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Lim, Rebecca; Callister, Robert J. & Brichta, Alan M. "An increase in glycinergic quantal amplitude and frequency during early vestibular compensation in mouse" Published in the *Journal of Neurophysiology*, Vol. 103, Issue 1, (2010).

Available from: <http://dx.doi.org/10.1152/jn.91223.2008>

Accessed from: <http://hdl.handle.net/1959.13/932385>

An increase in glycinergic quantal amplitude and frequency during early vestibular compensation in mouse.

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Abstract

The process of vestibular compensation includes both behavioral and neuronal recovery after unilateral loss of peripheral vestibular organs. The mechanisms that underlie this process are poorly understood. Previous research has shown the presence of both GABA_A and glycine receptors in the medial vestibular nuclei (MVN). It has been suggested that inhibitory transmission mediated by these receptors may have a role in recovery during vestibular compensation. This study investigated changes in fast inhibitory synaptic transmission of GABA_Aergic and glycinergic quantal events after unilateral labyrinthectomy (UL) at three different time points. Mice were anaesthetized and peripheral vestibular organs were removed from one side of the head. After recovery, transverse brainstem sections (300 µm) were prepared from mice that had undergone UL either; 4 hours, 2 days, or 7 days earlier. Our experiments do not show evidence for alterations in synaptic GABA_A receptor properties in MVN neurons after UL at any time point investigated. In contrast, during early vestibular compensation (4 hours post UL) there is a significant increase in the glycinergic quantal current amplitude in contralesional MVN neurons compared to control. Our results also show an increase in the frequency of glycinergic quantal events of both ipsi- and contralesional MVN neurons during this early period. We suggest that changes in both pre- and postsynaptic glycine receptor mediated inhibitory synaptic transmission after sensory loss is an important mechanism by which neuronal discharge patterns can be modulated.

30 **Introduction**

31

32 Vestibular compensation is a form of adult neuronal plasticity that occurs in response to the
33 unilateral damage or destruction of the peripheral vestibular organs. Initially, a broad set of
34 behavioral symptoms follow unilateral labyrinthectomy (UL) including, head tilt and postural
35 deficits toward the lesioned side, spontaneous nystagmus, and asymmetry of the vestibulo-
36 ocular reflex (VOR). Typically static symptoms that occur in the absence of movement, such
37 as head tilt and spontaneous nystagmus abate within a week (Darlington and Smith 2000).
38 However, dynamic symptoms such as asymmetry in the VOR have a longer time course of
39 recovery and are never fully restored (Aleisa et al. 2007; Maioli et al. 1983; Newlands et al.
40 2005).

41

42 The central mechanisms that underlie vestibular compensation are poorly understood. *In vivo*
43 studies have shown in the acute phase of vestibular compensation, immediately after UL,
44 neurons in the ipsilesional medial vestibular nucleus (MVN) become silent whereas
45 contralesional MVN neurons have higher discharge rates than control (Ris et al. 1995;
46 Shimazu and Precht 1965; Smith and Curthoys 1988a, b). Under normal conditions, type I
47 MVN neurons are inhibited during contralateral horizontal rotation. Contralateral neurons of
48 the commissural pathway are responsible for this inhibition (Kasahara et al. 1968; Mano et al.
49 1968; Shimazu and Precht 1966). Immediately after UL, the discharge rate of type I
50 ipsilesional MVN neurons is diminished due to both the removal of primary vestibular input
51 and increased inhibition from contralesional commissural neurons (Markham et al. 1977).
52 Approximately 2-3 days after UL, the discharge rates between ipsi- and contralesional sides
53 are comparable and by one week, discharge rates on both sides are equivalent to those prior
54 to ablation (Ris et al. 1995; Smith and Curthoys 1988a, b). It has been suggested, restoration
55 of pre-UL discharge rates may be mediated in part, by mutually inhibitory commissural
56 connections between the bilateral MVN (Bergquist et al. 2008; Galiana et al. 1984).

57

58 Inhibition in vestibular nucleus arises from commissural fibres (Precht et al. 1973),
59 cerebellum (Bagnall et al. 2009; de Zeeuw and Berrebi 1996), and inhibitory interneurons
60 (Holstein et al. 1999b). Inhibitory commissural neurons connecting the two MVN are
61 GABAergic and glycinergic (Bagnall et al. 2007; Holstein et al. 1999a; Precht et al. 1973),

