3D). The apparent theophylline-induced down regulation of iNOS may be important in diminishing long-term secondary neuronal damage in acute CNS injury. Inducible NOS is responsible for excess nitric oxide production in injured tissue and causes oxidative and nitrosative stress, which may be detrimental to neuronal survival (Emirandetti et al., 2010). Thus, suppression of iNOS expression after theophylline treatment combined with activation of BDNF and Bcl2 could be an early critical event in neuronal survival and recovery processes. Conversely, an activation of TXNIP and IL-1 β initiates the inflammatory process, which may be subsequently resolved by a cAMP/CREB-mediated neuronal survival and anti-inflammatory process. Perrone and colleagues (2010) recently demonstrated that TXNIP-NF κ B-IL-1 β axis is involved in recovery of partial sciatic nerve injury and primary Schwann cell activation. The findings in the present study that demonstrate elevated levels of the TXNIP-NF- κ B-IL-1 β pathway are in agreement with the findings reported by Perrone and colleagues (2010). TXNIP is an endogenous inhibitor thioredoxin, an anti-oxidant protein. TXNIP has been reported as a danger signal for cellular injury and metabolic dysregulation. It has been shown that activation of TXNIP can lead to innate immune responses via an induction of pro-IL-1 β and its maturation and secretion by NLRP3 inflammasomes (Zhou et al., 2010). The role of TXNIP and NLRP3 inflammasomes, which activates caspase-1 and cleaves pro-IL1 β to form the mature protein, in recovery of respiratory function in the C2H model has yet to be investigated. Furthermore, we also realize that the induction of pro inflammatory genes is measured at the message level and may not always have a direct correlation with protein levels. However, we have recently shown that protein and mRNA levels of these proinflammatory genes correlate positively in partial sciatic nerve injury recovery (Sbai et al., 2010). Therefore we anticipate that the observation of increases in mRNA levels in proinflammatory genes in this study will also correlate with protein levels. Nonetheless, in future studies we will measure protein levels as part of a larger investigation.

BDNF is important for neuronal survival and plasticity in CNS injury including SCI (Nakajima, 2010). Involvement of BDNF in respiratory activity has been previously described in a form of respiratory plasticity known as longterm facilitation directly or indirectly by adenosine A_{2A} agonist-induced transactivation through a specific receptor (TrK) and mimics BDNF (Baker-Herman et al., 2004, Golder et al., 2008). However; the findings reported in the present study are the first to describe a drug-induced partial recovery of respiratory activity (in the CH2 model) mediated through activation of the transcription factor, cAMP responsive element (CRE) binding protein (CREB) and expression of neurotrophic factor (BDNF, GDNF) and survival factor Bcl2. We observed that BDNF protein is reduced after CH2; however, theophylline administration reverses the decrease and actually enhances BDNF expression. The levels however, do not reach those observed in naïve rats (Fig 8). The mRNA level is activated even prior to theophylline administration (Fig 3A). Both BDNF mRNA and protein expression remain up-regulated even after the termination of drug treatment, and this may play an important role in neuronal survival and plasticity in recovery of respiratory activity. Enhanced BDNF mRNA and protein expression combined with enhanced expression of GDNF and Bcl2 may be responsible for phrenic motor neuron (localized in C3-C6 spinal cord segments) survival and viability in SCI as described in this study. Nonetheless, we also observed that glial activation may persist and the inflammation resolution is incomplete. This is evident by our observation that there still exists hyper-cellularity of satellite cells (probably both glial and infiltrating blood monocytes) in the ipsilateral hemi spinal cord (Fig 8). It is likely that enhanced mRNA level of pro-inflammatory and pro-apoptotic TNF- α underlies the hyper cellularity observed (Fig 9). Activated astrocytes and microglia produce TNF- α in neural inflammation (Li et al., 2011). It must be pointed out however that, while glial activation is necessary for tissue healing and establishment of homeostasis after acute injury, chronic glia reactivity/activation

can sustain inflammation, tissue scarring, and impede neurite regeneration. This is true to some extent in the C2H model of SCI since we observed that respiratory recovery after theophylline treatment is only partial in agreement with previous studies (Nantwi et al., 1996; 2003) and BDNF expression itself does not achieve the level of sham-operated controls (Fig 8). These results suggest that BDNF may at least in part, play an important role in C2H to induce and sustain recovery of respiratory activity after injury. In addition, our results also show that prolonged glial activation, TNF- α expression and inflammation following theophylline treatment may persist in the C2 hemisectioned cord. The apparent persistence of TNF- α expression may limit the magnitude/extent of recovery post injury (Genovese et al., 2010). The latter results may offer insights into how to harness the apparently beneficial effects of neurotrophic factors (BDNF in particular) while attenuating the restrictive effect of TNF- α . This is because we observed a persistent TNF- α and iba-1 expression even after theophylline treatment (Fig 9B and Fig S3) indicating presence of activated microglia following injury to the spinal cord.

