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Neuroprotective effect of cocaine- and amphetamine-regulated transcript peptide in spinal cord injury in mice

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ABSTRACT

We explored the effect of cocaine- and amphetamine-regulated transcript peptide (CART), alone and in combination with methylprednisolone (MP), on the cellular pathology and locomotor recovery of mice following spinal cord injury (SCI). While cellular pathology was evaluated in terms of spinal cord histology and profile of astrocytes following immunolabeling with antibodies against glial fibrillary acidic protein (GFAP), locomotor recovery was monitored using hindlimb motor function scoring system. At 24 h post-SCI, there was a massive loss of motor function and cysts formation in the spinal cord. The SCI mice, following 3 days and onwards, showed a significant ($P < 0.001$) increase in the population and hypertrophy of GFAP + astrocytes, suggesting the occurrence of reactive astrogliosis. Intra-fourth ventricular administration of CART (54–102) or intravenous treatment with MP, dose dependently improved motor function score, while CART-antibody (intra-fourth ventricular) was ineffective. This neuroprotective effect of MP was potentiated by the subeffective dose of CART and antagonized by CART-antibody. CART or MP treatment not only prevented the cysts formation, but also significantly attenuated the population of GFAP + astrocytes at days 3, 7, 14, 21 and 28 post-SCI and the hypertrophy of astrocytes at day 14 and 28. The histological consequence of SCI, like cysts formation in the spinal cord, was rapidly improved by CART and/or MP. Taken together, the data suggest that CART may exert its neuroprotective effect via inhibition of post-SCI astrogliosis and participate in the MP mediated neuroprotection.

1. Introduction

Spinal cord injury (SCI) inflicts severe health costs associated with life-long disabilities. It occurs primarily due to mechanical trauma leading to secondary injury and eventual loss of tissue and functions (Oyinbo, 2011). At cellular level SCI causes astrogliosis, a characteristic cellular response that form barrier to axonal regeneration. Marked upregulation of glial fibrillary acidic protein (GFAP) is a major index of astrogliosis (Eng and Ghirnikar, 1994; Sofroniew and Vinters, 2010; Wang and Bordey, 2008). Therapeutic strategies aim at attenuation of astrogliosis during the initial phase after SCI (Labombarda et al., 2011; Liu et al., 2008; Vitellaro-Zuccarello et al., 2008). Methylprednisolone (MP) is extensively used as a model neuroprotective reference standard (Ates et al., 2006) and an important drug in clinical practice for the treatment of SCI (Tohda and Kuboyama, 2011). However, at higher doses, the steroid produces side effects like sepsis and pneumonia (Gerndt et al., 1997). To circumvent these complications, a range of other potential neuroprotective agents like progesterone, erythropoietin, alpha-melanocyte stimulating hormone, brain derived neurotropic factor (BDNF) are being investigated in preclinical trials (Labombarda et al., 2011; Lankhorst et al., 1999; Vitellaro-Zuccarello et al., 2008).

Cocaine- and amphetamine-regulated transcript peptide (CART) is abundantly expressed in the brain and spinal cord (Koylu et al., 1998; Kozsurek et al., 2007). A role for the peptide in energy metabolism, regulation of food intake and reward behavior is well

Abbreviations: ANOVA, Analysis of variance; BDNF, Brain derived neurotropic factor; CART, Cocaine- and amphetamine-regulated transcript peptide; GFAP, Glial fibrillary acidic protein; i.v., Intravenous; i.p., Intraperitoneal; MP, Methylprednisolone; MFS, Motor function score; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; SCI, Spinal cord injury; SEM, Standard error of mean.

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established (Kristensen et al., 1998; Kuhar et al., 2005; Rogge et al., 2008). Using ischemic brain injury model, Xu et al. (2006) proposed neuroprotective role of CART. Neurotrophic effects of CART may be mediated via upregulation of BDNF (Wu et al., 2006), activation of extracellular signal-regulated kinase signaling pathways (Jia et al., 2008) and antioxidant activity (Mao et al., 2007, 2012). Interestingly, a direct correlation between glucocorticoids and CART systems is well established (Balkan et al., 2001; Vicentic et al., 2004; Vrang et al., 2000).