62 thus the role of inhibitory receptors and their transmitters during vestibular compensation has
63 been investigated using a variety of techniques with inconsistent results. In situ hybridization,
64 western blots, and immunohistochemistry in rats have shown no changes in GABA_A,
65 GABA_B, or glycine receptor expression levels after UL (Eleore et al. 2005; Eleore et al.
66 2004; Gliddon et al. 2005a). Contrary evidence however, suggests enhanced GABA
67 transmission such as, increases in mRNA of the $\alpha 1$ subunit of the GABA_A receptor and for
68 GABA precursor enzymes, GAD65 and 67 after UL (Horii et al. 2003). Pharmacological
69 studies show modified sensitivity of discharge rate to inhibitory amino acid agonists and
70 antagonists during vestibular compensation (Johnston et al. 2001; Vibert et al. 2000;
71 Yamanaka et al. 2000). Furthermore, elevated GABA release after UL has been reported by
72 microdialysis (Bergquist et al. 2008), as well as increases in the number of GABA
73 immunoreactive neurons in the MVN (Tighilet and Lacour 2001). In this study we aim to
74 resolve the discrepant role of fast inhibitory transmission after UL, by investigating synaptic
75 GABA_A and glycine receptors in ipsi- and contralesional MVN neurons at three time points
76 after UL. Our results show evidence of changes in both presynaptic and postsynaptic
77 inhibitory glycinergic synaptic transmission that occur during early vestibular compensation.
78

79 **Materials and Methods**

80

81 *Unilateral Labyrinthectomy*

82

83 All experiments were conducted according to The University of Newcastle Animal Care and
84 Ethics Committee Regulations. Prior to surgery, mice were habituated to jelly pellets
85 containing pain medication, buprenorphine (Reckitt and Colman Pharmaceuticals, Australia).
86 A previous study in rodents has shown that inclusion of sublingual buprenorphine into jelly
87 cubes is an effective, non-invasive means of analgesic delivery (Volker et al. 2000).

88

89 Forty-four mice (C57BL6) approximately four weeks old of both sexes underwent UL to the
90 left ear in a procedure previously described by Gacek and Khertepal (1998). Briefly, under
91 2.5% isoflurane anesthesia, a subcutaneous injection of carprofen (rimadyl; Novartis, 2.5
92 mg/kg) was administered to minimize inflammation and pain responses. A postauricular
93 incision was made to expose the external ear canal. The tympanic membrane was ruptured
94 and the malleus and incus removed. A small corneal rust ring remover with 0.5mm burr
95 (Algerbrush II; The Alger Equipment Company, USA) was used to remove the promonotory
96 region of the tympanic bone, thereby better exposing the stapes and the intersecting stapedia
97 artery. The stapedia artery was coagulated on either side of the footplate using a unipolar
98 cautery. The stapes was then removed using fine forceps and a hook was inserted into the
99 oval window to out-fracture the bone. Forceps were used to remove the exposed horizontal
100 and anterior ampullae and a right-angled hook was used to disrupt the remaining vestibular
101 organs. The vestibule was filled with Gelfoam and the surgical incision closed using
102 interrupted sutures. Subcutaneous injections of buprenorphine (0.05mg/kg) and lactated
103 Ringer's solution (0.5-1ml) were administered 30 minutes prior to the removal of isoflurane
104 anesthesia. Mice recovered quickly from anesthesia and immediately displayed symptoms
105 consistent with damage to the peripheral organs including, head tilt, circling and barrel
106 rolling (Gacek and Khetarpal 1998). In all cases, a post-mortem examination of the bony
107 labyrinth confirmed horizontal and anterior ampullae destruction and that gelfoam had filled
108 much of the vestibular labyrinth.

109

110

In vitro slice preparation

Three time points were investigated after UL: 4 hours, 2 days, and 7 days. Mice were injected with an intraperitoneal injection of ketamine (150 mg/kg) and decapitated. The head was placed in ice-cold sucrose-based Ringer's solution containing (in mM): 236 sucrose, 25 NaHCO₃, 11 glucose, 2.5 KCl, 1 NaH₂PO₄, 1 MgCl₂, and 2.5 CaCl₂ (Camp et al. 2006). The brainstem containing MVN was dissected as previously described (Camp et al. 2006). Coronal sections (300 µm thick) containing MVN were cut using a Leica microtome (VT1000S). We made a small nick to the right ventral side of the section with micro scissors to identify the contralesional side. Sections were then transferred to a humidified oxygenated incubation chamber containing normal Ringer's solution same as the sucrose-based Ringer's solution described above, except 118 mM NaCl substituted for sucrose. Sections were incubated for 1 hour prior to recording.

