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Cell proliferation and survival in the vestibular nucleus following bilateral vestibular deafferentation in the adult rat

Yiwen Zheng, Chisako Masumura, Phoebe Chung, Cynthia L. Darlington, Paul F. Smith*

Department of Pharmacology and Toxicology, School of Medical Sciences, University of Otago Medical School, Frederick St, Dunedin, New Zealand

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ABSTRACT

Cell proliferation and neurogenesis in the brainstem vestibular nucleus complex (VNC) has previously been reported following unilateral vestibular neurectomy in the cat. In this study, we examined the rate of cell proliferation and survival in the adult rat VNC following bilateral vestibular deafferentation (BVD), using injections of bromodeoxyuridine (BrdU) and stereological cell counting. We measured cell proliferation at 24, 48, 72 h and 1 week following BVD and found that it was significantly greater than in sham controls ($P = 0.002$) and that it varied significantly over time ($P = 0.01$), peaking at 48 h in the BVD group. Of note was that sham surgery was also associated with an increase in cell proliferation, which changed over time. When we compared the survival of new cells at 1 month after BrdU injection, there was no significant difference in survival between the sham and BVD groups. These results raise questions about the potential functional significance of cell proliferation in the VNC following vestibular lesions.

Although it is now well accepted that neurogenesis occurs in the adult CNS, it has been reported mostly in known neurogenic regions of the brain such as the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus [5]. By contrast, there have been relatively few reports of neurogenesis in brainstem regions [6].

Following unilateral loss of vestibular function, mammals generate a severe ocular motor and postural syndrome from which they gradually recover in a process known as 'vestibular compensation' (see [9] for a review). The acute loss of vestibular nerve activity results in a collapse of spontaneous resting activity in the ipsilateral vestibular nucleus complex (VNC), leading to a variety of complex neurochemical changes (see [10] for a review). Increased cell proliferation has been reported in the VNC following unilateral surgical labyrinthectomy in the rat; however, the newly generated cells appeared to be microglia rather than neurons [1–3]. By contrast, Tighilet et al. [11] reported that neurogenesis occurs in the deafferented VNC of the adult cat after unilateral vestibular neurectomy. They showed that most of the new cells survived up to 1 month following the vestibular lesion and that at least some of them differentiated into neurons with a GABAergic phenotype. Consequently, Tighilet et al. [11] were particularly interested in whether these new neurons might have functional significance in the vestibular compensation process.

The effects of unilateral and bilateral lesions of the peripheral vestibular system are dramatically different. Whereas unilateral loss of vestibular function results in an imbalance in spontaneous resting activity between neurons in the ipsilateral and contralateral VNCs, which causes asymmetric activation of the vestibular reflexes, bilateral lesions result in a complete loss of vestibular function without ocular motor or postural asymmetries [9]. Therefore, we wondered whether cell proliferation and survival would also occur in the VNC following bilateral vestibular deafferentation (BVD) in the adult rat. In order to answer this question, we conducted a cell proliferation time course study using BrdU to label the proliferating cells at different time points following BVD and then evaluated cell survival at 1 month following the BrdU injection.

Data were obtained from 39 male Wistar rats (250–300 g). BVD was performed using an otolaryngological microscope and the methods described in detail previously (e.g., [14,15]). Briefly, animals were anesthetised with 200 μg/kg fentanyl citrate (i.p.) and 500 μg/kg medetomidine hydrochloride (i.p.). The tympanic bulla was exposed using a retro-auricular approach and the tympanic membrane, malleus and incus removed. The stapedial artery was cauterised and the horizontal and anterior semicircular canal ampullae drilled open. The contents of the canal ampullae and the utricle and saccule were aspirated and the temporal bone sealed with dental cement. Carprofen (5 mg/kg, s.c.) was used for post-operative analgesia. Sham surgery consisted of exposing the temporal bones and removing the tympanic membrane, malleus and incus without producing a vestibular lesion. All procedures were approved by the University of Otago Committee for the Care and Use of Laboratory Animals.

* Corresponding author. Fax: +64 3 479 9140.
E-mail address: paul.smith@stonebow.otago.ac.nz (P.F. Smith).

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For the proliferation study, 29 rats were divided into sham surgery ($n = 13$) or BVD groups ($n = 16$). Bromodeoxyuridine (BrdU, 300 mg/kg, i.p), which is incorporated in newly synthesised DNA in place of thymidine, was injected either at 24 h (sham $n = 3$, BVD = 4), 48 h (sham $n = 4$, BVD = 5), 72 h (sham $n = 3$, BVD = 4), or 1 week (sham $n = 3$, BVD = 3), following the sham or BVD surgery. At 24 h after the BrdU injection, the animals were anesthetised (300 mg/kg pentobarbital, i.p) and then perfused with 4% paraformaldehyde. Brains were dissected and serial 40 μm sections throughout the VNC collected using a random and systematic design. VNCs were collected from either the right or the left side with the two sides counterbalanced. Free floating immunohistochemistry was used to label the BrdU^{+ve} nuclei in the sections. Briefly, following DNA denaturation with 4 M HCl, the sections were neutralized with 0.18 M borax and incubated with a mouse monoclonal anti-BrdU antibody (1:40, Dako) overnight at 4 °C. The sections were then incubated with a HRP conjugated goat-anti-mouse secondary antibody (1:200, Sigma) and stained with DAB.

The total number of immunopositive BrdU cells was estimated using a modified fractionator method [4,7,12]. Basically, every 5th section throughout the VNC was examined and all BrdU positive cells in the VNC were counted in each section. The total number of BrdU positive cells per section was then averaged and multiplied by the number of sections throughout the VNC and the interval between the sections. Great care was taken not to count BrdU positive cells on the wall of any blood vessel.

