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Comparison of γ -aminobutyrate receptors in the medial vestibular nucleus of control and *Scn8a* mutant mice

Yizhe Sun^a, Donald A. Godfrey^a, Kejian Chen^{a,b}, Leslie K. Sprunger^{c,d}, and Allan M. Rubin^a

^aDivision of Otolaryngology and Dentistry, Department of Surgery, University of Toledo College of Medicine, Toledo, OH 43614

^cDepartment of Human Genetics, University of Michigan, Ann Arbor, MI 48109

Abstract

The Purkinje cells of the cerebellum provide inhibitory input to vestibular nucleus neurons, with γ -aminobutyrate (GABA) as neurotransmitter. Using extracellular recordings and bath application of agonists and antagonists, we compared GABA receptors in the medial vestibular nucleus of brain slices from *Scn8a* mutant mice of med^J type, in which there is greatly reduced spontaneous and evoked activity of Purkinje cells, to those in slices from control mice. Muscimol, an agonist at GABA_A receptors, produced a larger reduction of firing rate in neurons of mutant mice than in neurons of control mice, whereas there was no difference for baclofen, an agonist at GABA_B receptors. In most cases tested, the effects of muscimol and baclofen remained similar when synaptic transmission was blocked, suggesting that the effects were predominantly directly upon GABA receptors of the neurons being recorded from. The upregulation of GABA_A receptors was similar in magnitude to that previously found for rats with bilateral transection of the inferior cerebellar peduncle. It may relate in both cases to reduced Purkinje cell input to medial vestibular nucleus neurons. The lack of effect on GABA_B receptors suggests that the changes found with peduncle transection may have resulted from something more than reduced Purkinje cell activity, such as reduced concentrations of GABA, or that reduction of Purkinje cell activity in *Scn8a* mutant mice was insufficient to affect GABA_B receptors. Other possible explanations of the results cannot be excluded since the *Scn8a* mutation affects other neuron types besides Purkinje cells.

Keywords

Muscimol; baclofen; bicuculline; saclofen; Purkinje cells; cerebello-vestibular

1. Introduction

Previous studies have found evidence for projections from the cerebellum to the vestibular nuclear complex (VNC) that use γ -aminobutyrate (GABA) as neurotransmitter (Obata et al.,

Address for correspondence: Donald A. Godfrey, Division of Otolaryngology and Dentistry, Department of Surgery, University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, Ohio 43614, Telephone: 419-383-3571, FAX: 419-383-3096, E-mail: donald.godfrey@utoledo.edu.

^bPresent address: Hough Ear Institute, Oklahoma City, OK 73112

^dPresent address: Department of Veterinary & Comparative Anatomy, Pharmacology, & Physiology, Program in Neuroscience, Center for Integrated Biotechnology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

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1967; Fonnum et al., 1970; Houser et al., 1984; Holstein, 2000; Gliddon et al., 2005). In our previous studies, transection of the inferior cerebellar peduncle in rats led to decreased GABA concentrations in all vestibular nuclei, including 32 % reduction in the dorsal division of the medial vestibular nucleus (MVN) 7 days after bilateral transection (Sun et al., 2007). In association with this, we also found that bilateral transection of the inferior cerebellar peduncle in rats led by 7 days to upregulation of GABA_A and, to a lesser extent, GABA_B receptors in the MVN (Sun et al., 2002a). The major cerebellar GABAergic projections to the vestibular nuclei derive from Purkinje cells in parts of the cerebellar cortex (Obata et al., 1967; De Zeeuw and Berrebi, 1995; Holstein, 2000; Gliddon et al., 2005), although there may also be inputs from some GABAergic deep nuclear neurons (Diagne et al., 2001).

It has been previously reported that, in mice with a mutation in the *Scn8a* sodium channel gene resulting in greatly reduced expression of *Scn8a* transcript, there is reduction of both spontaneous and evoked activity in cerebellar Purkinje cells (Raman et al., 1997; Meisler et al., 2004). This should result in less functional GABAergic input to the VNC. We hypothesized that such mice may have changes of GABA receptor pharmacology in the MVN similar to those we found in rats with cerebellar peduncle transection, although the effect might be less because there is a reduction of Purkinje cell influence rather than its total removal. To test this hypothesis, we compared the effects of GABA_A and GABA_B receptor agonists in the MVN of *med^J* mice, in which there is 98% reduction in expression of the *Scn8a* transcript (Kohrman et al., 1996; Sprunger et al., 1999), to those in control mice. The effects of the GABA_A agonist muscimol and the GABA_B agonist baclofen on extracellularly-recorded spontaneous activity of MVN neurons were compared in brain slices of *med^J* and control mice.

