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RAPID COMMUNICATION

Synaptic Protein Expression in the Medial Temporal Lobe and Frontal Cortex Following Chronic Bilateral Vestibular Loss

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ABSTRACT: Several studies have reported that bilateral vestibular deafferentation (BVD) results in the disruption of place cell function and theta activity in the hippocampus. Recent magnetic resonance imaging (MRI) studies in humans demonstrated that bilateral but not unilateral vestibular loss is associated with a bilateral atrophy of the hippocampus. In this study we investigated whether BVD in rats resulted in changes in the expression of four proteins related to neuronal plasticity, synaptophysin, SNAP-25, drebrin and neurofilament-L, in the hippocampal subregions (CA1, CA2/3, the DG) and the entorhinal (EC), perirhinal (PRC) and frontal cortices (FC), using western blotting. At 6 months following BVD, there were no significant differences in the expression of synaptophysin in any region. There were also no significant differences in SNAP-25 expression in CA1, CA2/3, EC, PRC, or the FC; however, there was a significant increase in SNAP-25 expression in the DG compared to sham controls. Drebrin A and E expression was significantly reduced in the EC and drebrin A was significantly reduced in the FC of BVD animals. NF-L expression was not significantly different in CA1, CA2/3, DG, EC, or the PRC. However, its expression was significantly reduced in the FC of BVD animals. These data suggest that circumscribed neurochemical changes in SNAP-25, drebrin and NF-L expression occur in the DG, EC, and the FC over 6 months following BVD. © 2008 Wiley-Liss, Inc.

KEY WORDS: bilateral vestibular deafferentation; hippocampus; medial temporal lobe; synaptophysin; SNAP-25; drebrin; neurofilament-L

INTRODUCTION

Many studies have shown that loss of vestibular function in animals and humans results in deficits in spatial memory that persist long after a substantial amount of compensation for the vestibular reflex deficits has developed (e.g., Stackman and Herbert, 2002; Wallace et al., 2002; Brandt et al., 2005; Zheng et al., 2006; Zheng et al., 2007). Both in vivo and in vitro electrophysiological studies suggest that at least part of the mechanism for these cognitive deficits is the disruption of the normal function of place cells in the hippocampus, which appear to rely on vestibular input in order to construct memories of the environment (Stackman et al., 2002; Russell et al., 2003; Zheng et al., 2003; Russell et al., 2006). Brandt et al. (2005) have reported that bilateral vestibular deafferentation (BVD) in humans is associated with a ~17% bilateral decrease in the volume of the hippocampus, 8–10 years following the lesions. Clearly, the hippocampus undergoes some major functional and structural changes following vestibular lesions, which suggests that biochemical changes must be occurring either in direct response to the lesion or possibly as part of a compensatory process. To date, the only neurochemical studies that have been conducted in the hippocampus have shown a decrease in the expression of the NR1 and NR2A subunits of the *N*-methyl-*D*-aspartate (NMDA) receptor, and in neuronal nitric oxide synthase (nNOS) expression (Zheng et al., 2001; Liu et al., 2003a,b); however, these studies employed unilateral rather than bilateral lesions, which apparently do not produce hippocampal atrophy in humans (Hufner et al., 2007). Therefore, the aim of this study was to perform an initial investigation of possible neurochemical changes in the hippocampus and other related medial temporal lobe regions (the entorhinal and perirhinal cortices) following BVD in rats. Since electrical stimulation of the vestibular nerve as well as saccular stimulation using acoustic clicks, have been shown to activate the frontal lobes (de Waele et al., 2001; Miyamoto et al., 2007), the frontal cortex was also investigated for comparison.

