Propentofylline after focal cortical lesion in the rat: impact on functional recovery and basic fibroblast growth factor expression

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Abstract

The effects of propentofylline, a xanthine derivative and adenosine transport inhibitor, were evaluated following anteromedial cortex lesion in the rat. Propentofylline (2 x 10 mg/kg, intraperitoneally) was administered for 7 days post-insult and basic fibroblast growth factor (bFGF) immunoreactivity measured at designated time points in the peri-lesional cortex and ipsilateral dorsal striatum. The spatiotemporal pattern of bFGF expression was then compared to functional recovery patterns. Propentofylline-treated animals displayed increased bFGF expression in the peri-lesional cortex which may have contributed to the observed early facilitation of functional recovery. Drug administration did not, however, produce a change in bFGF expression in the ipsilateral dorsal striatum compared to saline-treated animals. These findings taken together with other positive findings regarding propentofylline, support the drug’s therapeutic potential.

Keywords: Growth factor; Cortical injury; Recovery of function; Somatosensory asymmetry; Cortical insult; Adenosine; Trauma

Annually, there are an estimated two million traumatic brain injuries in the United States, of which 366 000 require hospital admission and 75 000 result in death [13]. A significant number of brain injury survivors experience life-long disabilities, with functional recovery being variable and dependent on a variety of factors including age, time since injury and size of insult [12]. In addition, the post-injury neural response cascade, as well as its modulation, are important factors. Primary cell death and denervation of undamaged regions trigger this response cascade, which includes glial cell activation, growth factor upregulation and secondary cell death, followed by dendritic arborization and synaptogenesis [12]. The timing of this cascade may parallel post-injury critical or sensitive periods within which the recovery process is vulnerable.

A critical period, lasting from 12 h to 6 days post-insult, has been identified following unilateral electrolytic lesion of the anteromedial cortex in the rat [9,17]. During this time, certain experimental manipulations will affect functional outcome, while these same manipulations administered after this time do not impact the recovery process. We have previously reported that an important marker of the post-injury response cascade, basic fibroblast growth factor (bFGF), is elevated and peaks (on day 6) during this post-lesion critical period [3]. Upregulation of this marker in the ipsilateral striatum and cortex precedes recovery from somatosensory deficits, which is most dramatic after post-operative day 9 and complete by day 19. The early post-lesion expression of bFGF, through its neurotrophic properties, could be responsible for subsequent functional recovery.

bFGF is a polypeptide with potent neurotrophic effects on neurons and glial cells [20]. Following central nervous system (CNS) injury, however, bFGF has been found to upregulate [3], increasing mitogenesis in astrocytes, promoting survival and outgrowth of neurons and thereby alleviating injury-associated neurotoxicity [15]. The importance of this growth factor for functional recovery and neuroplasticity has been shown by Rowntree and Kolb [16], who blocked post-lesion bFGF expression and reported dendritic atrophy and impaired functional recovery. The exogenous administration of bFGF following CNS injury has been reported to increase cell survival [1], as well as facilitate recovery of function [11].
Given bFGF’s important post-injury role, it would be useful to identify a drug with an established safety profile in humans that could be administered following brain injury to not only increase bFGF, but also improve functional outcome. Pentoproyline (PROP: MWA 285, 3-methyl-1-(5-oxy-hexyl)-7-propylxantaine) is a xanthine derivative that has been shown to reduce the pathological activation of glial cells [18], while at the same time enhance nerve growth factor (NGF)[19], facilitate functional recovery [4] and decrease cell death following CNS insult [8]. Though it may seem counterintuitive that diminished glial cell activation could also result in increased NGF, a possible explanation for this is that perhaps not all glial cells pathologically activated due to CNS insult produce growth factors. Alternatively, the drug PROP may only reduce the pathological activation in astrocytes that are not upregulating to produce certain growth factors. Regardless, PROP’s apparent potentiation of NGF opens the possibility that it might upregulate other growth factors, such as bFGF. Indeed, it has been reported that both NGF and bFGF upregulate after CNS insult through the same mechanism in reactive astrocytes [14].