2. Results

2.1. Spontaneous activity rates

The MVN neurons of mutant mice had a lower average rate of spontaneous activity than those of control mice. The mean $\pm$ SEM for 39 neurons in mutant mice was 11.9 ± 0.68 , as compared to 16.8 ± 1.46 for 41 neurons in control mice ($P < 0.002$). There was no significant correlation between the rate of spontaneous firing and body weight for either group.

2.2. GABA_A receptors

Muscimol produced a larger reduction of firing rate in the MVN neurons of mutant mice than in those of control mice (Fig. 1). For both concentrations used, the reduction of firing rate was significantly larger in mutant than in control mice (Fig. 2). At 3 μ M, the mean $\pm$ SEM firing rate during muscimol, as a percentage of the firing rate before muscimol, was 66.9 ± 4.2 % in slices from mutant mice, as compared to 92.9 ± 2.3 % in slices from control mice. At 10 μ M, the values for mutant and control were 30.5 ± 8.9 % and 71.9 ± 11.3 %. There was no significant correlation between the magnitude of the muscimol effect and body weight for either group.

The effect of 10 μ M muscimol was blocked by 20 μ M bicuculline in both cases where this was tested. When applied alone, 20 μ M bicuculline produced a slight increase in firing rate of the MVN neurons of mutant mice. The mean $\pm$ SEM firing rate during bicuculline, as a percentage of the firing rate before bicuculline, was 116 ± 6 % for 11 MVN neurons in slices from mutant mice, as compared to 80 ± 16 % for 10 MVN neurons in slices from control mice ($P < 0.05$).

2.3. GABA_B receptors

Baclofen produced a similar reduction of firing rate in the MVN neurons of mutant and control mice at both concentrations used (Fig. 2).

The effect of 3 μ M baclofen was blocked by 200 μ M 2-OH-saclofen in both cases where this was tested. When applied alone, 200 μ M 2-OH-saclofen had little effect on firing rate of the MVN neurons of mutant or control mice. The mean $\pm$ SEM firing rate during 2-OH-saclofen, as a percentage of the firing rate before 2-OH-saclofen, was 91 ± 11 % for 13 MVN neurons in slices from mutant mice and 92 ± 14 % for 13 MVN neurons in slices from control mice.

2.4. GABA agonist effects in low calcium/high magnesium medium

To test the extent to which the GABA agonist effects were directly upon the MVN neurons being recorded from, rather than being mediated through effects on other neurons, synaptic transmission was temporarily blocked by substituting low calcium/high magnesium ACSF for control ACSF in some experiments, for 2 neurons from control mice and 5 from mutant mice. When the low calcium/high magnesium medium replaced control medium, the spontaneous firing rate of the 2 control neurons decreased to zero, after which the firing gradually recovered in one of the neurons. For 2 mutant neurons, the firing decreased to zero and gradually recovered; for 2 others, there was little change; for one neuron, the firing rate gradually decreased. For 3 mutant neurons, the effect of muscimol in low calcium/high magnesium ACSF was similar to its effect in control ACSF. For one control and one mutant neuron, the effect of baclofen in low calcium/high magnesium ACSF was similar to its effect in control ACSF. For a second mutant neuron, however, the effect of baclofen was less in low calcium/high magnesium ACSF than in control ACSF.