The expression of four proteins related to synaptic transmission and neuronal plasticity, synaptophysin, SNAP-25, drebrin and neurofilament-L (NF-L), was investigated using western blotting, and the frontal cortex was used for comparison with the medial temporal lobe areas. Synaptophysin is a synaptic vesicle glycoprotein that is involved in the release of neurotransmitter vesicles (Fuentes-Santamaria et al., 2007); SNAP-25 ("synaptosome-associated protein of 25 kDa") is a presynaptic nerve terminal protein involved in vesicle exocytosis (Delgardo-Martinez et al., 2007); drebrin ("developmentally regulated brain protein") is a neuronal F-actin binding protein involved in axonal and dendritic plasticity (Kobayashi et al., 2007); and NF-L is a member of the neurofilament protein family, which is often used as a biomarker for cell death and axonal loss

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(Petzold et al., 2007; Barry et al., 2007). These four proteins were chosen for investigation for two reasons: (1) as a precursor to a larger gene microarray analysis, to determine the likelihood that genes and proteins related to synaptic transmission may change in the medial temporal lobe following BVD, since none have been investigated in this context previously; and (2) because these four proteins have been implicated in synaptic plasticity processes, such as axonal terminal remodelling (SNAP-25; Osen-Sand et al., 1993), activity-dependent synapse formation (synaptophysin; Tarsa and Goda, 2002), dendritic spine morphogenesis (drebrin; Sekino et al., 2007) and axonal transportation (NF-L). The expression of any or all of these proteins might be expected to change in the areas of the brain affected by vestibular damage.

A complete bilateral BVD was performed on six rats anesthetized with 300 μg/kg fentanyl citrate (i.p.) and 300 μg/kg medetomidine hydrochloride (i.p.). Under microscopic control, the tympanic bulla was exposed using a retro-auricular approach and the tympanic membrane, malleus and incus were removed. The stapedial artery was cauterized and then the contents of the canal ampullae and the utricle and saccule were aspirated before the temporal bone was sealed with dental cement. Carprofen (5 mg/kg, s.c.) was used for postoperative analgesia. A further six rats received a sham surgical procedure under the same anesthesia, consisting of exposing the temporal bone and removing the tympanic membrane without producing a vestibular lesion. Our previous studies using temporal bone histology have confirmed that this BVD procedure produces a complete and permanent bilateral vestibular lesion (Zheng et al., 2006). A second control group, subjected to sham surgery without removal of the tympanic membrane, was not used because there is no evidence to date that damage to the auditory system can alter place cell function in the hippocampus (see Smith et al., 2005 for a discussion). Nonetheless, because removal of the tympanic membrane produced partial deafness in the control group, the neurochemical effects of BVD in comparison to that control group must reflect vestibular damage itself. All procedures were carried out in accordance with the regulations of the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals.

