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Vagus Nerve Stimulation May Protect GABAergic Neurons Following Traumatic Brain Injury in rats: an Immunocytochemical Study

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Abstract

Seizures and subclinical seizures occur following experimental brain injury in rats and may result from inhibitory neuron loss. This study numerically compares cortical and hippocampal Glutamic Acid Decarboxylase (GAD) positive neurons between sham Fluid Percussion Injury (FPI), FPI with sham Vagus Nerve Stimulation (VNS) and FPI with chronic intermittent VNS initiated at 24 hours post FPI in rats. Rats (n=8/group) were prepared for immunocytochemistry of GAD at 15 days post FPI. Serial sections were collected and GAD immunoreactive neurons were counted in the hippocampal hilus and two levels of the cerebral cortex. Numbers of quantifiable GAD cells in the rostral cerebral cortices were different between groups, both ipsilateral and contralateral to the FPI. Post hoc analysis of cell counts rostral to the ipsilateral epicenter, revealed a significant 26% reduction in the number of GAD cells/unit area of cerebral cortex following FPI. In the FPI-VNS group, this percentage loss was attenuated to only an 8.5% reduction, a value not significantly different from the sham group. In the contralateral side of the rostral cerebral cortex, FPI induced a significant 24% reduction in GAD cells/unit area; whereas, the VNS-treated rats showed no appreciable diminution of GAD cells rostral to the contralateral epicenter. Hippocampal analysis revealed a similar reduction of GAD cells in the FPI group; however, unlike the cortex this was not statistically significant. In the FPI-VNS group, a trend towards increased numbers of hilar GAD cells was observed, even over and above that of the sham FPI group; however, this was also not statistically significant. Together, these data suggest that VNS protects cortical GAD cells from death subsequent to FPI and may increase GAD cell counts in the hippocampal hilus of the injured brain.

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Keywords

GABA; fluid percussion injury; epilepsy; GAD; cerebral cortex; hippocampus; neuroprotection

1. Introduction

Our recent studies demonstrate that vagus nerve stimulation (VNS), a therapeutic approach to seizure control in humans (Schachter and Wheless, 2002; review), markedly facilitates the recovery of locomotor and cognitive function in rats that have been subjected to unilateral fluid percussion injury (FPI) (Smith et al., 2005,2006). The mechanism of this facilitation remains an ardent focus of our studies and we have hypothesized that interactions between gamma aminobutyric acid (GABA), norepinephrine (NE) and VNS may be involved in functional recovery and seizure suppression (Smith et al., 2005,2006).

Seizure disorders often develop in the weeks following traumatic brain injury in humans (Frey, 2003; Pitkanen and McIntosh, 2006) and may exacerbate associated morbidity (Asikainen et al., 1998). Seizure activities, including subclinical seizures verified with electrophysiology, also occur following experimental brain injury in rats (Santhakumar et al., 2001; D'Ambrosio et al., 2004). Seizure activity has been recorded at one and two weeks following fluid percussion injury (FPI) with seizure foci first detected in the cerebral cortices adjacent to the injury site. However, by 27–28 weeks post injury, seizure activities manifest as hippocampus-initiated focal seizures (D'Ambrosio et al., 2005).

Data from previous studies suggest that the seizure suppressive effects of VNS may be partially subserved by the locus coeruleus (LC; Krahl et al., 1998), a pontine nucleus that produces the majority of NE in the brain. We, and others, have helped establish that NE is a primary determinant of seizure severity in both genetic as well as non-genetic seizure models (Kilian and Frey, 1973; Maynert et al., 1975; Buterbaugh and London, 1977; Jobe et al., 1986; Browning, 1987; Clough et al., 1994; Weinshenker and Szot, 2002). Generally, reductions in NE promote increased severity of seizures whereas elevated NE can suppress seizure activity. Moreover, an antiepileptogenic role of NE has been demonstrated in models of epileptogenesis including electrogenic kindling (McIntyre, 1980). A now classic study by Naritoku et al (1995) showed that VNS causes an increase in fos labeling in the LC, presumably as a result of intense neuronal activation. We have shown a similarly increased activation in the LC as a consequence of a mixed variety of seizure evoking stimuli (Eells et al., 1997). Finally, our recent microdialysis/HPLC studies demonstrate that VNS increases NE release within the hippocampal formation and the cerebral cortex (Roosevelt, et al., in press). Thus, hypothetically, VNS may promote recovery of function via seizure suppressive effects in part through activation of NE release into the brain. This hypothesis remains under investigation.

