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TYPE B GABA RECEPTORS CONTRIBUTE TO THE RESTORATION OF BALANCE DURING VESTIBULAR COMPENSATION IN MICE

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Abstract—Following unilateral vestibular damage (UVD), vestibular compensation restores both static and dynamic vestibular reflexes. The cerebellar cortex provides powerful GABAergic inhibitory input to the vestibular nuclei which is necessary for compensation. Metabotropic GABA type B ($GABA_B$) receptors in the vestibular nuclei are thought to be involved. However, the contribution of $GABA_B$ receptors may differ between static and dynamic compensation. We tested static and dynamic postural reflexes and gait in young mice, while they compensated for UVD caused by injection of air into the vestibular labyrinth. The effects of an agonist (baclofen), an antagonist (CGP56433A) and a positive allosteric modulator (CGP7930) of the $GABA_B$ receptor were evaluated during compensation. Static postural reflexes recovered very rapidly in our model, and baclofen slightly accelerated recovery. However, CGP56433A significantly impaired static compensation. Dynamic reflexes were evaluated by balance-beam performance and by gait; both showed significant decrements following UVD and performance improved over the next 2 days. Both CGP56433A and baclofen temporarily impaired the ability to walk on a balance beam after UVD. Two days later, there were no longer any significant effects of drug treatments on balance-beam performance. Baclofen slightly accelerated the recovery of stride length on a flat surface, but CGP7930 worsened the gait impairment following UVD. Using immunohistochemistry, we confirmed that $GABA_B$ receptors are abundantly expressed on the vestibulospinal neurons of Deiters in mice. Our results suggest that $GABA_B$ receptors contribute to the compensation of static vestibular reflexes following unilateral peripheral damage. We also conclude that impairment of the first stage of compensation, static recovery, does not necessarily result in an impairment of dynamic recovery in the long term.

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Abbreviations: ANOVA, analysis of variance; CN, cochlear nucleus; DAB, diaminobenzedine; $GABA_B$, metabotropic GABA type B; IgG, immunoglobulin G; In, nucleus interpositus; L, lateral cerebellar nucleus; LTP, long-term potentiation; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; N, nodulus; PBS, phosphate-buffered saline; PFL, paraflocculus; Ph, nucleus prepositus hypoglossi; UVD, unilateral vestibular damage; VOR, vestibulo-ocular reflex.

Key words: posture, vestibular reflexes, gait, Deiters' nucleus, cerebellum, locomotion.

After the vestibular labyrinth is damaged on one side, postural reflexes, gaze stability, and normal locomotion are largely restored by vestibular compensation (Schaefer and Meyer, 1974; Curthoys and Halmagyi, 1995). Despite a long history of investigation, compensation is poorly understood, particularly for movements of the limbs. It is useful to consider two main processes in compensation: First, tonic limb muscle tone and gaze stability (with the subject stationary) are restored ("static compensation"). Second, the dynamic reflexes that operate during locomotion and during head movements must be recalibrated ("dynamic compensation"). Although dramatic, vestibular compensation is not perfect. In fact, some 30% of patients with unilateral vestibular damage (UVD) fail to recover even approximately normal function (Curthoys and Halmagyi, 1999).

Compensation is thought to involve plasticity within the vestibular nuclei of the brainstem (Fetter and Zee, 1988; Darlington et al., 2001; Broussard and Hong, 2003; Bergquist et al., 2008). The vestibular nuclei are the targets of a powerful, tonic GABAergic input from Purkinje cells (Ito, 1984) as well as from the inhibitory vestibular commissure. Following UVD, GABA levels become asymmetric (Bergquist et al., 2008). Metabotropic GABA type B ($GABA_B$) receptors are present in all subdivisions of the vestibular nuclei (Holstein et al., 1992; Eleore et al., 2005) and have been proposed to contribute to compensation (Johnston et al., 2001). Presynaptic $GABA_B$ receptors can inhibit the release of both excitatory and inhibitory neurotransmitters (Mouginot and Gahwiler, 1996; Aroniadou-Anderjaska et al., 2000; Yamada et al., 2000; Wang et al., 2003). Postsynaptically, $GABA_B$-receptor activation has multiple effects; these include hyperpolarization (Seabrook et al., 1990; Luscher et al., 1997), which can trigger intrinsic plasticity (Nelson et al., 2003). The varied intracellular targets of $GABA_B$ receptors position them to participate in multiple plasticity mechanisms via second-messenger systems in both pre-and postsynaptic terminals.

In the vestibular nuclei and in the cerebellum, resting discharge rates are high, suggesting that pharmacological agents that depend on tonic activation of neurotransmitter receptors (such as modulators) may have more pronounced effects in cerebellar and vestibular networks than in other brain regions. If so, therapeutic intervention at $GABA_B$ receptors could improve the rate and/or the final outcome of compensation. The antagonist CGP56433A has been reported to exacerbate both static and dynamic vestibular symptoms during compensation for hemilabyrinthectomy in rats, while

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the agonist baclofen can cause transient improvement (Magnusson et al., 2002). In brain slices, suppression of firing due to $GABA_B$ activation is reduced on the damaged side, a few hours following hemilabyrinthectomy (Yamanaka et al., 2000), and this reduction is believed to contribute to static compensation. Other brainstem mechanisms involving $GABA_B$ receptors have also been proposed (Peterson et al., 1996). The cerebellar cortex, including the flocculus, contains a high density of $GABA_B$ receptors (Bowery et al., 1987). The flocculus is thought to contribute to compensation, possibly by modifying vestibular neuronal excitability (Johnston et al., 2002).