Following surgery, the animals were allowed to recover for 6 months. Although the vestibulo-ocular and vestibulo-spinal reflexes never recover to normal following BVD, by 6 months the animals have recovered from the severe, acute symptoms of BVD and some compensation has occurred (see Smith and Curthoys, 1989 for a review). The animals were decapitated without anesthesia, and the hippocampal subregions (CA1, CA2/3 and the dentate gyrus (DG)), entorhinal cortex (EC), perirhinal cortex (PRC), and frontal cortex (FC) dissected. The protein concentrations in the samples were equalized to 2 mg/ml. Tissue homogenates were then mixed with gel loading buffer (50 mM Tris-HCl, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue) in a ratio of 1:1 and then boiled for 5 min. About 10 μl of each sample was loaded in each well on 7.5–10% SDS-polyacrylamide mini-gels. Pre-stained protein (10–250 kDa; Bio-Rad, Precision Plus: Dual color) was used as molecular weight markers on each gel. Samples were electrophoresed with a 90 V variable current (Bio-Rad, PowerPack 3000) until protein flattened at the stacking/resolving interface, and 180 V thereafter. Protein was blotted on polyvinylidene-difluoride (PVDF) membranes using a Trans-Blot cell (2.5 L; Bio-Rad) filled with transfer buffer (0.25% tris base, 1.2% glycine, 16.6% absolute methanol w/v in distilled H_2O) and 10 V variable current for ~18 h. (Bio-Rad PowerPack 3000). Nonspecific IgG/M binding was blocked by incubation with 5% nonfat dried milk protein (Pams) and 0.1% bovine serum albumin (BSA) (Sigma) for 7 h at 4°C. The membranes were then incubated at a dilution of 1:5,000 with an affinity-purified IgM monoclonal mouse antibody to synaptophysin (Chemicon, MAB329), an IgG monoclonal mouse antibody to SNAP-25 (Sigma, S5187), an IgG polyclonal rabbit antibody to drebrin (Sigma, D3816), or an IgG polyclonal goat antibody to NF-L (Santa Cruz Biotechnology, sc-12966), overnight at 4°C in TTBS (10% 50 mM TBS pH 7.6, 0.1% Tween-20). Secondary antibodies (see below) were diluted 1:5,000 in 5 mM TTBS and incubated for 5 h at 4°C (goat-antirabbit IgG-HRP: Sigma, A6154; donkey-antigoat IgG-HRP: Santa Cruz Biotechnology, SC-2,020; goat-antimouse IgM: Sigma, A8786; goat-antimouse IgG: Sigma, A4416). Immuno-detection was performed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, NZ). Hyperfilms (Amersham Biosciences, NZ) were analyzed by densitometry to determine the quantity of protein expressed in each group using a calibrated imaging densitometer (Bio-Rad) and a PowerPC Mac running OS 9.2 and Quantity One software. Results were expressed as volume of the band, i.e., optical density × area of the band. An IgG monoclonal antibody against β-actin (Santa Cruz Biotechnology, SC-69879, 1:5,000; secondary: goat antimouse IgG, Sigma, A4416, 1:5,000) was used as a loading control to ensure that the same amount of protein was loaded in each lane, and the density of each target band was then expressed as a percentage of its corresponding loading control. Although we realized that it is possible that actin itself might change as a result of BVD, we felt that it was essential to use a loading control and our exploratory regression analyses suggested that any changes in actin expression were unlikely to account for changes in the target protein expression ($R^2 = 0.087$).

Statistical analysis employed planned, single pairwise *t*-tests for each brain region (BVD vs. sham) with the significance level set at 0.05; this procedure was used in preference to analysis of variance (ANOVA) because the comparisons for each protein were independent of one another and they were within the same gel (Quinn and Keough, 2002).

BVD resulted in a significant increase in SNAP-25 expression in the DG ($t(10) = 2.87$, $P = 0.02$; Fig. 1), but not CA1, CA2/3, EC, PRC or the FC (data not shown). There were no significant differences in the expression of synaptophysin in CA1, CA2/3, the DG, EC, PRC or the FC (data not shown).

Drebrin is expressed in the brain in two isoforms and therefore always appears as a double band at approx. 110 kDa ("drebrin A") and ~95 kDa ("drebrin B") (Shirao, 1995;

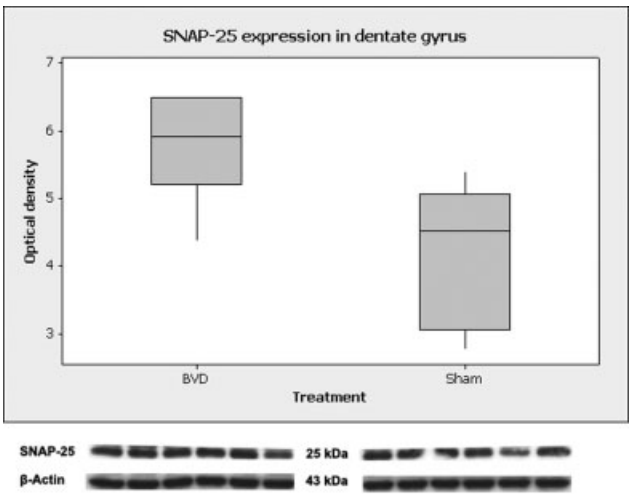

FIGURE 1. Boxplot showing the mean, interquartile range, and range for protein expression of SNAP-25 in the DG. Representative blots are shown below. BVD, bilateral vestibular deafferentation.