Electrophysiology

Voltage clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) were made from MVN neurons that were located within the rostral half of the nucleus. They were visualized using infra-red differential interference contrast (IR-DIC) optics. Observations were made at room temperature (23-25°C) on slices that were continually superfused with normal Ringer's solution oxygenated with Carbogen (95% O₂ - 5% CO₂). Room temperature was selected so that direct comparison could be made with data previously reported by this laboratory (Camp et al. 2006) and with the majority of other studies of inhibitory postsynaptic quantal currents, also recorded at room temperature (21-23°C) (Graham et al. 2006; Lim et al. 1999; Lu et al. 2008; O'Brien and Berger 1999, also see discussion). Patch electrodes (borosilicate glass; 3-5 MOhms) were filled with a CsCl-based internal solution containing (in mM): CsCl 130, MgCl₂ 1, EGTA 10, HEPES 10, ATP-Mg 2, GTP-Na 0.2. To isolate inhibitory mIPSCs, antagonists of sodium dependent action potentials (tetrodotoxin, TTX; 1 µM; Alomone, Jerusalem) and glutamatergic AMPA receptors (6-cyano-7-nitroquinoxaline-2,3-dione, CNQX; 10 µM; Sigma, Australia) were added to the perfusate. Drugs were applied for a minimum of 3 minutes prior to data acquisition. Neurons were held at a membrane potential of -70mV, thus the normal Mg²⁺ block of the N-methyl-D-aspartate (NMDA) receptor prevents activation of NMDA receptors from contributing to synaptic

events. GABA_Aergic mIPSCs were isolated by the addition of the glycine receptor antagonist, strychnine (1 μ M; Sigma, Australia). In experiments isolating glycinergic mIPSCs, bicuculline (5 μ M; Tocris, UK) was added to the TTX and CNQX perfusate. In some cells, the frequency of glycinergic mIPSCs was low. If in the presence of bicuculline, the frequency was less than 0.03 Hz (<1 event per 30 sec), we classified these neurons as receiving GABA_Aergic-only synaptic inputs and we analyzed mIPSC data previously recorded in the presence of TTX and CNQX only.

Series resistance was routinely monitored before, during, and after the addition of drugs. Data were rejected if series resistance changed by more than 20%. Synaptic currents were recorded and filtered online (2-5 kHz, sampling rate 5-10 kHz) using a Multiclamp 700A amplifier (Axon CNS, MDS Analytical Technologies), ITC1600 digital interface (Instrutech) and Axograph X (AxoGraph Scientific, Sydney, Australia) or Axopatch 1D amplifier (Axon Instruments), an ITC-16 digital interface (Instrutech) and Axograph 4.8 (Axon Instruments). No significant difference was observed between the two data acquisition systems.

Data analysis

Quantal synaptic events were detected using the sliding template function of Axograph X (see Clements and Bekkers 1997), using parameters previously determined by Camp et al., (2006). Peak amplitude, kinetics (rise time; 10-90% of peak amplitude and decay time constant; single exponential 20-80% of decay phase), and frequency were measured for each neuron. The decay-time of mIPSCs was best fit by a single exponential. Quantal events within a minimum of 3 minutes recording time were used to calculate the frequency. To calculate the inter-event interval cumulative probability distribution for cells in the 4 hour post-UL group, the data was normalized and pooled using Igor Pro graphical analysis package (v6.0.2, Wavemetrics). Mann-Whitney U non-parametric tests (SPSS Statistics 17.0; Chicago) were used for comparisons of mIPSC characteristics. Data are presented as mean $\pm$ standard error.

Results

Behavioural observations after UL

After UL, all mice showed typical behaviors consistent with damage to the peripheral vestibular organs; including head tilt and postural deficits toward the lesioned side, circular ambulation and occasional barrel rolling. While we did not measure the degree of spontaneous nystagmus, we video-recorded and observed the behavior of mice during the week after UL. Within 3 days of UL, there was a significant improvement in head tilt and postural lean as well as reduced circling and rolling behaviors. One week after UL, with the exception of a slight head tilt, mice displayed few asymmetric behaviors. The gait was normal and sure-footed and some mice were observed to crawl upside-down on the underside of their cage cover with no apparent impairment. The timecourse of improvements in posture and gait were consistent with those described previously by Gacek and Kheterpal (1998).

Electrophysiological recordings

Recordings were obtained from a total of 181 mouse MVN neurons (52 mice including control animals). GABA_Aergic and glycinergic mIPSCs were recorded from MVN neurons at three time points after UL; 4 hours, 2 days, and 7 days. Previously, it has been shown that only 30% of MVN neurons receive glycinergic input (Camp et al. 2006). However, to allow comparisons of quantal event properties for both GABA_Aergic and glycinergic mIPSCs, with approximately equal sample sizes, we increased the glycinergic data set.