Further studies using combination drug therapies that will endeavor to enhance BDNF expression and simultaneously inhibit TNF- α (and/or microglial inactivation) may be important to restore near complete respiratory function in the SCI. In this regard, it is relevant that in a previous study on the therapeutic potential of theophylline to alleviate respiratory deficits in chronic SCI using the C2H model, Nantwi and colleagues (Nantwi et al., 2003b; Nantwi and Goshgarian, 2005) reported that the drug was ineffective. Based on the present finding that TNF- α levels are enhanced in injury, we speculate that over time (in chronic injury), enhanced levels of TNF- α may persist and can potentially limit the efficacy of the drug in improving respiratory function. In fact, it has been previously suggested that TNF- α is important in chronic inflammation (Tzeng et al., 1999; Paterniti et al., 2011). Furthermore, a recent clinical study on the efficacy of the drug to improve inspiratory capacity provided mixed results in the beneficial effects of theophylline in improving function in a population of chronic SCI patients (Tzelepis et al., 2008). The apparent ineffectiveness of theophylline to improve respiratory function in chronic injury remains intriguing. Based on our data we propose that a combinatorial approach that will specifically target (inhibit) TNF- α expression while enhancing BDNF, Bcl2 and/or GDNF expression may be an attractive strategy to further improve function after injury. In this case, we propose that lithium, an inhibitor of protein kinase glycogen synthase kinase (GSK) 3 β and activator of CREB phosphorylation, may be utilized either alone or sequentially after theophylline treatment in improving SCI recovery. The fact that both drugs appear to activate CREB phosphorylation, albeit via different molecular signaling pathways makes this an attractive line of investigation. Thus far, initial studies assessing effects of lithium chloride in restoring function in the C2H model are promising (Nantwi et al., 2011).

In conclusion, we provide the first evidence that drug-induced respiratory recovery in the C2H model is associated with induction of both neurotrophic factors and pro-inflammatory genes. BDNF (and Bcl2) may play a crucial role in motor neuron survival and neuronal plasticity. Simultaneously, a near complete resolution of inflammation and gliosis may prevent glial scarring and an accumulation of inhibitory extracellular matrix components that prohibit axonal and dendritic reconnection in the healing process (Alilain et al., 2011). In this respect, targeted inhibition of TNF- α may be important. Thus, our results provide potential gene targets and intracellular signals that might be successfully exploited for gene and drug therapies to elevate respiration in upper cervical cord injury and paralyzed patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Theophylline induces functional recovery.
2. Theophylline induces TXNIP and IL-1 β and suppresses iNOS in C3-C6 segments.
3. Theophylline enhances expression BDNF, GDNF and Bcl2
4. Theophylline activates CREB and NF κ B.

Fig 1. Electrophysiologic tracings

In each set of tracings, the upper tracing represents the raw neurogram of activity in the right hemidiaphragm (RHD) and the lower tracing, activity in the left hemidiaphragm (LHD) **A.** Representative tracings from a SH rat (48 hr, SHAM Day 2). **B.** Tracings from a C2 hemisected rat after 48 hr (C2H Day 2). Note the total quiescence in the LHD, indicating a functionally complete hemisection. **C.** After theophylline treatment (C2H-T Day 5) recovered activity in the LHD is evident (compare with upper tracings in B). **D.** After weaning from theophylline, recovered activity in the LHD is persistent (C2H-T Day 17). In all representative groups of rats, respiratory activity was monitored under conditions of spontaneous-respiration. For clarity and consistency, identical recording settings were maintained throughout A–D.

Fig 2. Messenger RNA expression analysis for neurotrophic and pro-inflammatory genes in SHAM operated SCI

Real Time qPCR for neurotrophic factors (BDNF, GDNF, Bcl2) and pro-inflammatory (TXNIP, IL-1 β , iNOS) gene expression in the ipsilateral (left) hemi spinal cord (expressed as Fold mRNA Change) compared with the contralateral (right) hemi spinal cord (C3-C6). Gene expression was normalized by actin. There was no difference in the mRNA level for the above genes between the left and right cord in sham-operated rats 2 days after surgery (SHAM Day 2). The data are expressed as means $\pm$ se of n=5 for each of the left and right cord, $p>0.4$, not significant).