Certain aspects of brain pathology also appear to correlate with seizure development subsequent to experimental FPI. For example, glial fibrillary acidic protein (GFAP) staining is prominent at 2–3 weeks post FPI in the ipsilateral cerebral cortex; whereas, at 27–28 weeks following injury, significant GFAP elaboration is observed in the temporal lobe compared to a 2–3 week time point (D'Ambrosio et al., 2005). Additionally, there is no apparent hippocampal shrinkage at 2–3 weeks after FPI; however, varying degrees of hippocampal and temporal lobe asymmetry are present at 27–28 weeks. Structural changes, thought to be related to increasing seizure propensity, are also apparent in mossy fiber systems of the hippocampal dentate gyrus subsequent to FPI (Santhakumar et al., 2001). Furthermore, FPI is associated with a loss of up to 50% of hilar neurons in the ipsilateral hippocampus and a pronounced period of hyper-excitability in hippocampal pyramidal neurons (Lowenstein et al., 1992; Toth et al., 1997; Santhakumar et al., 2001). We, and others, have shown a significant loss of CA3-

CA1 pyramidal neurons over two weeks following experimental FPI in rats (Smith et al., 2005; Anderson et al., 2005) that apparently coincides with the temporal profile of excitability (Santhakumar et al., 2001). Continued loss of pyramidal cells in the Cornu Amonis of the human hippocampus after brain injury has also been described (Kotapka et al., 1992; Maxwell et al., 2002). Cortical neuron loss, as may be expected, both in and around the epicenter of experimental brain injury have been widely described (McIntosh et al., 1989; Smith et al., 1997). These events, particularly the loss of cortical neurons and derangement of their circuitry, loss of hilar neurons (including, presumably, inhibitory GABA neurons), mossy fiber sprouting, and resulting hyper excitability of the pyramidal cells, appear to set the stage for predisposition to seizures. Thus, the development of post-traumatic seizures that occurs following brain injury may involve a loss of GABAergic cells and inhibitory tone in the brain.

A role for the inhibitory neurotransmitter GABA in VNS-mediated seizure suppression in humans undergoing VNS therapy is also suggested. At both high and low amplitude VNS, total GABA levels in the cerebrospinal fluid of VNS patients are significantly increased (Ben-Menachem et al., 1995). Additionally, single photon emission computed tomography (SPECT) of GABA_A receptor density in the hippocampus following 1-year of VNS therapy showed a significant normalization of GABA-receptor density that correlated with seizure reduction (Marrosu et al., 2003). Regulation of GABAergic signaling involves the synthesis of GABA from glutamate by the enzyme glutamic acid decarboxylase (GAD). Two isoforms of GAD exist: GAD₆₅, which is predominantly located in membranes and axon terminals, and GAD₆₇, which is distributed widely in cells (Soghomonian and Martin, 1998). The majority of GABAergic cells express both isoforms and thus either or both forms of GAD can be used with immunocytochemistry to label GABAergic neurons. Insofar as VNS is associated with an increased steady state GABA level in humans being treated for epilepsy, we hypothesize that the VNS may have a protective effect on GAD neurons in the cerebral cortex as well as the hippocampus that may otherwise be jeopardized in traumatic brain injury.