Sekino et al., 2007). Consistent with the evidence that this is a specific reaction, drebrin appeared as a double band in this study and the expression of the 95 kDa band ("drebrin B") was significantly reduced in the EC and FC in BVD animals ($t(10) = -3.26$, $P < 0.01$, and $t(10) = -2.75$, $P = 0.02$, respectively; Fig. 2A,B). The expression of the 110 kDa band ("drebrin A") was also significantly reduced in the EC of BVD animals ($t(10) = -2.30$, $P < 0.05$; Fig. 2A). However, drebrin expression was not significantly different in CA1, CA2/3 or the PRC.

NF-L expression was not significantly different in CA1, CA2/3, DG, EC, or PRC. However, its expression was significantly reduced in the FC of BVD animals ($t(10) = -4.31$, $P < 0.01$; Fig. 3).

Although a few previous studies have investigated changes in the expression of NMDA receptor subunits and NOS in the hippocampus following unilateral vestibular lesions (Zheng et al., 2001; Liu et al., 2003a,b; Zheng et al., 2003), to the best of our knowledge no previous study has investigated neurochemical changes in the hippocampus and related medial temporal lobe regions following BVD. Here we report for the first time that chronic BVD is associated with an increase in SNAP-25 expression in the DG, a decrease in drebrin in the EC (drebrin A and E) and FC (drebrin E), and a decrease in NF-L in the FC. These results suggest a very circumscribed pattern of neurochemical change in the medial temporal lobe and FC following BVD. Interestingly, although striking electrophysiological changes have been found in CA1 following BVD (Stackman et al., 2002; Russell et al., 2003; Zheng et al., 2003; Russell et al., 2006), no changes in the expression of these proteins were found in this region. Consistent with this, previous studies of NMDA receptor subunits and NOS following unilateral vestibular lesions, have found changes mainly in the CA2/3 region and the DG (Zheng et al., 2001; Liu et al., 2003a,b). It is possible that the changes that underlie the electrophysiological disruption of place cell function following BVD may be subtle and localized outside CA1, in areas such as CA2/3, the DG and EC. The functional significance of the increase in the expression of SNAP-25 in the DG, the decreases in drebrin in the EC and FC, and the decrease in NF-L expression in the FC following BVD, is unclear at this stage. The increased SNAP-25 expression in the DG suggests changes in the docking and fusion of synaptic vesicles prior to neurotransmitter secretion. It is possible that this is an adaptive response to changes in hippocampal computation following BVD (Russell et al., 2003a,b; Russell et al., 2006). The finding of reduced debrin expression in the EC may reflect a loss of