GABA_Aergic mIPSCs during vestibular compensation

A total of 89 neurons from 19 mice receiving GABA_Aergic inputs were recorded. These were a combination of exclusively GABA_Aergic and mixed inhibitory inputs (Camp et al. 2006). Figure 1A shows representative data of GABA_Aergic mIPSCs from a control neuron. The upper panel (figure 1A) is a continuous trace of GABA_Aergic mIPSCs in the presence of TTX, CNQX and strychnine. Figure 1A, inset, is the averaged mIPSC profile (n = 159 mIPSCs), while the lower panel in figure 1A is the amplitude distribution of GABA_Aergic

mIPSCs for this neuron. All other panels share the same layout. Figure 1 (B-C) shows representative data of GABA_A mIPSCs from both ipsilesional (B1-B3) and contralesional (C1-C3) MVN neurons at each of the three time points (4 hours: B1 and C1; 2 days: B2 and C2; 7 days: B3 and C3) after UL. Our results showed that although there appeared to be an increase in GABA_A mIPSC amplitude in ipsilesional MVN neurons, at 4 hours post UL it was not significant. Group data (figure 1D) showed that after UL there was no significant change in the mean GABA mIPSC amplitude compared to control for either ipsi- or contralesional MVN neurons, or between the ipsi- and contralesional sides. Analysis of other quantal parameters including rise time, decay time, and frequency (see Table 1) also showed no significant changes in the kinetics or frequency of GABA_A mIPSC quantal events between experimental and control MVN neurons, or between ipsi- and contralesional MVN neurons at any time point investigated after UL.

Glycinergic mIPSCs during vestibular compensation

Glycinergic mIPSCs were recorded from a total of 92 neurons (25 mice). These mIPSCs were from a population of glycine only and mixed inhibitory inputs. In comparison to GABA_A mIPSCs, glycinergic mIPSCs have larger mean amplitudes, faster kinetics, and lower frequencies, consistent with results previously reported (Camp et al. 2006). Figure 2A shows representative data of glycinergic mIPSCs from a control neuron. The upper panel (figure 2A) is a continuous trace of glycinergic mIPSCs in the presence of TTX, CNQX, and bicuculline. Figure 2A, inset, is the averaged mIPSC profile (n = 63 mIPSCs), while figure 2A lower is the amplitude distribution of glycinergic mIPSCs for this neuron. All other panels share the same layout. Figure 2 (B-C) shows representative data of glycinergic mIPSCs after UL and is presented in the same format as GABAergic mIPSCs described above. Four hours after UL there is a significant increase in the mean amplitude of glycinergic mIPSCs in contralesional MVN neurons compared to control (70.2 ± 16.1 pA vs 37.3 ± 6.6 pA, $p < 0.05$; Figure 2D). This increase in amplitude of glycinergic mIPSCs from contralesional MVN neurons, represents almost a 2-fold increase compared to control (figure 2C1 versus 2A) and indicates a change in postsynaptic receptor subunit composition or cluster size.

An increase in mean glycine receptor amplitude in contralesional MVN neurons, 4 hours post-UL, may be due to the alteration of glycine receptor subunits. This would be seen as a change in decay-time kinetics of mIPSCs. A comparison of decay time constants between contralesional and control MVN neurons shows no significant change 4 hours after UL (5.9 ± 0.5 ms vs 6.0 ± 0.7 ms; $p > 0.05$). Similarly, there was no significant difference in the decay-time kinetics between ipsi- and contralesional neurons or control neurons at any time point after UL (see Table 2). Therefore, these data suggest that UL does not modify glycine receptor subunit composition. Alternatively, as mentioned above, the increase in glycine receptor amplitude may be due to changes in the size of glycine receptor clusters. It is possible that there is an increase in the size of the 'synaptic' glycine receptor cluster area that would have been difficult to detect in previous immunolabelling studies. Confirmation, however, would require labelling of both pre- and postsynaptic specializations, and is beyond the scope of this study.

Our results also show a shift in the amplitude distribution of glycinergic mIPSCs in contralesional MVN neurons at 4 hours post-UL toward larger quantal events compared to control. For example, the cell illustrated in figure 2C1 shows a typical increased mean amplitude and skewed amplitude distribution (filled histogram) when compared to control (open histogram; figure 2C1 vs figure 2A; mean mIPSC amplitude: -73.7 pA vs -31.7 pA, range: -17 to -343 pA vs -9.8 to -93.2 pA). This shift toward larger quantal events after UL, suggests there may be a preferential activation of receptors from large synaptic sites (Lim et al. 2003). This possibility is supported by changes in the frequency of glycinergic mIPSCs addressed below.