2. Results

GAD immunohistochemistry

Figure 1 depicts representative photomicrographs of GAD_{65/67}-like immunopositive cells within the cerebral cortex (panels A and C) and within the hippocampus (panels B and D). The areas used for counting the GAD_{65/67}-like cells in the cerebral cortices and the hippocampal hilar region are graphically depicted. Counting of GAD_{65/67}-like cells was performed on live/online images in order to insure identity criteria of profiles. Our lab has found this to be a more advantageous approach when using 30 μ m sections because it allows the investigators to focus throughout the section and qualify or disqualify profiles that may be questionable at only one focal plane (such as occurs in image capture).

GAD cell semi-quantitative morphometry

Results of GAD_{65/67}-like morphometrics in the cerebral cortices of the rat brain following sham FPI and FPI with and without VNS are shown in Figure 2. The number of quantifiable GAD_{65/67}-like cells in the rostral cerebral cortex was significantly different between the sham-FPI, FPI-no VNS, and FPI-VNS groups in both the ipsilateral and contralateral sides of the brain (ANOVA, ipsilateral side, $F(2,21) = 4.303$, $p=0.027$; contralateral side, $F(2,21) = 3.896$, $p=0.036$). Post hoc analysis of cell counts 3mm rostral to the ipsilateral presumptive epicenter, revealed an approximate 26% reduction in the number of GAD_{65/67}-like cells/unit area of cerebral cortex in the FPI-sham VNS group ($p<0.05$). In the VNS-treated group, this percentage loss in the rostral cortex was attenuated to only an 8.5% reduction, a value not significantly different from the sham-FPI group. In the contralateral side of the rostral cerebral cortex, FPI also induced an approximate 24% reduction in GAD_{65/67}-like cells/unit area compared to the

sham-FPI group ($p<0.05$); whereas, the VNS-treated rats showed no appreciable diminution of GAD_{65/67}-like cells rostral to the contralateral epicenter compared to the sham-FPI group ($p>0.05$). The mean number of GAD_{65/67}-like cells was significantly higher in the FPI-VNS group compared to the FPI-no VNS group in both the ipsilateral and contralateral sides ($p<0.05$) of the rostral cortex (see Figure 2).

The number of quantifiable GAD_{65/67}-like cells in the cerebral cortex in the lateral surround of the lesioned ipsilateral epicenter, although showing a similar tendency in diminution of GAD_{65/67}-like cells to the rostral cortex, were not found to be significantly different between groups. Cell counts in the contralateral cortex at the level of the epicenter and complementary to the site of the ipsilateral cerebral cortices in the lateral surround of the epicenter, though showing a tendency similar to the lesioned side, also did not show a statistically significant diminution of GAD_{65/67}-like cells following FPI.

Results of cell counts in the hippocampal hilar region are shown in Figure 3, and are expressed per measured unit area of the hippocampus, rather than standardized to 1 square mm. This method was used to avoid bias that may result from the possibility of slightly different hippocampal surface areas examined between rats (even though all sections were obtained at Bregma -4.0). Results of hilar quantifications showed that the number of GAD_{65/67}-like cells per unit area of hippocampus were not significantly different between groups (ANOVA, ipsilateral side, $F(2,21) = 2.966$, $p = 0.073$; contralateral side, $F(2,21) = 1.328$, $p = 0.286$). However, there was a strong tendency for the FPI-no VNS animals to have fewer GAD_{65/67}-like cells counts in the ipsilateral hilus. Herein, a 26% reduction in hilar GAD_{65/67}-like cell counts was observed following FPI, although not statistically significant, this trend towards a diminution was similar to that seen in cortical GAD_{65/67}-like cell counts after FPI.

Additionally, the FPI-VNS rats actually showed an opposite tendency towards an increased number of GAD_{65/67}-like cells in the hippocampal hilus ipsilateral to FPI. In the ipsilateral hilus of the FPI-VNS rats, a 37% increase in numbers of GAD_{65/67}-like cells was observed, a number that was over and above that of even the sham-lesioned animals, although this was not statistically significant by ANOVA ($p=0.073$). This finding warrants further investigation. There were no statistically significant differences in the comparisons between treatment groups in the hilus contralateral to the FPI.