Static and dynamic compensation have different mechanistic requirements. Static compensation likely requires a tonic increase in the activity of secondary vestibular neurons on the damaged side (Smith and Curthoys, 1988). Dynamic compensation requires not only the restoration of tonic activity, but also recalibration of the transmission of vestibular sensory signals that change during movement, that is, dynamic signals. Signal transmission by the inhibitory commissural pathways is thought to be important in dynamic compensation (Precht et al., 1966; Galiana et al., 1984). Signal transmission by excitatory brainstem pathways is also modified during dynamic compensation (Broussard and Hong, 2003; Farrow and Broussard, 2003). Cerebellar motor learning may participate in the restoration of dynamic reflex function (Broussard et al., 1999; Murai et al., 2004; Faulstich et al., 2006; Beraneck et al., 2008). Finally, recent evidence suggests that $GABA_B$ receptors may contribute to cerebellar motor learning (Titley et al., 2009). Except for one study describing circling (Gacek and Khetarpal, 1998), there has been little investigation of static or dynamic compensation of postural reflexes or gait in the mouse model.

We tested both static and dynamic vestibular function in young mice that had mild unilateral damage caused by injection of air into the vestibular labyrinth. We measured the effects of an agonist (R-baclofen) and an antagonist (CGP56433A) on compensation. We also tested a positive allosteric modulator (CGP7930) which in the presence of GABA, enhances the activity of the $GABA_B$ receptor (Urwyler et al., 2001).

No evidence has yet been obtained for any long-term effects of $GABA_B$ drugs on vestibular compensation. One hypothesis suggests that if compensation fails to occur during an early, critical period it will fail to occur in the long term (Newlands et al., 2005; Lacour, 2006). If this is the case, then interference with compensation at an early stage should prevent complete recovery at later stages. We have made an attempt to test this prediction.

EXPERIMENTAL PROCEDURES

Animals

A total of 68 male C57/Bl6 mice, either bred in our facility or purchased from Taconic Farms (New York), were used in this study (Table 1). Mice were on a normal light cycle with continuous access to food and water. All experiments were initiated between 7:30 and 10:30 AM. All procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee at the University Health Network.

Immunohistochemistry

$GABA_B$ receptors were labeled using immunohistochemistry in four mice, 42–48 days old. Mice were anesthetized with 0.1 ml of sodium pentobarbital (24 mg/mL ip; CDMV, St. Hyacinthe, Canada) and perfused through the heart, at a flow rate of 6 ml/min, with 30 ml of 0.1 M phosphate-buffered saline (PBS) followed by 30 ml of 4% paraformaldehyde in PBS. Both solutions were at room temperature. The brain was removed and stored at 4 °C in 4% paraformaldehyde/PBS for 24 h, then cryoprotected in 15% sucrose/PBS followed by 30% sucrose/PBS. Coronal sections through the brainstem, 40 μm thick, were cut on a sliding freezing microtome (American Optical, Buffalo, NY). For immunohistochemistry, sections were rinsed in PBS (all rinses were 3×10 min at room temperature with gentle agitation). Non-specific binding was blocked by incubating in a solution of 0.1 M PBS with 1% bovine serum albumin, 1.5% normal goat serum (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) and 0.5% Triton X-100 (Sigma Chemical Company, St. Louis, MO) for 30 min. Sections were incubated overnight at 4 °C in that same solution with the addition of a rabbit antiserum to the $GABA_B$ receptor (Santa Cruz Biotechnology, Santa Cruz, CA, $GABA_B$ R2 (H-300): s.c.-28792, 1:400), rinsed, and incubated with the Vector anti-rabbit IgG secondary antibody (0.5%, following manufacturer's instructions) in PBS with T-X and NGS, and then in the Vector ABC reagent according to manufacturer's instructions. Immunoreactivity was visualized with the DAB/glucose oxidase method (Shu et al., 1988). Sections were mounted on slides, air-dried, dehydrated in graded ethanol solutions, cleared with Histosol (National Diagnostics) and coverslipped. Digital images of selected sections were captured with a SPOT camera mounted on a Leitz Dialux 20 microscope. Control sections were processed identically, except that the primary antibody was omitted from the first incubation solution. No immunostaining was seen on the control sections.

UVD test groups and protocols

For the UVD study, mice were 1–3 months old and weighed 15–23 g; this size range was chosen because small individuals recovered rapidly from anesthesia. Thirty-six mice were included in the experimental groups, and 14 additional mice died or were excluded following surgery (see Table 1). The remaining mice were used for preliminary tests, as described below.

Drug dosages and activity levels

Recovery from vestibular damage is thought to be affected by the amount of locomotor activity experienced post-lesion (Mathog and Peppard, 1982). In addition, baclofen has been shown to interact with isoflurane (Sugimura et al., 2002) and CGP56433A can increase locomotor activity in the rat (Slattery et al., 2005). Appropriate drug doses for this study were chosen based on previously published results and on preliminary testing. Our goal was to select the maximum dose that would not alter activity levels following isoflurane anesthesia. We anesthetized a separate group of five mice for 1.75 h and then immediately injected one dose of the drug to be tested. The mouse was then observed for the next 3.5 hours, noting any obvious differences in activity levels. These mice are included in Table 1, along with the range that was tested for each drug. For CGP56433A, we tested only 5 mg/kg, because that dose was higher than the dose that is known to exacerbate vestibular signs following compensation (Magnusson et al., 2002).

After a dose was chosen for each drug, we verified that activity was not affected by the chosen dose, in another group of eight mice (see Table 1). In this group, mice were given repeated