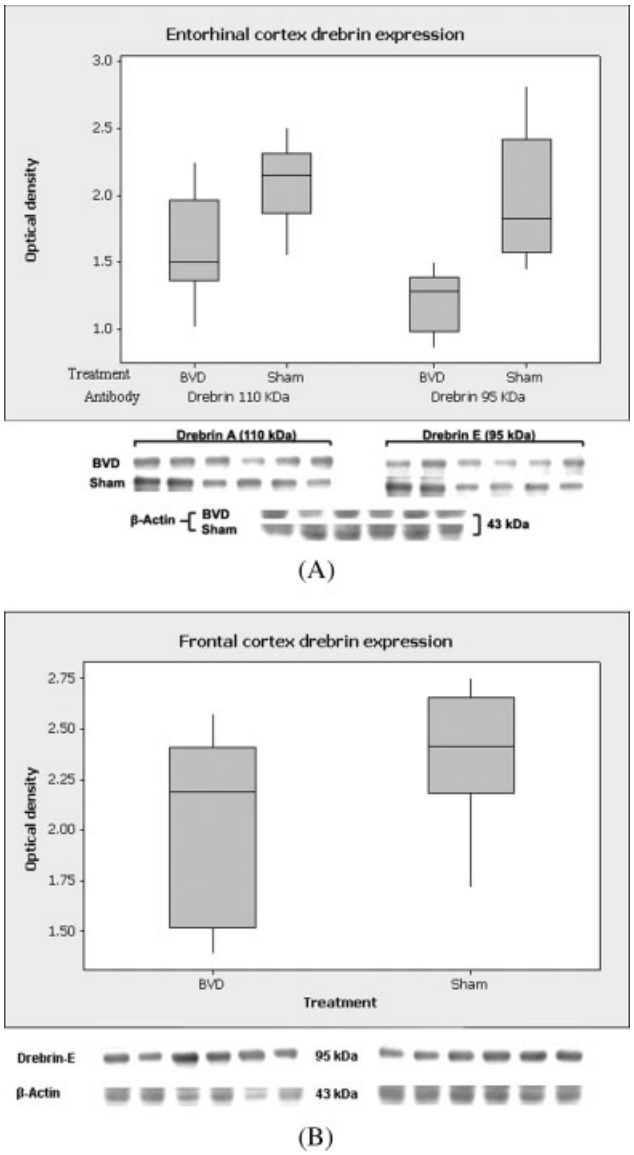

FIGURE 2. Boxplots showing the mean, interquartile range and range for protein expression of (A) drebrin E (95 kDa) and A (110 KDa) in the EC and (B) drebrin A in the FC. Representative blots are shown below. BVD, bilateral vestibular deafferentation.

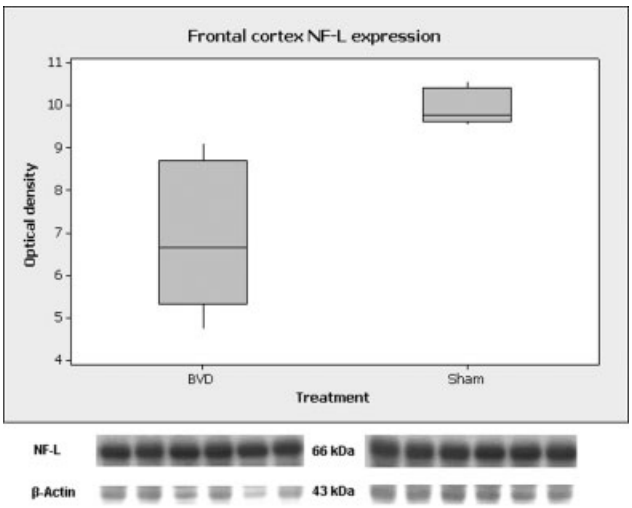

FIGURE 3. Boxplot showing the mean, interquartile range and range for protein expression of NF-L in the FC. Representative blots are shown below. BVD, bilateral vestibular deafferentation.

dendritic spines and/or a reduction in the extent of dendritic arborization in that region. This is consistent with our previous data relating to NOS, which was reduced in the EC following unilateral vestibular damage (Liu et al., 2004). Some isoforms of NOS contain a postsynaptic-density (PSD-95) binding motif that localizes with PSD proteins including actin (Brenman et al., 1996), and by extension, drebrin, which is an F-actin side-binding protein (Shirao, 1995; Sekino et al., 2007). The decrease in drebrin and NF-L expression in the FC is difficult to interpret at present, since the FC has not been studied previously in the context of BVD. Nonetheless, the current results suggest that synaptic remodeling occurs outside the hippocampus in areas of the brain that also participate in contextual information processing and working memory (see Eichenbaum, 2000; D'Esposito, 2007). Perhaps the most important conclusion of the present study is that although areas of the hippocampus such as CA1 seem to undergo very large changes in electrophysiological function, in terms of place cell activity (Stackman et al., 2002; Russell et al., 2003a) theta rhythm (Russell et al., 2006) and electrical excitability (Zheng et al., 2003), following vestibular damage, the neurochemical changes associated with them appear to be more subtle and distributed across many brain regions related to CA1.

Western blotting has a lower sensitivity to detect changes in protein levels compared to immunohistochemistry; therefore, it is possible that, in areas where we did not detect changes in synaptophysin, SNAP-25, drebrin or NF-L, smaller changes occurred that were below the resolution of the method we used. Only future immunohistochemical studies can determine whether this is the case. It is also possible that more severe neurochemical changes occur earlier following BVD and that by 6 months substantial compensation has occurred; nonetheless, it should be noted that place cell dysfunction and spatial memory impairment have been demonstrated in rats several months following BVD (Russell et al., 2003; Zheng et al., 2007).

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