We hypothesized that CART may play a role in preventing harmful effects triggered by neural damage, and promote axonal regeneration. In mouse model of SCI following contusion, we monitored the locomotor recovery using motor function scoring (MFS) scale (Farooque, 2000). Following SCI, while MP, a reference standard neuroprotective agent widely used in preclinical studies, was administered by intravenous (i.v.) route, CART or its antibody was administered into fourth ventricle. With a view to finding out the interactions, if any, CART or CART-antibody was administered prior to MP immediately following SCI. Sections of the spinal cord were stained with Luxol fast blue-cresyl violet solutions to evaluate the tissue damage following SCI, and eventual recovery, with or without various treatments. Further, to probe the underlying mechanism, we monitored the astrogliosis by GFAP immunolabeling of astrocytes in ventral gray horn of spinal cord.

2. Materials and methods

2.1. Subjects

Adult male Swiss-albino mice (25–30 g) were group housed and given free access to food (Trimurti Feeds, Nagpur, India) and drinking water. Following fourth ventricular cannulation and SCI surgery, the mice were housed one animal per cage and were maintained on a 12-12 h light–dark cycle, in controlled temperature (25 ± 2 °C) and relative humidity (50–70%). All surgical procedures were performed under aseptic conditions. The experimental procedures were approved by the Institutional Animal Ethics Committee.

2.2. Cannulation in the fourth ventricle

The stereotaxic placement of cannula targeted at the fourth ventricle was performed as previously described by Bharne et al. (2011). Briefly, mice were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) injected subcutaneously. A permanent stainless steel guide cannula prepared in house (Kokare et al., 2011) was placed aseptically into the fourth ventricle, using stereotaxic coordinates, −5.88 mm posterior and 3.80 mm ventral with respect to bregma (Paxinos and Franklin, 2001). The guide cannula was secured using dental cement and mounting screws anchored to the skull. A stainless steel dummy cannula was used to occlude the guide cannula when not in use (Kokare et al., 2011). Animals were allowed a recovery period of 7 days.

2.3. Surgical procedure for SCI

We employed the weight-drop contusion method to induce and evaluate SCI in mice. In brief, animals were anesthetized and thoraco-lumbar vertebral region was demarcated. Using the intrascapular space as a reference point, the skin and subcutaneous tissues in the thoracic T10-T12 region were incised. The paravertebral muscle fascia was penetrated, and muscles were peeled laterally using blunt dissection forceps. The spinal cord segment at T10-T12 level was exposed by total laminectomy. The animals were subjected to an impact of 5 g weight (stainless steel rod, 3 mm diameter tip, Impactor device for rat and mice, VJ Instruments, Karanja, India) dropped vertically on the center of the exposed spinal cord from the height of 2 cm and allowed to remain for 30 s. In sham-operated mice, the similar procedure was carried out, except that the spinal cord contusion was not performed. The incision was sutured layer to layer using chromic catgut sutures.

Necessary post-operative care of the animals was taken as previously described (Bharne et al., 2011). Briefly, the mice were treated with gentamicin (40 mg/kg, intramuscular) twice daily during the first 3 days as prophylaxis against urinary tract infection. The mice were also injected subcutaneously with 1 ml lactated Ringer's, daily for a period of 10 days. Drinking water, softened chow and regular pellets were provided *ad libitum* in the cages. Bladders were emptied manually twice a day until bladder function returned to normal.

Separate sets of sham- and SCI-operated animals were used for behavioral and immunocytochemical/histological studies detailed below.

2.4. Drug treatments and experimental outline

CART (54–102), and its monoclonal antibody developed in mouse (Thim et al., 1998), were reconstituted in double distilled water and stored as stock solutions at −20 °C. The stock solution was diluted in aCSF containing 0.1% bovine serum albumin and administered via fourth ventricle. Sodium succinate salt of MP (Solu-Medrol; Pharmacia and Upjohn Company, USA) was dissolved in normal saline and administered via tail vein.