3. Discussion

Recent research in our labs has demonstrated profound effects of VNS on the recovery of behavioral function following FPI of the rat brain (Smith et al., 2005,2006). The mechanism(s) of VNS facilitated recovery of function remain to be discovered, although we hypothesize that it may include protection of the injured brain from seizures. We presently describe an intriguing interaction between FPI, VNS and the number of GABAergic neurons in the cerebral cortices and possibly the hippocampal formation in a rat model of traumatic brain injury.

We show that FPI induces a significant loss of GAD_{65/67}-like immunoreactive cells within the cerebral cortices, especially rostral to the presumptive epicenter of the FPI. This loss is present both ipsilateral and contralateral to the FPI lesion. However, in the lateral surround at the epicenter (where there was observable lesion cavity in a majority of the rats), this diminution of GAD neurons, while following the same trend, was not statistically significant.

The most intriguing finding of this study was an apparent sparing of GAD_{65/67}-like neurons in the rostral cerebral cortex of FPI lesioned animals that were subjected to VNS therapy. FPI was found to induce a 26% reduction in the number of GAD_{65/67}-like cells in the rostral cerebral cortex; whereas, in animals receiving VNS, this diminution was on the order of 8%. This difference was statistically significant and it suggests that VNS has an overall protective effect

on GABAergic neurons that would otherwise be destroyed in FPI. Similar findings were observed in the hippocampal hilus near the epicenter although this failed to reach statistical significance ($p=0.073$). Nonetheless, semi-quantitative morphometry in the hippocampal hilus ipsilateral to the FPI also revealed an approximate 26% trend towards reduction in the number of GAD_{65/67}-like cells at two weeks after FPI, a diminution similar to that observed in the rostral cerebral cortex. Perhaps more interesting in the hippocampal hilus was the number of GAD_{65/67}-like cells counted in the VNS treated FPI lesioned animals. This quantification suggested a 32% increased number of GAD_{65/67}-like cells in the VNS treated FPI group, over and above even that observed in the sham-FPI group. Although this finding was not statistically significant by ANOVA ($p=0.073$), it suggests that VNS may actually promote increased numbers of GAD_{65/67}-like cells in the hippocampal hilus. Such an increase could result from either increased neurogenesis over all, or an increased commitment of newly born neurons into a GABAergic phenotype.

There appears to be an interaction between VNS and GABA within the central nervous system of humans undergoing VNS for the treatment of epilepsy. For example, total GABA levels in the cerebrospinal fluid of VNS patients are significantly increased (Ben-Menachem et al., 1995). Additionally, GABA_A receptor density in the hippocampus following 1-year of VNS therapy is shown to be normalized and correlated with seizure reduction (Marrosu et al., 2003). Though speculative, the present findings suggest that VNS-related sparing of GABAergic neurons from loss due to FPI may contribute to subclinical seizure suppression and, in so doing, facilitate the recovery of function following brain injury in rats.

In addition, the mechanisms of VNS' benefit in seizure control and recovery of function following brain injury may involve effects of NE. Previous studies show that NE is a determinant of seizure severity in a variety of seizure models ranging from genetic seizure prone models (Kilian and Frey, 1973; Maynert et al., 1975; Buterbaugh and London, 1977; Jobe et al., 1986; Browning, 1987; Clough et al., 1994; Weinshenker and Szot, 2002) to kindling epileptogenesis (McIntyre, 1980). Moreover, there is abundant evidence that the recovery of function following experimental brain injury is facilitated by NE (Boyeson and Feeney, 1990; McIntosh, 1993) and retarded by the lack of it (Goldstein, 1997). We have recently found that VNS increases the levels NE levels in the brain (Roosevelt et al., in press) adding further support to a notion of VNS induced NE release and complimentary facilitation of recovery of function after experimental brain injury.