Given the neuroprotective effects of PROP and the significance of bFGF after CNS injury, we hypothesized that PROP administration following brain insult would increase bFGF expression and facilitate functional recovery.

To test this, male Long–Evans hooded rats (Harlan–Gibco, Indianapolis, IN) weighing 220–270 g on the day of surgery, were maintained on a 12/12 h light/dark cycle (lights on 06:00–18:00 h), with food/water provided ad libitum and temperature controlled at 21 ± 1°C. Daily handling began 24 h after arrival, when animals were group housed. One week later, animals were individually housed.

Under equithesin anesthesia (0.46 ml/100 g; a mixture of 140 mg/kg chloral hydrate and 30 mg/kg pentobarbital) animals were placed in a stereotaxic apparatus (Stoelting Instruments) and sustained a unilateral electrolytic lesion of the anteromedial cortex [17] as has been described elsewhere [3].

Rats were randomly assigned to the drug (PROP, graciously donated by A. Sickmüller and A. Fabel from Aventis, formerly Hoeschst Marion Roussel) or no drug (saline vehicle) groups. A 7 day treatment regimen (twice daily 0.9% saline or PROP (10 mg/kg)) began 24 h following surgery. This dose was chosen because others have found it to be neuroprotective [5,8] and to facilitate functional recovery [4] following ischemia.

Somatosensory asymmetry and behavioral recovery were assessed beginning 24 h after the lesion and before drug administration, using the bilateral tactile stimulation tests developed to detect behavioral deficits easily and reliably in the animals’ home cage [17]. The bilateral tactile stimulation tests are administered sequentially: the first test detects the presence of a somatosensory response bias, while the second test assesses the severity or magnitude of that deficit. These tests were administered pre- and post-operatively according to methods described previously [3,17]. Briefly, distinct sizes of adhesive-backed paper were placed bilaterally on the radial aspect of each forelimb, after which, rats were returned to their home cage and the latency to contact and remove each stimulus with the teeth was recorded. Animals displaying an ipsilateral asymmetry in response to equally sized adhesive stimuli were given the second, more sensitive test to determine the magnitude of their asymmetry (e.g. deficit). This was done by varying the size of the contralateral and ipsilateral stimuli. When the size of the non-preferred (contralateral) stimulus was sufficiently larger than the size of the preferred (ipsilateral) stimulus, the ipsilateral response preference was neutralized or reversed. The contralateral/ipsilateral stimulus size ratio that reverses any lesion-induced preference is used as an estimate of the magnitude of the somatosensory asymmetry. The greater the contralateral/ipsilateral stimulus size ratio required to reverse the ipsilateral bias, the more impaired the animal is.

Animals were sacrificed for immunohistochemistry at one of the following times: 2, 4, 6, 8 or 29 days after surgery. Basic procedures for immunohistochemistry have been described in detail elsewhere [3,16]. Briefly, following an overdose of Nembutal, rats were perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted and post-fixed in the 4% paraformaldehyde solution for 48 h after which 50 μm coronal sections were taken using a Vibratome®.

Sections were incubated in blocking solution (0.3% H2O2 in PBS) for 30 min. Slices were then rinsed in PBS and incubated overnight in primary antibody (rabbit anti-bFGF F3393, Sigma) at a 1:800 dilution. Next, slices were rinsed in PBS and incubated for 1 h in the secondary antibody (Vector, biotinylated goat anti-rabbit Ig, BA-1000) diluted to 1:200. Sections were rinsed in PBS, then incubated for 1 h in A&B reagent solution (Vector, A&B reagent avidin-biotin HRP, PK-6100) at a dilution of 1:400. This was followed by rinses in PBS then Tris. Lastly, a standard diaminobenzidine solution was used with a nickel ammonium sulfate intensification procedure (PBS/Dab/Nickel: 1 ml7 μg/25 μg) preceding rinses in Tris. Sections were mounted onto slides, dehydrated and coverslipped after a rinse in histoclear. As a control for non-specific binding, a few slices were incubated overnight in block solution and were exposed to all of the above, except for the primary antibody.

