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Principal component analysis suggests subtle changes in glutamate receptor subunit expression in the rat hippocampus following bilateral vestibular deafferentation

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HIGHLIGHTS

- Bilateral vestibular loss causes hippocampal dysfunction.
- Hippocampal glutamatergic neurotransmission might be expected to change.
- The results of previous studies are inconsistent.
- PCA was used to investigate the relationship between glutamate receptor subunits.
- Changes in principal components were found in rats with bilateral vestibular loss.

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ABSTRACT

Previous studies have shown that lesions of the peripheral vestibular system result in electrophysiological dysfunction in the hippocampus. Given the importance of glutamate as a neurotransmitter in the hippocampus, it was predicted that bilateral vestibular deafferentation (BVD) would alter the expression of NMDA and AMPA receptors in this area of the brain. However, the results of studies conducted to date are inconsistent. In this study, we performed principal component analysis (PCA) on the expression of the NR1, NR2B, GluR1, GluR2 and GluR3 glutamate receptor subunits, as well as calmodulin kinase IIα (CaMKIIα) and phosphorylated CaMKIIα (pCaMKIIα), in the rat CA1, CA2/3 and dentate gyrus (DG) subregions of the hippocampus, at 6 months following BVD, using western blotting. The expressions of the different glutamate receptor subunits, in terms of NMDA versus AMPA receptor subunits, as well as CaMKIIα and pCaMKIIα, were tightly correlated, and this was shown again the loading plots. However, the pattern of the contributions of each protein to the first 2 principal components appeared to be inverted for the BVD group compared to the sham group.

Previous studies have shown that bilateral lesions of the peripheral vestibular system result in abnormal electrophysiological function in the hippocampus, including disordered place cell firing and theta rhythm [6,7,9]. However, the neurochemical bases of these functional changes remain unknown. Given the importance of glutamatergic synaptic transmission in the hippocampus, it might be predicted that vestibular lesions would alter the expression and function of glutamate receptors. However, the evidence to support this prediction is inconsistent. Previous studies using unilateral vestibular deafferentation (UVD) in rats, showed that the expression of the NR1 and NR2A subunits of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, decreased in the ipsilateral CA2/3 region at 2 weeks post-UVD, while the expression of the NR2A subunit was also reduced in the contralateral CA2/3 region at the same time point [3]. By contrast, the expression of the NR2A subunit was increased in the CA1 region at 10 h following UVD [3]. However, this study did not investigate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits, GluR1–GluR4, and the longest post-operative time point was 2 weeks. Besnard et al. [1] used a sequential UVD procedure, involving intratympanic sodium arsanilate injections (i.e., one ear, followed several weeks later by the other ear), and measured NMDA receptor density and affinity in the hippocampus using receptor autoradiography. They observed a significant increase in the NMDA receptor B_{max} and a decrease in K_d.

More recently, we investigated the expression of several glutamate receptor subunits (NR1, NR2B, GluR1, GluR2 and GluR3), calmodulin kinase IIα (CaMKIIα) and phosphorylated CaMKIIα

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(pCaMKIIα) in the CA1, CA2/3 and dentate gyrus (DG) subregions of the hippocampus, at various time points following bilateral vestibular deafferentation (BVD), using western blotting [13]. To our surprise, we found no significant effects of BVD on the expression of these glutamate receptor subunits.

One possibility is that changes occur in the relationships between the different glutamate receptor subunits, which are not obvious at the level of any one subunit, and therefore such changes are not detectable using univariate statistical analyses. In order to further explore this possibility, we used the multivariate statistical method, principal component analysis (PCA), to determine whether there were linear combinations of the protein expressions of interest that accounted for most of the variation in the data, and whether the contributions of the proteins to those components changed as a result of bilateral vestibular lesions.

The data on which these analyses were based have been previously published [13] and in this paper they have been subjected to further multivariate statistical analysis. At the beginning of the study, male Wistar rats (300–500 g) were randomly allocated to BVD or sham surgery at 6 months post-surgery ($n = 14$ for the BVD group and 12 for the sham group). For the 6 month time point, BVD or sham animals were divided into those with or without spatial forced alternation in T maze training, to determine whether spatial learning experience had any effect on hippocampal glutamate receptor expression. Our previous study showed that the effects of T maze training were not significantly different for the BVD and sham animals [13]; therefore, for the purposes of the PCA, the data were pooled to provide a sufficiently large sample size ($n = 14$ for the BVD group and $n = 12$ for the sham group). Animals were maintained on a 12:12 h light:dark cycle at 22 °C and housed in individual cages. 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 and were approved by that Committee.

The animals were anaesthetised using 300 mg/kg fentanyl citrate (i.p.) and 300 mg/kg medetomidine hydrochloride (i.p.) and BVD surgery was performed under microscopic control as detailed previously [11–13]. Following surgery, the animals recovered for 6 months. All of the animals receiving a BVD exhibited the postural and locomotor behavioural symptoms that are characteristic of bilateral vestibular loss [13].