Fig 3. Messenger RNA expression analysis of neurotrophic and pro-inflammatory genes in C2H before (Day 2) and after treatment with theophylline (C2H-T Day 5)

A. The mRNA level of BDNF, GDNF, and Bcl2 in the ipsilateral hemi spinal cord (fold mRNA change) compared with the contralateral hemi spinal cord 2 days after C2 hemisection (C2H Day 2). The ipsilateral hemi spinal cord had significantly higher mRNA levels than the contralateral hemi spinal cord segment. **B.** The expression of pro-inflammatory TXNIP and iNOS was significantly higher in the ipsilateral hemi spinal cord but the level of IL-1 β did not reach a significantly higher value. **C.** Theophylline administration for 3 days only 2 days after C2 hemisection (C2H-T Day 5) further induces neurotrophic factor mRNA expression (BDNF, GDNF, Bcl2). **D.** Pro-inflammatory gene expression for TXNIP and IL-1 β is significantly increased but the iNOS level is reduced. All data (A–D) are presented as mean $\pm$ se; n=6 per group).

Fig 4. Messenger RNA expression analysis of neurotrophic and pro-survival genes in SHAM, C2H-UnT and after treatment with theophylline (C2H-T Day 5)

A. The mRNA level of BDNF in the ipsilateral hemi spinal cord (hemi-SC) C2H-UnT and C2H-T when compared with SHAM control at Day 5 rats. The ipsilateral hemi-SC in both C2H-UnT (2.72 ± 0.5) and C2H-T (5.45 ± 1.55) had significantly higher mRNA levels than the ipsilateral hemi spinal cord in SHAM control ($p < 0.05$, $n = 5$). The difference between C2H-UnT and C2H-T was not significant ($p = 0.12$). **B.** The mRNA level of BDNF in the contralateral hemi-SC (fold mRNA change) in C2H-UnT and C2H-T compared with SHAM control at Day 5 animals. The C2H-UnT cord had a significant decrease in BDNF level (0.17 ± 0.16 , $p < 0.01$, $n = 5$) while C2H-T (0.65 ± 0.49 , $p = 0.31$, $n = 5$) was not significant compared with corresponding contralateral hemi-SC in SHAM control. However, the difference between C2H-UnT and C2H-T was not significant ($p = 0.4$). **C.** The mRNA level of Bcl-2 in the ipsilateral hemi-SC: The ipsilateral hemi-SC in both C2H-UnT (3.2 ± 0.78) and C2H-T (2.22 ± 0.33) had significantly higher mRNA levels than the ipsilateral hemi-SC in SHAM control ($p < 0.05$, $n = 5$) while C2H-UnT vs. C2H-T was insignificant ($p = 0.23$). **D.** The mRNA level of Bcl-2 in the contralateral hemi-SC: The contralateral hemi-SC in both C2H-UnT (0.27 ± 0.13) and C2H-T (0.07 ± 0.03) had significantly lower mRNA levels than the SHAM contralateral ($p < 0.01$, $n = 5$) while C2H-UnT vs. C2H-T was insignificant ($p = 0.24$).

Fig 5. EMSA and Western blot of CREB in C2 hemisected spinal cord segments

A. The DNA binding activity of CREB in spinal cord segments was measured by EMSA using nuclear extracts (5 μ g) and biotin-labeled DNA probes with or without a competitive cold DNA probe as described in the Methods Section. Protein-DNA complexes were detected by streptavidin-HRP and ECL in Cell Bioscience EM. Free CRE probe moved faster towards the bottom of the gel (P, probe alone) and complex of the DNA probe with nuclear factor retarded their mobility. Ipsilateral hemi-SC C3-C6 segments showed increased CREB-DNA complex compared with the corresponding contralateral segments. In the presence of competitive (C) cold probes, the reactive band is abolished indicating specificity

of the CREB-DNA binding. **B.** Thirty μ g nuclear proteins were analyzed by Western blotting on 12% PAGE for CREB. Total CREB level increased in the ipsilateral segments than the contralateral cord. **C.** Similarly, densitometric analysis of the phosphorylation of CREB at Ser 133 results in $\sim 1.6 \pm 0.17$ fold increase ($p < 0.01$, $n = 5$) in the ipsilateral hemi-SC compared to the contralateral segments. We also observed an additional band below pCREB, which represents another transcription factor, pATF, closely related to pCREB and cross-reacting with the phospho-CREB antibody. All figures (A–C) are representative of $n = 5$.

Fig 6. EMSA of NF-κB in C2 hemisected spinal cord segments

The DNA binding activity of NF-κB in spinal cord segments was measured by EMSA using nuclear extracts (5 μg) of C2H Day 5, and biotin-labeled κB DNA probes with or without a competitive cold DNA probe as described in Methods. Protein-DNA complexes were detected by streptavidin-HRP and ECL in Cell Bioscience EM. Free κB probe moved faster towards the bottom of the gel (P, probe alone) and the complex of the DNA probe with nuclear factors retarded their mobility. Ipsilateral hemi-SC shows increased NF-κB-DNA complex compared with the contralateral hemi-SC. In the presence of competitive (C) cold probes, the reactive band is abolished indicating specificity of the NF-κB-DNA binding. Band densitometric analysis reveals ~2.16±0.29 fold increase in NF-κB-DNA binding in the ipsilateral cord compared to the contralateral cord ($p<0.01$, $n=5$).