bFGF immunopositive cells from two consecutive coronal sections (+1.60 mm and +1.20 mm relative to bregma) were quantified using a 400 × 300 μm rectangular grid superimposed on the ipsilateral peri-lesional frontal cortex, areas 1&2 and the dorsal striatum [3,16]. Quantification was done in these regions because plasticity therein has been associated with functional recovery [10,16]. These grids were visualized with a 20 × objective on a VANOX–T Olympus microscope (Olympus Optical Co. LTD., Tokyo, Japan) with a high performance CCD monochrome camera (model 4912–5010, COHU Inc., San Diego, CA). For the peri-lesional cortex, the grid was placed directly dorsal to the horn of the corpus callosum and lateral to the lesion. For the dorsal striatum, the grid was placed...
directly ventral to the horn of the corpus callosum. To account for the thickness of the tissue, cells were visualized and quantified by focusing up and down through the tissue. Only cells displaying the morphology of reactive astrocytes were counted in the two consecutive coronal sections. The mean of these two counts for each brain regions was used for statistical analyses.

The following groups were analyzed. Notations include condition, day of sacrifice and group size in parentheses: PROP-treated, D2 (n = 2), D4 (n = 4), D6 (n = 6), D8 (n = 7) and D29 (n = 3), and saline-treated, D2 (n = 2), D4 (n = 3), D6 (n = 4), D8 (n = 4) and D29 (n = 3).

bFGF immunoreactivity was assessed throughout and beyond the critical period to determine if there were any early drug-associated changes in lesion-induced growth factor expression, the persistence of such effects and how these correlated with behavioral change. Statistical analyses were done as previously reported [3], whereby only behavioral testing scores obtained on or immediately before the day of sacrifice were used for analyses.

To determine whether the administration of PROP affected responsivity to adhesive stimuli, latencies (in seconds) to contact the first adhesive stimulus over days were analyzed. A one-factor ANOVA performed on each of the testing days (1, 5, 7, 9, 12, 14, 19, 23 and 29) did not reveal a significant difference among groups. Contact times were within 5 s, on average, regardless of day or group.

A two-factor ANOVA assessing the magnitude of asymmetry revealed a significant difference over days \( F_{4,37} = 19.290, P < 0.0001 \). One day after surgery (Fig. 1), the magnitude of asymmetry for both groups was equivalent (saline = 3.643 ± 0.808 vs. PROP = 4.0 ± 0.839). This degree of asymmetry is in accordance with previously published data from this laboratory [3]. By day 5, however, PROP-treated animals displayed a significantly diminished deficit compared to the saline-treated animals (one-factor ANOVA, \( P = 0.0020 \): PROP = 1.429 ± 0.354 vs. saline = 3.071 ± 0.272). This early facilitation was maintained through day 7. At time points beyond this, however, recovery patterns between the two groups were indistinguishable. Thus, PROP administration was associated with an early facilitation of functional recovery (post-op days 5 and 7), but not a significant difference overall.

A two-factor ANOVA assessing cortical bFGF expression revealed a significant difference among groups \( (F_{1,33} = 12.509, P = 0.0012) \), over days \( (F_{5,33} = 14.694, P < 0.0001) \), and a significant group \( \times \) day interaction \( (F_{5,33} = 4.530, P = 0.0030) \). Up to 4 days after surgery, cortical bFGF levels were equivalent in both PROP and saline-treated animals. However, at day 6, bFGF levels peaked in PROP-treated animals to a level significantly greater than that seen in saline-treated controls (one-factor ANOVA, \( P = 0.0010 \): PROP = 12.583 ± 1.457 vs. saline = 3.125 ± 0.625). After this time and through day 29, bFGF expression in PROP-treated animals approached the level of saline-treated controls (Figs. 2A and 3A).