While the above results suggest evidence for postsynaptic modulation of synaptic strength, our data also imply presynaptic effects. This may be mediated by changes in the probability of transmitter release, as evidenced by alterations in mIPSC frequency. While we did not observe a change in GABA_A mIPSC frequency after UL (Table 1), there is however, a significant increase in glycine mIPSC frequency in both ipsi- and contralesional MVN neurons, 4 hours after UL compared to control (ipsilesion 0.6 ± 0.2 Hz, contralesion 0.7 ± 0.2 Hz, control 0.2 ± 0.03 Hz; $p < 0.01$; Figure 3A). The increased frequency of mIPSCs reflects a decrease in the inter-event intervals of quantal events. Figure 3B illustrates a higher

proportion of short latency inter-event intervals in ipsi- and contralesional MVN neurons compared to control, 4 hours post-UL. Between 75 and 85% of inter-event intervals are shorter than 2.5 sec in ipsi- and contralesional MVN neurons, whereas <60% of inter-event intervals are less than 2.5s in control neurons. This increase in the frequency of glycinergic mIPSCs most likely reflects an increase in the probability of presynaptic release of glycine onto MVN neurons within 4 hours of UL. While this increase in frequency of glycinergic mIPSCs in contralesional MVN neurons, 4 hours post-UL, is not correlated with increases in mIPSC amplitude, it should be emphasized that presynaptic modulation of synaptic strength has not been reported during vestibular compensation.

In summary, while our results show no change in GABA_A receptor function in MVN neurons one week post UL, however we observed a distinct, brief alteration in inhibitory glycinergic transmission four hours after UL. Modifications in both pre- and postsynaptic transmission, such as frequency of quantal events and mean current amplitude, respectively, suggest there is more than one mechanism responsible for initiating and/or maintaining vestibular compensation.

Discussion

Asymmetric discharge rates in MVN neurons immediately after UL are not only due to loss of primary afferent input but also a concomitant change in fast inhibitory synaptic drive mediated by GABA_A and glycine receptors (Bergquist et al. 2008; Gliddon et al. 2005b). Whether inhibitory synaptic connections have a role in restoring discharge rates between the two sides has not yet been established. This study has investigated fast inhibitory synaptic transmission in MVN neurons at various time points after UL, to determine their role in vestibular compensation.

Synaptic inhibition in mouse MVN is mediated by both GABA_A and glycine receptors (Camp et al. 2006). It has been proposed that recovery from the asymmetric discharge rate of MVN neurons after UL is partially due to GABAergic and glycinergic commissural connections between the two sides (Bergquist et al. 2008). It is possible therefore, that altered inhibitory synaptic transmission, due to changes in neurotransmitter release and/or functional

modifications of postsynaptic receptors could differentially modulate the discharge rate of MVN neurons during vestibular compensation.

GABA_A receptor mediated mIPSCs

Studies have reported that MVN neurons have an altered sensitivity to GABA receptor agonists and antagonists after UL (Johnston et al. 2001; Vibert et al. 2000; Yamanaka et al. 2000), which may be reflected in functional changes of receptor properties. It was an aim of this study to determine whether UL could induce a change in inhibitory receptor function. These potential changes would influence the discharge rate of MVN neurons and consequently help explain previous pharmacological results. To determine whether receptor properties were altered after UL, we recorded quantal synaptic currents, at three time points after UL. Our results indicate that properties of GABA_A receptors, including amplitude, kinetics, and mIPSC frequency in labyrinthectomized animals were not significantly altered compared to control or between ipsi- and contralesional sides. Thus, we see no evidence for an alteration in subunit composition that may account for reduced agonist sensitivity reported previously (Johnston et al. 2001; Vibert et al. 2000; Yamanaka et al. 2000).

The properties of GABA_A postsynaptic receptors are comparable with those previously reported by Camp et al., (2006) with the exception of lower frequency of GABA_Aergic events in this study. The lower frequency might be due to sampling neurons that receive both GABA_A-only and mixed GABA_Aergic-glycinergic inputs, while the data reported by Camp et al., (2006) represents the frequency of mIPSCs that originate from GABA_A-only inputs to MVN neurons. Therefore, neurons that possess GABA_A-only receptors may have a greater number of GABAergic inhibitory inputs or a higher rate of GABA release, *i.e.*, higher mIPSC frequency, than MVN neurons that have both GABA_A and glycine receptors, but this has yet to be determined.

Our results did not show any changes in GABA_A receptor-mediated synaptic transmission after UL and is consistent with previous immunohistochemical and western blot studies of GABA_A receptor expression (Eleore et al. 2005; Eleore et al. 2004). These results however, are in contrast with those of Horii et al., (2003) which show an increase in GABA_A receptor

alpha-1 subunit after UL. Higher concentrations of GABA_A agonist muscimol are required to attenuate discharge rate of MVN neurons after UL (Vibert et al. 2000; Yamanaka et al. 2000) suggesting alterations in GABA_A receptors. Both these studies may reflect an upregulation of extrasynaptic receptors, not addressed in this study.