3. Discussion

3.1. Comparison to results for rats with cerebellar peduncle transection

Our results suggest that GABA_A receptors in the MVN are up-regulated in mice with greatly reduced expression of *Scn8a* sodium channels, similarly to our findings in rats with bilateral transection of the inferior cerebellar peduncle (Sun et al., 2002a). However, there was no significant effect on GABA_B receptors in mutant mice, whereas an effect was found in lesioned rats, although it was smaller than the effect on GABA_A receptors. Since the mutant mice have reduced Purkinje cell activity (Raman et al., 1997), our results may suggest that this significant reduction of activity in the cerebellar neurons providing a major GABAergic input leads to a compensating increase in responsiveness of MVN neuron GABA_A receptors, although our methods cannot determine to what extent this represents an increase in number, affinity, and/or efficacy of the receptors. The similarity in the relative magnitudes of the muscimol effects on control and mutant mice as compared to those in control and lesioned rats (Sun et al., 2002a) may be coincidental, but it would be consistent with the possibilities that the effects of cerebellar peduncle transections on MVN GABA_A receptors are entirely accounted for by their removal of Purkinje cell input, and that the amount of reduction of Purkinje cell activity in the mutant mice is large enough to give an effect on GABA_A receptors comparable to that of total removal of their input to the MVN. The lack of effect on GABA_B receptors may represent a difference between the effects of reduced Purkinje cell activity and total removal of Purkinje cell input, including reduced GABA concentrations in the MVN in the latter case, as well as possible GABAergic input from deep cerebellar nuclei (Diagne et al., 2001). Further, greatly reduced expression of *Scn8a* sodium channels likely affects to some extent activity in other neurons as well (Meisler et al., 2004). These may include neurons in the VNC (Bäurle et al., 1997; Holstein, 2000; Gliddon et al., 2005), both ipsilateral and contralateral (Furuya and Koizumi, 1998; Holstein et al., 1999) to the recording site, and neurons outside the VNC, such as inferior olivary complex neurons (Balaban, 1988; De Zeeuw and Berrebi, 1995), that may provide GABAergic input. Changes of activity in some or all of such other GABAergic neurons in mutant mice could contribute to effects on GABA receptors in the MVN. Available evidence suggests that the reduced expression of *Scn8a* sodium channels in mutant mice has the most striking effects on neurons with bursting, or complex spiking, patterns of activity. These include

just a few types of neurons, such as cerebellar Purkinje cells (Raman et al., 1997) and dorsal cochlear nucleus cartwheel cells (Chen et al., 1999; see next section), and do not include neurons of the VNC (Sun et al., 2002b) or inferior olive (Llinás and Yarom, 1986). Nevertheless, if there are sizeable changes in activity of such other neurons in mutant mice, they would complicate the interpretation of our findings for agonist and antagonist effects. They also might contribute to our finding of generally decreased spontaneous firing rates in mutant mouse slices as compared to those from control mice, whereas a decrease in the inhibitory GABAergic cerebello-vestibular input might have been expected to result in increased rates of spontaneous activity. This last finding resembles that reported for mutant mice with degeneration of almost all Purkinje cells, wherein there was a decrease of spontaneous and stimulus-driven activity of VNC neurons in the mutant mice instead of the expected increase (Bährle et al., 1997).

Differences between effects in mutant mice and lesioned rats could also relate to differences between mice and rats. Although the responsiveness to muscimol was not very different between mice and rats, being slightly less in mouse MVN than in rat MVN, the responsiveness to baclofen in control mouse MVN was much greater than that in rat MVN. The spontaneous firing rates of MVN neurons were generally similar in slices from control mice and rats, but the level of ongoing GABAergic activity may differ since both the GABA_A and GABA_B antagonists led to increased neuronal firing rates in control rat MVN but not in control mouse MVN.

As in our study on lesioned rats (Sun et al., 2002a), heterogeneity of effects on different mouse MVN neurons could relate to recordings being from a variety of neuron types with different relative innervations from Purkinje cells, or other neurons affected by the mutation, as opposed to unaffected sources of innervation. Based on the experiments with synaptic blockade by low calcium with high magnesium, the effects found here for mouse MVN, like those for rat MVN (Sun et al., 2002a), appear to be predominantly related to GABA receptors on the neurons being recorded from rather than receptors on other neurons that influence the recorded neurons.

3.2. Comparison to results for mouse dorsal cochlear nucleus

For the auditory division of the eighth nerve sensory systems, the primary brain nucleus, and thus the counterpart of the VNC, is the cochlear nucleus. We have previously examined neural activity in the dorsal subdivision of the cochlear nucleus of *med^J* mutant and control mice (Chen et al., 1999). In the dorsal cochlear nucleus, three patterns of spontaneous activity were found – regular, irregular, and bursting. Although the bursting patterns, which are associated with cartwheel cells, were virtually absent from the dorsal cochlear nucleus in slices of *med^J* mutant mice, regular and irregular patterns remained. This is consistent with a concept that the *Scn8a* mutation particularly affects the activity of neurons with bursting patterns of activity, as with the cerebellar Purkinje cells. The mean firing rates of the dorsal cochlear nucleus neurons with regular patterns were possibly slightly (15%) lower in slices from mutant than from control mice, but the difference was not statistically significant. For MVN neurons, which all displayed regular patterns, the mean firing rates were about 30% lower in mutant than in control mice, and this difference was statistically significant. This difference between the mutation effects on MVN and dorsal cochlear nucleus might relate to the much greater cerebellar innervation of the MVN than of the dorsal cochlear nucleus. Although the change in neural activity is opposite to what might have been expected from reduction of a chronic inhibitory Purkinje cell influence, it might result from compensatory changes in other neurons when the Purkinje cell influence is reduced (Bährle et al., 1997) or, based on a previous report (Nelson et al., 2003), from decreased intrinsic excitability of neurons when their inhibitory input is decreased.