Thus, in summary, the present study shows that VNS has an effect to prevent the loss of GABA neurons within the cerebral cortex, and possibly the hippocampal formation, following FPI and in-so-doing, may facilitate the recovery of behavioral function. Further studies are warranted to investigate whether this finding is related to NE in the brain.

4. Methods

Twenty-four male Long Evans hooded rats (Charles Rivers, Wilmington, MA) received VNS implants (Neuroprosthetic Pulse generator Model 102, Cyberonics Inc.) using previously detailed methods (Smith et al., 2005, 2006). FPI was performed on 16 anesthetized rats also using previously detailed methods (Smith et al., 2005, 2006). Briefly, one week following left vagus nerve electrode implantation in all 24 rats, 16 rats were exposed to unilateral FPI. A 4 mm craniotomy (epicenter: AP = -4.4 mm, ML = -2.4 mm) was made over the left hemisphere. Eight animals received a craniotomy but did not receive an injury (sham-FPI). Animals subjected to FPI were assigned to either the VNS (FPI-VNS) or no VNS treatment (FPI-no VNS) groups by pair-wise matching of their initial index of severity. The indices of severity were obtained by summing the durations of apnea and unconsciousness. This stratified assignment procedure was done to insure that similarly-affected animals were placed within

each of the FPI treatment groups. After assignment, there were no group differences found in the indices of severity: Apnea [$F(1,15) = 0.807$, $p = 0.384$] (FPI-VNS: mean = 20.00 sec, SD = 12.07; FPI-no VNS: mean = 15.38 sec, SD = 8.14); duration of unconsciousness [$F(1,15) = 0.101$, $p = 0.755$] (FPI-VNS: mean = 153.87 sec, SD = 66.87; FPI-no VNS: mean = 144.87 sec, SD = 44.19). Following surgery, VNS animals received a 0.5 mA intensity, 30 sec train of 0.5 ms biphasic pulses delivered at 20 Hz, initiated 24 hr post-injury, every 30 min for the 14-day duration of the study. Animal care, surgical and anesthetic protocols were conducted in strict accordance with Federal regulations as outlined in the *NIH Guide for the Care and Use of Laboratory Animals*. After surgeries, animals were subjected to behavioral testing in order to assess functional recovery (reported in Smith et al., 2006). The Southern Illinois University Institutional Animal Care and Use committee have approved all experimental procedures on animals.

Neurohistology

On day 15 post-injury, the animals were anesthetized and transcardially perfused with cold 0.1 M (Na^+) phosphate buffered saline (PBS, pH 7.3) followed immediately by 4% paraformaldehyde in (Na^+) PBS. Brains were post-fixed for 24 hours, and cryoprotected in 30% sucrose for 2 days. Serial sets of coronal 30 μm frozen-sections were collected from each brain starting 1.00 mm posterior to bregma and ending at 4.00 mm posterior to bregma (Paxinos & Watson, 1998). Two sets of 10 serial sections were used for GAD immunocytochemistry. Briefly, floating sections were incubated in 1.5% normal goat serum (blocking serum) in (K^+) PBS and then incubated in a rabbit anti-GAD_{65/67} primary antibody (dilution 1:2,000 in (K^+) PBS., Sigma, St. Louis, MO) for 24 hours with gentle agitation (shaker). Sections were then processed using an ABC substrate kit (Vector Labs) according to kit directions. Visualization of the final reaction product was made using a solution of 0.04% nickel-intensified 3'-5' diaminobenzidine solution (4.4%) in Tris Buffered Saline (TBS). Sections were mounted onto gel-dipped slides, dehydrated, and cover slipped. Slides were coded so as to insure a blind analysis and were visualized with brightfield microscopy at 20x magnification (Olympus, BH2 with video capture).