Given the lack of change in GABA_A-receptor mediated quantal transmission in mouse, how does this reconcile with previous findings regarding increases in GABA immunoreactivity and release after UL? Studies in cats have shown an increase in the number of GABA immunoreactive neurons in MVN after UL (Tighilet and Lacour 2001). Alterations in the number of inhibitory GABAergic neurons may be a means by which neuronal excitability is modulated. It is unknown however, whether these are inhibitory interneurons operating within the MVN or inhibitory projection neurons. If it were the former, we would have expected to see an increase in GABA_A receptor-mediated quantal events on MVN neurons, but this was not the case. We suggest, therefore, that these may be neurons projecting out of the nucleus. However, we cannot discount the possibility that increased GABAergic neurons may be acting on GABA_B receptors that are also known to modulate discharge rate of MVN neurons (Johnston et al. 2001; Yamanaka et al. 2000).

A possible reason we did not observe a change in GABA_A receptor-mediated synaptic transmission, post-UL, may be due to highly effective GABA uptake mechanisms. GABA transporters appear to minimize fluctuations in GABA concentrations from the MVN as suggested in a recent microdialysis study by Bergquist et al., (2008). Indeed, increased mRNA expression of GABA transporter, GAT, has been shown in MVN, 6-50 hours post-UL (Horii et al. 2003) and supports this notion. It should be noted that Bergquist et al., (2008) report a brief increase in GABA release at 24 hours post-UL that may be due to the transporters being transiently overwhelmed. While not significant, our results suggest at 4 hours, there is elevated GABA release as indicated by an increase in the frequency of GABA_A synaptic currents. However, since we did not record at a 24 hour post UL time point we cannot confirm the findings of Bergquist et al., (2008) .

Our study has shown that despite the predominance of GABA_Aergic synaptic transmission in the MVN, there is no change in postsynaptic GABA_A receptor function, subunit composition,

or presynaptic release probability after sensory ablation. Therefore, our results strongly suggest that vestibular compensation in the MVN does not involve alterations in GABA_Aergic synaptic input during the first week after UL.

Glycine-receptor mediated mIPSCs

In contrast to our GABA_A receptor results described above, we have shown that inhibitory glycinergic synaptic transmission is altered during the early phase of vestibular compensation. We found a significant increase in the amplitude of glycine receptor mediated mIPSCs in contralesional MVN neurons, 4 hours after UL. This indicates postsynaptic receptor changes, such as alterations in receptor cluster size or the up-regulation of receptor subunits. A previous report (Eleore et al. 2004) found no differences in the immunofluorescence of the inhibitory receptor anchoring protein gephyrin, or glycine receptor $\alpha 1$, $\alpha 2$, $\alpha 3$, β subunits between ipsi- and contralesional MVN. It should be noted however, that study represents immunofluorescence of a total population of receptors both synaptic and extrasynaptic, and did not quantify possible ultrastructural changes in receptor cluster area, or alteration of receptor number or somato-dendritic localisation. Given the rapid lateral diffusion of glycine receptors between extrasynaptic and synaptic domains (30 sec to < 2 mins), movement of glycine receptors to the synapse may be a means of modulating inhibition on a fast timescale (Dahan et al. 2003; Meier et al. 2000).

While the effects of UL were induced *in vivo*, at physiological temperature, the effects of sensory removal on inhibitory synaptic transmission were investigated *in vitro* in a brain slice recording. Recordings from brain slices were done at room temperature to allow direct comparisons of data between this, and other laboratories. The vast majority of papers that record inhibitory quantal synaptic currents have been done at room temperature (see Jonas et al. 1998; Lu et al. 2008; Nusser et al. 1998; Sebe et al. 2003). The advantage of recording quantal synaptic currents at room temperature allows stable physiological recordings, particularly important when investigating changes that occur following application of various antagonists, such as bicuculline and strychnine. Furthermore, voltage clamp of neurons with extensive dendritic trees is potentially problematic due to space clamp distortions, however recording at room temperature can reduce these errors (Williams and Mitchell 2008). As a

consequence of recording at room temperature, it is established that synaptic currents have slower kinetics, smaller magnitude, and lower frequencies than those recorded at physiological temperature (Awatramani et al. 2004; Fatt and Katz 1952; Zhang and Trussell 1994). However, it has been shown that the proportion of time that glycine receptors translocate between synaptic and extrasynaptic domains is not significantly different between room temperature and 37°C (Dahan et al. 2003). Therefore, our findings that glycinergic mIPSC amplitude and frequency increase during early vestibular compensation, reported here at room temperature, would presumably be even greater in brain slices at, or near, physiological temperatures and *in vivo* though this has yet to be established.