For the cell survival study, an additional 10 rats (sham, $n = 5$ and BVD, $n = 5$) were injected with 300 mg/kg, i.p BrdU at 72 h post-op. and they were sacrificed at 1 month after the BrdU injection. The 72 h post-op. time point was chosen based on the peak proliferation time reported in the cat [11]. Tissue was processed and counts of BrdU^{+ve} cells in the VNC were quantified using the methods described above. The survival rate was calculated by comparing the number of BrdU^{+ve} cells labelled at 72 h post-op. and surviving at 1 month, with the number at 24 h after the BrdU injection.

Cell counting data are often not normally distributed [8]. After testing for normality and equality of variance, it was determined that the data were non-normal using an Anderson–Darling test and consequently a square root transformation was performed on the proliferation data [8]. After confirming that normality had been achieved, a 2 factor ANOVA with surgery and time as between group factors was performed [8]. In the case of a significant effect, Tukey's post hoc comparisons were used. For the survival data, transformation did not result in normality; therefore, a non-parametric Mann–Whitney U-test was used [8]. $P < 0.05$ was considered significant (Table 1).

For the proliferation study, BVD animals demonstrated a significant increase in cell proliferation ($F(1,21) = 12.84$, $P = 0.002$), although some proliferation did occur in the VNC even after the sham surgery (Figs. 1A and 2), however the interaction between surgery and time was not significant. Post hoc analysis indicated that the only significant pairwise differences were between the 1 week sham condition and the BVD 24 h ($t = -3.68$, $P < 0.03$) and BVD 48 h conditions ($t = -5.03$, $P = 0.01$). Although the number of BrdU labelled cells was not calculated separately for the right and left VNCs, there was no obvious difference between the two sides.

Table 1
The results of a 2 way ANOVA on the cell proliferation data. The surgery factor had two levels, BVD and sham; the time factor had four levels, 24, 48, 72 h and 1 week.

Variable and source of variation	F-Value (df)	P-Value
Surgery	$F(1,21) = 12.84$	$P = 0.002^*$
Time	$F(3,21) = 4.63$	$P = 0.012^*$
Surgery × time	$F(3,21) = 0.42$	$P = 0.743$

* Significant difference.

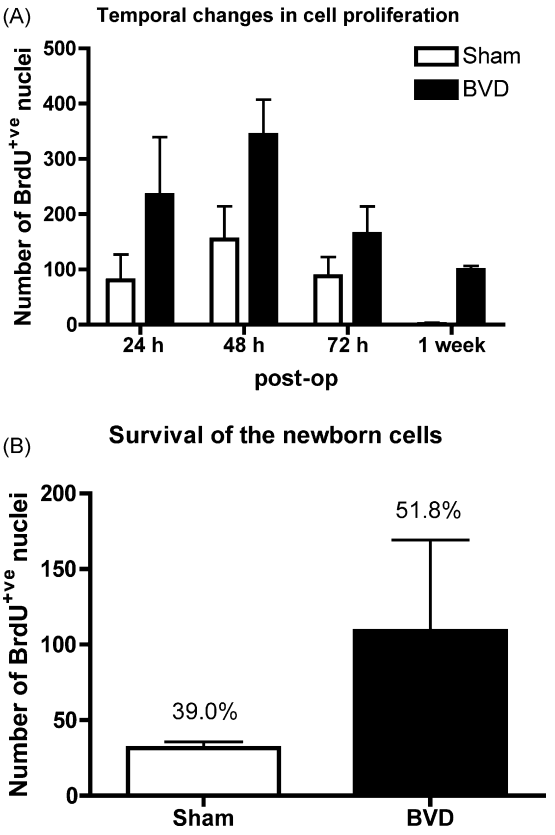

Fig. 1. (A) Number of BrdU^{+ve} nuclei in the VNC at 24, 48, 74 h and 1 week following BVD or sham surgery. (B) Percentage survival of newborn cells at 1 month after BrdU injection compared to 24 h after BrdU injection. Bars represent means ± 1 SE.

Analysis of the survival of new cells at 1 month indicated no significant difference between the sham and BVD groups, due to the large degree of variability in the number of BrdU positive cells in the BVD animals (Fig. 1B).

The results of this study confirm that BVD, like unilateral vestibular lesions, does result in an increase in cell proliferation in the rat VNC. In this case, proliferation started to increase at 24 h, peaked at 48 h and declined toward 1 week post-op. This pattern is slightly different from the results of Tighilet et al. [11], using BrdU labelling in the VNC of the cat after unilateral vestibular neurectomy, where the peak proliferation time was at 72 h post-op. This discrepancy could be due to the use of bilateral versus unilateral vestibular lesions or to a species difference. In the case of bilateral vestibular lesions, the VNC may respond more quickly. It was particularly interesting that even the sham surgical procedure, which consisted only of removing the tympanic membrane and drilling on the temporal bone, also resulted in cell proliferation, although to a lesser extent than the BVD. This result, and the complex pattern of change in cell proliferation in the BVD and sham groups over time, suggests that it may be very important to use separate sham controls for every time point when studying cell proliferation and neurogenesis in the VNC.

Because we did not undertake double immunolabelling in this study, we do not know whether any of the new cells we identified differentiated into neurons. It is possible that they were part of the microglial reaction described previously following unilateral vestibular lesions [1–3]. Nonetheless, approximately 50% of the newly generated cells survived for up to 1 month following BVD and therefore on the basis of the work of Tighilet et al. [11], it is likely that at least some of these cells differentiated into neurons. One reason for not undertaking double immunolabelling to identify the phenotypes of the new cells, was that the percentage