3.3. GABA receptors in vestibular compensation

Previous studies have suggested an important role for the cerebellum in vestibular compensation (McCabe and Ryu, 1969; Kitahara et al., 1997), and changes have been found in both GABA_A and GABA_B receptor responsiveness of MVN neurons after unilateral labyrinthectomy (Yamanaka et al., 2000; Johnston et al., 2001). Our results for mutant mice and for rats with inferior cerebellar peduncle transection (Sun et al., 2002a) are consistent with an important role of cerebellar Purkinje cells in plasticity of GABA receptors in the VNC. Increased responsiveness of GABA_A receptors on MVN neurons has also been reported in aged rats, in which there is loss of cerebellar Purkinje cells and MVN neurons (Him et al., 2001). However, recent evidence does not strongly support an important role of GABA receptor changes in the development of vestibular compensation (Eleore et al., 2005; Gliddon et al., 2005a,b,c). Previous studies with mutant mice suggest that changes in other neurons besides cerebellar Purkinje cells greatly complicate the interpretation of cerebellar effects on the VNC (Grüsser-Cornehls et al., 1995; Bärle et al., 1997).

4. Experimental procedure

All experimental procedures were in accordance with the NIH guide for the care and use of laboratory animals and approved by the University of Toledo College of Medicine Institutional Animal Care and Use Committee.

4.1. Animal subjects

Results are based on experiments using adult mice. Ages of individual mice were not recorded, but they ranged between 4 and 10 months: 17 wild type control mice, weighing 17-41 (average 29.2) grams, and 20 *med^J* mutant mice, weighing 13-22 (average 17.4) grams. Experiments using mutant mice were interspersed with those using control mice to guard against any time-dependent effects on experimental results.

Scn8a^{med-J} (*med^J*) mice carry a splice site mutation in the *Scn8a* gene which reduces Na_v1.6 mRNA and protein to between 5% of the normal level on a C57BL/6J strain background and 10% of normal on a C3H strain background (Kearney et al., 2002). Congenic lines of *med^J* mice on the C57BL/6J background and the C3H background were obtained from the laboratory of Dr. M.H. Meisler at the University of Michigan. Both colonies carry the closely linked, dominant Caracul mutation in cis with the *med^J* mutation, permitting the identification of heterozygous *med^J/+* mice by their wavy hair. Breeding colonies of both lines were maintained at the University of Toledo by backcrossing heterozygous *med^J/+* mice to the appropriate inbred strain. Experiments were carried out on F1 homozygotes obtained from crosses between the two strains, as these mice survive to adulthood with a reproducible phenotype (Sprunger et al., 1999). Homozygotes (approximately 25% of the offspring) were identified by their phenotypic abnormalities (ataxia, tremor, dystonia, muscle atrophy) and wavy coats. Wild type (+/+) controls were recognized by the lack of neurological abnormalities and absence of wavy hair. The neurogenic muscle atrophy in the *med^J* homozygotes is the major contributor to their lower average body weight, which begins to diverge from that of their unaffected littermates beginning in the third postnatal week, coincident with the appearance of the movement disorder. Despite their deficits, however, mutant mice are able to eat and drink unassisted, groom themselves normally, and the females can reproduce and care for their pups. Although a formal long-term survival analysis has not been done for this mutant line, they typically live to at least one year of age, with many surviving 15-18 months.