Previous studies have not addressed the possibility that alterations in *presynaptic* signalling at glycinergic synapses may also be involved in vestibular compensation. Our results showed a significant increase in the mean frequency of glycinergic mIPSCs in both, ipsi- and contralesional MVN neurons, 4 hours post-UL. This suggests an elevated rate of quantal glycine release, and is a presynaptic means of modulating synaptic strength (Lim et al. 2003). Increased presynaptic glycine release, together with larger amplitude glycinergic postsynaptic currents would provide elevated levels of inhibition to contralesional MVN during a period of intensive excitation immediately following UL, seen *in vivo* (Smith and Curthoys 1988a).

What is the potential role of enhanced glycinergic inhibition ipsilesionally early in vestibular compensation? Here we discuss two possibilities. First, inhibition may exert a modulatory affect on discharge rate by an unusual mechanism known as firing rate potentiation (FRP; (Nelson et al. 2003). Initially, neuronal discharge is prevented by stimulation of inhibitory fibers, or application of GABA or glycine. Later, release from this inhibition results in long lasting potentiation of discharge rate (Nelson et al. 2003). Increased glycinergic drive ipsilesionally therefore, may act to enhance and restore, low spontaneous discharge rates that are evident after UL.

Second, the involvement of excitatory NMDA receptors in synaptic plasticity (Bliss and Collingridge 1993), has led to the proposal that they also contribute to mechanisms underlying vestibular compensation (Flohr and Luneburg 1993; Kinney et al. 1994; Vidal et al. 1996). It should be noted that NMDA receptors require glycine as a co-agonist. Since we

have shown an increase in the frequency of quantal glycinergic currents, this raises the possibility that elevated levels of glycine might “spillover” to activate and/or potentiate NMDA receptors. Indeed, blockade of glycine uptake has been shown to potentiate NMDA receptors in the brainstem (Lim et al. 2004). Like GABA transporters (Bergquist et al. 2008), it is possible that glycine transporters are similarly overwhelmed during vestibular compensation resulting in activation of NMDA receptors. Further experiments are required to determine the effect of increased glycine release on NMDA receptors after UL.

An interplay between excitation and inhibition is necessary to maintain complex neuronal discharge patterns. Removing a significant sensory input induces profound changes in neuronal signaling and processing as seen in the visual system. This model in particular has been used to study homeostatic plasticity. Most studies however, have focused on synaptic scaling of excitatory inputs, that is, an alteration of mEPSC amplitude as a result of sensory deprivation (Desai et al. 2002; Goel and Lee 2007; Turrigiano et al. 1998). Fewer studies of homeostatic plasticity have addressed how deprivation of sensory inputs affects inhibitory synaptic connections. In layer IV of the visual cortex, two days of monocular deprivation significantly potentiates the inhibitory synaptic connection between fast spiking basket cells and star pyramidal cells and results in a significantly reduced discharge rate of star pyramidal cells (Maffei et al. 2006). Our data shows that permanent loss of sensory input can also enhance inhibitory synaptic transmission. This enhancement of glycine receptor-mediated synaptic transmission coincides with a reduction in discharge rates of MVN neurons. Modulation of inhibitory inputs appears to be an important mechanism of altering neuronal excitability. *In vivo* patch clamp methods will be necessary to unequivocally establish the influence of inhibitory GABA_A and glycine receptors on the discharge rate of MVN neurons after UL.

Acknowledgements

We are grateful to Drs Angela Kindig, Aaron Camp, and Elizabeth LaMont, Iain Stitt, and Belinda Harris for their assistance. This work was supported by the National Health and Medical Research Council of Australia Grant 401249, and the Garnett Passe and Rodney Williams Memorial Foundation.

Figure 1. GABA_A receptor-mediated mIPSCs after unilateral labyrinthectomy.

Panel A. Representative current trace (*upper*), amplitude distribution (*lower*) and averaged quantal events (*inset*) of control GABA_Aergic mIPSCs (see Methods). The layout of panel A is the same for the panels in *B* (1, 2, 3) and *C* (1, 2, 3).

Representative **ipsilesional** recordings of GABA_A mIPSCs at 4 hours (*B1*), 2 days (*B2*) and 7 days (*B3*). Representative **contralesional** recordings of GABA_A mIPSCs at 4 hours (*C1*), 2 days (*C2*) and 7 days (*C3*). *D.* Bar graph shows the average GABA_A receptor mediated mIPSC amplitude recorded from ipsi- and contralesional MVN neurons at 4 hours, 2 days, and 7 days post UL. There were no significant differences at any time point post UL. Data are expressed as mean $\pm$ SEM.