Mice were divided into different groups as described below and all the treatments were given between 0900 and 1200 h. Group I: sham-operated ($n = 5$), Group II: mice subjected to SCI ($n = 5$), Group III: SCI mice injected with aCSF ($n = 8$)/saline ($n = 6$), Group IV: treated with single bolus injection of CART (0.1, 0.3 and 0.5 nM/mouse, intra-fourth ventricle, $n = 8$ in each group) 30 min following SCI, Group V: treated with CART-antibody (Dilution 1:500, 5 μl/mouse, intra-fourth ventricle) 30 min following SCI ($n = 7$), Group VI: single bolus injection of MP (20, 30 or 40 mg/kg, i.v., $n = 6$ in each group) 30 min following SCI, Group VII: treated with single bolus injection of CART (0.1 nM/mouse) or CART-antibody (Dilution 1:500, 5 μl/mouse) 30 min after injury and followed by MP treatment (30 or 40 mg/kg, i.v., $n = 8$ in each group) 15 min later. While CART, or its antibody, was administered into the fourth ventricle, MP was injected via tail vein in all protocols. The doses used in the present study were selected on the basis of dose dependent effect of CART, CART-antibody and MP. Hindlimb motor function was observed and scored to evaluate the locomotor recovery of each SCI mouse one day prior to injury and on days 1, 4, 7, 10, 14, 21 and 28 post-injury.

2.5. Assessment of locomotor recovery

While different assays have been employed to screen the hindlimb motor functions of mice following SCI (Basso et al., 2006; Pajoohesh-Ganji et al., 2010; Ung et al., 2007), we adopted the hindlimb MFS system proposed by Farooque (2000) and standardized in our laboratory (Bharne et al., 2011).

This test is based on detection of movement of ankle, knee and hip joints in freely exploring animal in the open field. We also monitored the ability of mice to walk on bars of different widths. The test detects minor deficits that may be otherwise missed in open field and other assessment methods (Farooque, 2000). Individual animal was allowed to freely explore the open and well-illuminated arena (0.7×0.9 m), and observed for 1 min. Parameters like the movements in the hip, knee, and ankle joints, plantar placement, coordination between forelimbs and hindlimb as well as weight bearing capacity were carefully observed and the performance of the mouse was scored accordingly. Briefly, the score 0 was given to the animals if they showed no movement. If barely visible movement at any hindlimb joint (hip, knee or ankle) was observed, then animals were scored 1. Similarly, if movement of one or more hindlimb joints in one or both limbs, but no coordination, alternate stepping movements or weight bearing were observed, then animals were scored 2. Score 3 was given to the animals showing alternate stepping and forward propulsive movements of the hindlimb, but no capability of weight bearing. In these animals, the hindlimbs were externally rotated and they used hindlimbs for forward propulsion. The animals showing ability to bear weight on their hindlimbs and could walk with some deficit (slight external rotation of one or both limbs and/or hip instability) were scored 4. Herein, the plantar placement of the hindlimb was observed. However, if this deficit in animals was not observed, and the animals showed reduced mobility, then they were scored 5.

Those mice with score 5 were then placed on the bars of different widths (2, 1.5, 1, 0.7 and 0.5 cm) and the walking of the animal without any slips, in at least two trials, was recorded. In addition, even if mice showed inability to walk on the bars and tried to climb under the bar, it was considered as a failure. Herein, the animals were scored 6, if they were able to walk on bars of width 2 cm. Similarly, scores 7, 8, 9 and 10 were given to animals walking on the bars of 1.5, 1, 0.7 and 0.5 cm widths, respectively. During the study, some mortality was observed (<10%) across the different groups; data from such animals were not considered for the statistical analysis.

The effect of CART on spontaneous locomotor activity of individual mouse was monitored using an actophotometer (VJ Instruments, Karanja, Maharashtra, India). An actophotometer records the interruption of intersecting photocell beams evenly spaced along the opposite walls of the test chamber of diameter 40 cm. Two 5 min tests were conducted, one immediately following administration of CART (0.5 nM/mouse, intra-fourth ventricular), and other after an interval of 24 h.

At the end of each experiment, the mice were euthanized using high dose of thiopentone sodium [70 mg/kg; intraperitoneal (i.p.)] and their brains were removed, sectioned, stained with cresyl violet, and the injection sites targeted at fourth ventricle were verified under a light microscope as described in our earlier studies (Bharne et al., 2011). The data of animals with incorrect placement were excluded from the study.

2.6. Immunohistochemistry

Spinal cord sections at days 3, 7, 14, 21 and 28, post-SCI time points from different treatment groups and sham-operated mice were processed for GFAP immunostaining ($n = 5$ mice per group). Briefly, mice were anesthetized using