Semiquantitative morphometry

Assessment and counting of GAD_{65/67}-like positive neurons in layer four of the cerebral cortex utilized a transparent grid template containing a fixed number of squares with consistent dimensions (100² μm). The 12 square grid was positioned on the computer monitor to overlay a predetermined and consistent area of the cerebral cortex that was observable on the monitor screen of each serial brain section under study. Over each section, the grid was positioned at 10x, and then magnified to 20x for cell counting. Initial positioning of the grid template was consistently the same between all brain sections and the treatment groups under study were blind to the observers. Following positioning, two independent investigators counted the number of GAD_{65/67}-like positive neurons within 6 of the 12 squares using a systematic random sampling method of grid square selection (Elias & Hyde, 1988). For comparison purposes, the number of GAD_{65/67}-like cells per unit of cortical surface area was standardized to one square millimeter. Serial sections were collected, processed, and counted immediately rostral to the lesioned area (Bregma -1mm) and at the presumptive epicenter (Bregma -4mm) of the FPI lesion. These counts were made on a minimum of 6 sections per brain area per each of 8 animals per group. Cell counts in the sections taken at the epicenter were made on the cortical area in the lateral surround of the lesion, not immediately beneath the epicenter. This was done because a majority of the brains had obvious lesions of the cortical tissue immediately beneath the epicenter.

An alternative method was used to quantify the number of GAD_{65/67}-like cells within the hilar region of the hippocampus, near the presumptive epicenter of the FPI. These counts were made

on the same brain sections as the near-epicenter cortical cell count sections. Briefly, the total number of GAD_{65/67}-like positive neurons were counted within the hilar region of each section, specifically, an area isolated between the superior and inferior blades of the dentate gyrus, extending to the medial edge of the CA3 pyramidal cell layer of the hippocampus. Due to possible alteration in hippocampal size as a result of FPI, the hippocampi of both the ipsilateral and contralateral sides were also measured for total surface area. Thus, rather than using absolute hilar neuron counts which may have been biased as a result of size changes, the number of GAD_{65/67}-like cells in the hilus were standardized per unit surface area of the hippocampi on each side.

Statistical analyses were conducted using one-way ANOVA followed by Fischer's Least Squared Differences post hoc tests.

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Figure 1. GAD_{65/67} immunopositive cells in the brain. Representative photomicrographs of GAD_{65/67}-like immunopositive cells within the cerebral cortex (panels A and C) and within the hippocampus (panels B and D) are shown. Panels C and D graphically depict the regions used for counting the GAD cells in the cerebral cortices. GAD_{65/67} positive cells are indicated by dark arrows. The grid over panel C represents the region of the cerebral cortex quantified in each brain section. Systematic random sampling was used to quantify 6 grid squares. Labeled neurons that intersected the top and left borders of each counted square were not included in counts, whereas, those that intersected the bottom and right borders were included. Panel D depicts the region of the hippocampal hilus quantified in each section. These quantifications were standardized to measured total hippocampal area.

Figure 2.

The effect of sham-FPI, FPI-no VNS and FPI-VNS on the number of GAD_{65/67}-like immunoreactive cells in the cerebral cortices following experimental injury. Data are expressed as GAD_{65/67}-like cell number per 1mm unit area in the rostral and caudal regions, respective to the injury site. Analysis revealed a significant difference in number of GAD_{65/67}-like positive cells in the rostral cortex, left (injured) and right (uninjured) cortices between the sham-FPI and FPI-no VNS groups (a, $p < 0.05$), and the FPI-VNS and the FPI-no VNS animals (b, $p < 0.05$). Sham-FPI and FPI-VNS animals did not significantly differ in the rostral cortex. No significant differences were revealed in the caudal extent of the cortex.

Figure 3.

The effect of sham-FPI, FPI-no VNS and FPI-VNS on the number of GAD_{65/67}-like immunoreactive cells in the hippocampal hilus following experimental injury. No significant differences were revealed in the hilar GAD cell numbers by ANOVA ($p=0.073$).