In contrast to that seen in the cortex, striatal bFGF expression was equivalent on all days for the PROP and saline-treated groups (Fig. 2B and 3B). Although, a two-factor ANOVA revealed that there was a significant difference over days \( (F_{5,33} = 16.347, P < 0.0001) \), reflecting that both groups changed over time.

All animals sustained equivalent damage to the intended cortical regions (medial precentral, anterior cingulate, medial agranular, prelimbic and infralimbic), as was deter-

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**Fig. 1.** Mean (SEM) magnitude of asymmetry plotted over days following a unilateral anteromedial cortex lesion. Facilitated functional recovery was observed in the PROP-treated animals compared to saline-treated animals during the first post-surgery week.

**Fig. 2.** Mean (SEM) number of bFGF positive astrocytes quantified from an area \((400 \, \mu m \times 300 \, \mu m)\) situated immediately lateral to the anteromedial cortex lesion (peri-lesional cortex (A) and immediately ventral to the horn of the corpus callosum in the dorsal striatum (B)). Error bars depict the standard error of the mean for each group. bFGF levels were elevated in the peri-lesional cortex for PROP-treated animals on post-operative day 6, prior to the attainment of functional recovery. The expression of bFGF in the ipsilateral dorsal striatum was not significantly different from saline-treated animals on all testing days.
mired microscopically by at least one person blind to the experimental condition. Lesions were consistent with the criteria established in previously published data [17] and have been depicted elsewhere [3].

Following anteromedial cortex lesion, there was a region-specific and drug-associated increase in bFGF expression. Specifically, PROP administration led to an early increase in bFGF immunoreactivity in the peri-lesional cortex on day 4, which peaked on day 6. This coincided with early facilitation of functional recovery on post-operative days 5 and 7. Thus, the positive functional effects of PROP administration may have stemmed from the early and sustained upregulation of bFGF in the peri-lesional cortex. Unlike the cortex, bFGF expression in the dorsal striatum was not significantly altered by PROP, with treated and untreated groups exhibiting peak expression on days 6–8.

That PROP administration was associated with increased bFGF expression in the peri-lesional cortex and an early facilitation of functional recovery is not surprising given the importance of this structure and this growth factor for functional recovery. For example, when a GABA agonist was administered into the peri-lesional cortex following unilateral anteromedial cortex lesion, functional recovery was blocked [10]. Rowntree and Kolb also reported that blocking bFGF expression in the peri-lesional cortex produced dendritic atrophy, as well as diminished functional recovery [16]. Overall, bFGF upregulation has been shown to alleviate injury-induced neurotoxicity [15] and be important for functional recovery and neuroplasticity [7,15].

Interestingly, the early functional improvement observed in PROP-treated animals in the present study did not lead to better overall recovery. This is an important issue when considered in light of human traumatic brain injury where early functional improvement may be critical for better ultimate outcome. Specifically, survivors of traumatic brain injury exhibit numerous physical, cognitive, and psychosocial deficits [6], the presence of which can be associated with failure to return to work [2], as well as re-establish social contacts. Thus, magnifying the duration or degree of functional deficits in humans could diminish the patient’s quality of life in both the short- and long-term. In contrast, quality of life could be improved if certain attributes (e.g., duration or degree) of post-injury impairment were lessened.

In conclusion, the present study delineates the spatial and temporal patterns of bFGF expression and functional recovery following anteromedial cortex lesion in PROP-treated animals. As has been shown using other models of brain insult, PROP administration was associated with an early facilitation of functional recovery [4]. During the post-lesion critical period, a clear temporal relationship exists between this behavioral improvement and the drug-associated increase in bFGF expression in the peri-lesional cortex. Taken together, these data support the potential therapeutic role of PROP following brain insult.

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