Figure 2. Glycine receptor-mediated mIPSCs after unilateral labyrinthectomy.

Panel A. Representative current trace (*upper*), amplitude distribution (*lower*) and averaged quantal events (*inset*) of control glycinergic mIPSCs (see Methods). The layout of panel A is the same for the panels in *B* (1, 2, 3) and *C* (1, 2, 3).

Representative **ipsilesional** recordings of glycinergic mIPSCs at 4 hours (*B1*), 2 days (*B2*) and 7 days (*B3*). Representative **contralesional** recordings of glycinergic mIPSCs at 4 hours (*C1*), 2 days (*C2*) and 7 days (*C3*). *D.* Bar graph shows the average glycinergic receptor mediated mIPSC amplitude recorded from ipsi- and contralesional MVN neurons at 4 hours, 2 days, and 7 days post UL. There was a significant increase in amplitude of glycinergic mIPSCs contralesionally, 4 hours post UL. * $p < 0.05$. Data are expressed as mean $\pm$ SEM.

Figure 3. Increase in glycine receptor-mediated mIPSC frequency during early vestibular compensation.

A. Bar graph shows the mean frequency of glycine mIPSCs from ipsi- and contralesional MVN neurons at 4 hours, 2 days, and 7 days post UL. At 4 hours post UL, both ipsi- and contralesional neurons had significantly elevated mean frequency compared to control. There was also a significant decrease in glycinergic mIPSC mean frequency of ipsilesional MVN neuron at 2 days post UL compared to the 4 hour time point. By 7 days post UL, there were no significant differences in frequency compared to control. Data are expressed as mean $\pm$ SEM.

B. Cumulative inter-event interval distribution of glycinergic quantal events from ipsi- and contralesional MVN neurons that underwent labyrinthectomy 4 hours earlier compared to control. Vertical line at 2.5 s indicates that 75 to 85% of inter-event intervals are less than 2.5 s in both ipsi- and contralesional MVN neurons, while less than 60% of inter-event intervals are less than 2.5 s in control MVN neurons.

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B₁

C₁

B₂

C₂

B₃

C₃

D

A

B

Table 1. *Quantal parameters of GABA_A ergic mIPSCs.*

	Control	4 hours post UL		2 days post UL		7 days post UL	
		Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Amplitude (pA)	31.9± 4.0	44.0± 6.9	34.9± 3.2	28.2± 2.6	30.8± 2.5	26.9± 2.9	29.2± 3.5
Rise Time (ms)	0.9± 0.05	0.8± 0.03	0.9± 0.08	0.9± 0.05	0.9± 0.06	1.0± 0.1	1.0± 0.05
Decay time (ms)	8.3± 0.7	7.6± 0.6	7.6± 0.6	7.5± 0.3	7.9± 0.5	8.2± 0.7	8.0± 0.4
Frequency (Hz)	0.5± 0.2	1.0± 0.3	1.4± 0.5	0.8± 0.2	1.0± 0.5	0.8± 0.3	0.7± 0.2
CV (Amplitude)	0.5± 0.04	0.5± 0.03	0.6± 0.07	0.5± 0.02	0.5 ±0.04	0.5± 0.03	0.5± 0.03
Number of neurons	11	13	12	16	13	11	13

Values are means ± SEM, Ipsi; ipsilesional, Contra; contralesional.

Table 2. *Quantal parameters of glycinergic mIPSCs.*

	Control	4 hours post UL		2 days post UL		7 days post UL	
		Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Amplitude (pA)	37.3± 6.6	45.1± 6.0	70.2± 16.1*	57.5± 11.2	53.5± 7.8	42.9± 7.1	42.1± 7.3
Rise Time (ms)	1.0± 0.1	0.9± 0.07	0.8± 0.05	0.7± 0.05	0.8± 0.04	0.9± 0.07	0.9± 0.05
Decay time (ms)	5.9± 0.5	6.5± 0.9	6.0± 0.7	5.7± 0.6	5.2± 0.4	6.5± 0.5	7.1± 1.0
Frequency (Hz)	0.2± 0.03	0.6± 0.2*	0.7± 0.2*	0.1± 0.03	0.5± 0.2	0.3± 0.1	0.4± 0.2
CV (Amplitude)	0.6± 0.05	0.7± 0.04	0.6± 0.06	0.7± 0.05	0.7± 0.06	0.8± 0.1	0.7± 0.07
Number of Neurons	14	12	11	11	13	19	12

Values are means ± SEM, Ipsi; ipsilesional, Contra; contralesional.