At the designated time point post-op., the animals were decapitated without anaesthesia, and the hippocampal subregions (CA1, CA2/3 and the dentate gyrus (DG)), were dissected out using the methods described previously [12], and stored in a −80 °C freezer until use. At the time of processing, tissue buffer (containing Complete Proteinase Inhibitor, 50 mM Tris–HCl pH 7.6) was added to the samples on ice, then the tissue was homogenised using ultrasonification (Sonifier cell disrupter B-30, Branson Sonic Power Co.) and centrifuged at 12,000 × *g* for 10 min at 4 °C. The protein concentration in the supernatant was measured using the Bradford method and equalised, then the supernatants were 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 boiled for 5 min. Ten μg of protein from each sample was loaded in each well on a 7.5% SDS–polyacrylamide mini-gel and pre-stained protein markers (10–250 kDa; Bio-Rad, Precision Plus: Dual colour) were used as molecular weight markers on each gel. In order to control for between gel variations, an internal standard made of pooled cerebellar samples from sham rats was loaded on each gel. The 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. The proteins were transferred to polyvinylidene-difluoride (PVDF) membranes using a transblotting apparatus (2.5 L; Bio-Rad). The transfer was performed overnight in transfer buffer (25% methanol, 1.5% glycine and 0.3% Tris-base) at a 10 V variable current (Bio-Rad PowerPack 3000). Non-specific IgG binding was blocked by incubation with 5% dried milk protein (Pams) and 0.1% bovine serum albumin (BSA) (Sigma) for 6–7 h at 4 °C. The membranes were then incubated with affinity-purified polyclonal goat antibodies raised against GluR1, GluR2 and GluR3, and affinity-purified polyclonal rabbit antibodies raised against NR1, NR2B, CaMKIIα and pCaMKIIα, overnight at 4 °C (see antibody details in [13]). The specificity of these antibodies has been demonstrated in previous studies [13] and the dilutions were optimised for the current study. The secondary antibodies were anti-goat IgG linked to horseradish peroxidase and anti-rabbit IgG linked to horseradish peroxidase. Detection was performed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, NZ). Hyperfilms (Amersham Biosciences, NZ) were analysed 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 the volume of the band, i.e., optical density × area of the band. An antibody against β-actin was used as a loading control and exploratory regression analyses performed in our laboratory have shown that any changes in β-actin expression were unlikely to account for changes in the target protein expression ($R^2 = 0.087$) [12]. The volume of each target band was then normalised to its corresponding loading control and then the internal standard within each gel. It was expected that the protein levels measured would reflect both the intra-cytoplasmic and membrane receptor subunits together.

The data from the CA1, CA2/3 and DG hippocampal subregions were combined, expressed as *z* scores and principal component analysis (PCA) was carried out based on the correlation matrix, using SPSS 20 and Minitab 16 [4,5,10]. The Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy was >0.5, suggesting that the sample was of adequate size [4,5,10]. Exactly the same statistical analyses were carried out on the data from the sham and BVD animals. A Scree plot, representing the proportion of the total variance in the data accounted for by each principal component, was used to assess the importance of the principal components (i.e., 'eigenvalues') and a loading plot to evaluate the contribution (i.e., 'eigenvectors') of each variable (protein in this case) to the first 2 components [4,5,10]. The loading plots in PCA can therefore be used to assess the relationships between different variables for the different principal components [4,5,10]. Unlike Factor Analysis, which assumes a formal statistical model, PCA is an exploratory statistical method that does not make formal assumptions about the data, and therefore significance tests were not employed [4,5,10]. Cluster analysis using the Ward's minimal variance algorithm on the correlation coefficient distance, was used to assess the degree to which the expression of the 7 neurochemical variables varied together [4,5,10]. A dendrogram was used to display this graphically.

For the sham group, the first 2 PCs accounted for 75.1% of the variation in the data, compared to 76% for the BVD group. In both cases, 4 PCs were needed to account for 97% of the variation in the data. While the Scree plots were virtually identical (data not shown), the loading plots were different. The dendrogram for the cluster analysis showed that the expressions of the different glutamate receptor subunits, in terms of NMDA versus AMPA subunits, as well as CaMKIIα and pCaMKIIα, were tightly correlated (Fig. 1), and this was shown again in the loading plots (Fig. 2). However, the pattern of the contributions of each protein to the first 2 PCs appeared to be inverted for the BVD group compared to the sham group (see Fig. 2).

The results of this study suggest that while the expression of individual AMPA and NMDA glutamate receptor subunits, and CaMKIIα/pCaMKIIα, in the hippocampus, was not significantly different between BVD and sham animals at 6 months post-op. [13],