4.2. Electrophysiology and pharmacology

Procedures for brain slice electrophysiology and pharmacology were generally similar to those used in our previous studies (Chen et al., 1999; Sun et al., 2002a,b). For preparation of brain

slices, each mouse was anesthetized with sodium pentobarbital (52 mg/kg, i.p.) and decapitated. The brain stem was exposed while rinsing the head in chilled oxygenated artificial cerebrospinal fluid (ACSF), then rapidly removed and immersed in chilled ACSF prebubbled with 95% O₂ and 5% CO₂. After removing the cerebellum, a block of tissue containing the VNC was glued to an agar support, which had been glued to the cutting stage of a vibratome, and serially sectioned into 350-500 µm-thick transverse slices. Slices containing the MVN were immediately transferred onto a nylon mesh in an interface chamber that was perfused at about 1.2 ml/min with ACSF consisting of (mM): KH₂PO₄ 1.25, KCl 5.0, MgSO₄ 2.0, CaCl₂ 2.0, NaCl 124, glucose 10 and NaHCO₃ 26. The interval of time between decapitation and placement of slices into the chamber was 13 min or less. Humidified 95% O₂, 5% CO₂ gas flowed continuously over the slices. Temperatures of ACSF and gas were maintained at 32-34°C by a temperature-controlled water bath.

After a recovery period of usually 2 h, slices were explored with 2-3 µm tip diameter glass micropipettes filled with 1 M NaCl (resistance 5-10 megohms). Boundaries of the MVN were determined visually with a dissecting microscope using both transmitted and reflected light, similarly to our previous study on rats (Sun et al., 2002a,b). Recordings were from the mid portion of the rostrocaudal extent of the MVN, and mostly from its dorsal part since there was more certainty of the dorsal limit of the nucleus, next to the fourth ventricle, than the ventral limit. Extracellular discharges of spontaneously active neurons in the MVN were displayed, recorded on magnetic tape, and also led into a digital computer interface for storage of continuous counts of discharges in consecutive 1 sec periods. Spike counts and measurements of intervals were done before, during, and after bath application of drugs, with a time resolution of 0.1 msec, and saved as binary files for further calculations. Firing rates were plotted as the average for each 30 sec period vs. time.

One to 3 different concentrations of drugs were applied into the ACSF and tested on each recorded neuron. The concentrations used were based on our previous study in rats (Sun et al., 2002a) and preliminary experiments on control mice. Drugs were selected through a series of stopcocks, with an approximately one-minute time delay between the change of a solution and 50% rise of concentration in the chamber (Godfrey and Waller, 1992). Agonists and antagonists used (all from RBI) were the GABA_A agonist muscimol hydrobromide (3-10 µM), the GABA_A antagonist (-)-bicuculline methochloride (20 µM), the GABA_B agonist (±)-baclofen (0.03-3 µM), and the GABA_B antagonist 2-OH-saclofen (200 µM). When the blocking of agonist effect by an antagonist was tested, the antagonist was applied for 3-6 min before and throughout the time of application of the agonist. In some experiments, low calcium/high magnesium ACSF, containing 0.2 mM calcium and 7.8 mM magnesium, was temporarily substituted for control ACSF to block synaptic transmission in order to test the extent to which effects were directly upon the MVN neurons being recorded from.

4.3. Data analysis

All data are from recordings of regularly firing neurons in the MVN, which comprise the vast majority of MVN neurons present in slices (Sun et al., 2002b). Mean firing rates of neurons were compared before and after application of GABA-related chemicals. The magnitude of each drug effect was calculated as the firing rate during the 30 sec period showing maximal change from control within 6 min of the start of drug application, divided by the average firing rate for the 3 min period immediately preceding drug application. Statistical comparisons between groups were made by t-tests, with significance taken as $p < 0.05$.

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Abbreviations

ACSF	artificial cerebrospinal fluid
GABA	γ -aminobutyrate or γ -aminobutyric acid
MVN	medial vestibular nucleus
VNC	vestibular nuclear complex

Figure 1.

Firing rates of an MVN neuron from a control mouse and one from a med^J mutant mouse during presentation of two concentrations of muscimol. Bar width of each histogram plot is 30 sec. Times of application of muscimol in the perfusion medium are marked by horizontal and vertical lines.

Figure 2.

Responses to muscimol and baclofen in MVN neurons of control and *med^J* mutant mice. Data, as mean $\pm$ SEM, were pooled from all experiments for control and mutant mice. For muscimol, mean values for control and mutant mice, respectively, at each concentration, based on 9 measurements in each case, were 93% and 67% at 3 μ M and 72% and 30% at 10 μ M. For baclofen, mean values for control and mutant mice, respectively, at each concentration were 58% and 57% for 7 and 5 measurements at 0.1 μ M and 48% and 44% for 8 and 5 measurements at 0.3 μ M. Statistically significant differences between control and mutant responses are indicated by ** $P < 0.001$ and * $P = 0.01$.