Fig 7. Messenger RNA expression analysis for neurotrophic and pro-inflammatory genes in C2H-T Day 17 rat cervical spinal cord segments

A. The mean BDNF expression in the ipsilateral cord remains elevated significantly (2.2 ± 0.43 folds, $n=5$, $p=0.05$) when compared with the contralateral cord. All other genes are normalized to the level of the contralateral cord. **B.** The mRNA level of BDNF in the ipsilateral hemi-SC (fold mRNA change) in C2H-UnT and C2H-T when compared with SHAM control at Day 17 animals. The ipsilateral hemi-SC in both C2H-UnT (8.12 ± 2.44 , $p=0.05$, $n=5$) and C2H-T (12.09 ± 8.8 , $p=0.05$) had higher mRNA levels when compared with the ipsilateral hemi-SC in SHAM. The difference between C2H-UNT and C2H-T was not significant ($p=0.7$). **C.** The mRNA level of BDNF in the contralateral hemi-SC: The

C2H-UnT hemi-SC had an increase in BDNF mRNA level (5.85 ± 2.99 , $p=0.38$, $n=5$) while C2H-T (0.35 ± 0.14 , $p<0.12$, $n=5$) vs. corresponding contralateral hemi-SC in SHAM controls. However, the difference between C2H-UnT and C2H-T was not significant ($p=0.23$).

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Fig 8. IHC of BDNF expression in ipsilateral segments of Sham and C2 hemisected cords
A. IHC staining of BDNF in sham-operated left spinal cord (SHAM Day 2); **B.** C2H Day 2; **C.** C2H Day 5; and **D.** C2H Day 17. There is an apparent reduction in BDNF level in the ipsilateral hemi-SC at C2H Day 2 compared with SHAM Day 2. Theophylline treatment for 3 days enhances BDNF expression at Day 5 (C2H-T Day 5), which persists at C2H-T Day 17. However, BDNF staining is higher in Sham operated spinal cord than C2 hemisected cords. Thick arrows (yellow) indicate BDNF staining in the spinal cord neurons. Thin arrows (green) indicate glial cell nuclei in the spinal cord. Note the enhanced level of cellular nuclei in the C2H (Day 2) compared to sham control. We counted nuclei in at least 4 different areas in all sections. There was ~1.43 fold higher in number of nuclei in the C2 hemisected cord than in the sham operated cord. A representative of n=3 is shown here. (Mag. =400 $\times$, bar=50 μ m).

Fig 9. Effect of theophylline on the mRNA level of pro-inflammatory cytokine TNF- α in C2 hemisected spinal cord
(A) Real Time qPCR for pro-inflammatory TNF- α gene expression in the ipsilateral cord segments (C3-C6). There was no difference in the mRNA level of TNF- α between left hemi-SC and right hemi-SC in SHAM Day 2 and left C2H Day 2 animals. However, theophylline treatment for 3 days (C2H-T Day 5), significantly increased TNF- α expression in the ipsilateral hemi-SC (4.7 ± 1.5 fold, $p=0.03$, $n=6$) compared with the contralateral hemi-SC. Interestingly, even after weaning from theophylline for 12 days, there was significantly enhanced TNF- α expression (1.5 ± 0.14 fold, $p=0.03$, $n=6$) when compared with the contralateral cord. **B.** The mRNA level of TNF- α in the ipsilateral hemi-SC (fold

mRNA change) in C2H-UnT and C2H-T compared with SHAM control at Day 17 animals: The ipsilateral hemi-SC in both C2H-UnT (4.03 ± 0.88 , $p < 0.05$, $n = 5$) and C2H-T (3.25 ± 0.25 , $p = 0.02$, $n = 5$) had higher mRNA levels than the ipsilateral hemi-SC in SHAM control. The difference between C2H-UnT and C2H-T was not significant ($p = 0.35$). C. The mRNA level of TNF- α in the contralateral hemi-SC: The C2H-UnT hemi spinal cord had no significant change in BDNF mRNA level (0.45 ± 0.88 , $p = 0.21$, $n = 5$) while C2H-T (0.2 ± 0.01 , $p < 0.01$, $n = 5$) vs. corresponding contralateral hemi-SC in SHAM. However, the difference between C2H-UnT and C2H-T was not significant ($p = 0.11$).