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Roles of Nitric Oxide in Vestibular Compensation

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The effects of nitric oxide on the vestibular function recovery following unilateral labyrinthectomy (UL) were studied. Sprague-Dawley male rats, treated with nitric oxide liberating agent sodium nitroprusside (SNP) and NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), were subjected to destruction of the unilateral vestibular apparatus, and then spontaneous nystagmus was observed in the rat. To explore the effects of nitric oxide on the neuronal excitability, whole cell patch clamp technique was applied on isolated medial vestibular nuclear neurons. The frequency of spontaneous nystagmus in SNP treated rats was lesser than that of spontaneous nystagmus in control animals. In contrast, pre-UL treatment with L-NAME resulted in a significant increase in spontaneous nystagmus frequency. In addition, SNP increased the frequency of spontaneous action potential in isolated medial vestibular nuclear neurons. Potassium currents of the vestibular nuclear neurons were inhibited by SNP. After blockade of calcium dependent potassium currents by high EGTA (11 mM) in a pipette solution, SNP did not inhibit outward potassium currents. 1H-[1,2,4] oxadiazolo [4,3-a] quinozalin-1-one (ODQ), a specific inhibitor of soluble guanylyl cyclase, inhibited the effects of SNP on the spontaneous firing and the potassium current. These results suggest that nitric oxide after unilateral labyrinthectomy would help to facilitate vestibular compensation by inhibiting calcium-dependent potassium currents through increasing intracellular cGMP, and consequently would increase excitability in ipsilateral vestibular nuclear neurons.

Key Words: Vestibular compensation, Nitric oxide, Spontaneous action potential, Potassium current

INTRODUCTION

Vestibular compensation is a process of partial behavioral recovery that occurs following lesions to the vestibular inner ear. After unilateral labyrinthectomy (UL), the resting activity in the ipsilateral vestibular nucleus complex is markedly decreased, resulting in an imbalance in neuronal activity between the ipsilateral and contralateral vestibular nucleus complexes. This imbalance causes severe postural and oculomotor disturbances. Despite of permanent loss of the ipsilateral vestibular peripheral inputs, many but not all of these symptoms over time gradually disappear along with the recovery of resting activity in the ipsilateral medial vestibular nuclear neurons. This process of progressive rebalancing is due to the functional reorganization of the central vestibular system and is one of the main features of vestibular compensation. Vestibular compensation appears to be relatively independent of any recovery in the peripheral vestibular system, and therefore is attributed to plasticity in the central nervous system. However, the biochemical basis of the compensation is still poorly understood (Smith & Darlington, 1988; De waele et al, 1990; Smith & Darlington, 1991; Dieringer, 1995; Ris & Godaux, 1998).

Glutamate is an excitatory neurotransmitter in the nervous system and plays an important role not only in the synapses between primary vestibular afferent neurons and vestibular nuclear neurons, but also in those between vestibular nuclei and the cerebellum. The fact that excitatory amino acid receptors such as the *N*-methyl-D-aspartate (NMDA) receptor, a specific type of glutamate receptor, and associated second messengers have been implicated in vestibular compensation suggests that other NMDA receptor-related neuromodulators, such as nitric oxide (NO), are also likely to be involved (Sanson et al, 1992; Darlington et al, 1996). Nitric oxide is a diffusible gas synthesized from L-arginine by the enzyme, nitric oxide synthase (NOS), and released from neurons by simple diffusion and acts on intracellular targets in other neurons via the activation of guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP) (Dawson et al, 1992).

Flugel et al. (1994) were the first to investigate NO in the context of vestibular compensation and reported that the selective NOS inhibitor, L-NAME, delivered post-UL into the dorsal lymph sac of the frog, delayed vestibular compensation. Kitahara et al. (1999), using rats, showed that changes in NOS expression occur in the cerebellar flocculus following UL and that post-UL injection of

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ABBREVIATIONS: NO, nitric oxide; SNP, sodium nitroprusside; UL, unilateral labyrinthectomy; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a] quinozalin-1-one; 4-AP, 4-aminopyridine; NMDA, *N*-methyl-D-aspartate; L-NAME, N omega-nitro-L-arginine methyl ester.

L-NAME into the flocculus also inhibited the compensation process. However, Paterson et al. (2000), in guinea pigs, reported that only pre-UL system administration, not post-UL administration, of L-NAME increase the rate of spontaneous nystagmus compensation.

Although many authors have reported that NMDA receptors play an important role in vestibular recovery following UL, only a few have concentrated on the role of nitric oxide which acts in conjunction with NMDA receptors in vestibular compensation. Thus, the present study was to determine the action of nitric oxide in vestibular compensation process by examining the effects of SNP and L-NAME on the frequency of spontaneous nystagmus fo llowing UL. In addition, we examined the effects of nitric oxide on spontaneous action potential firing and potassium currents using the whole-cell patch clamp technique under current and voltage clamp recording in medial vestibular nuclear neurons.

METHODS

Unilateral labyrinthectomy and behavioral study

Adult Sprague-Dawley male rats, weighing about 200 g, were used. Animals were anesthetized with pentobarbital sodium. After a longitudinal skin incision was made along the medial side of the angle of the mandible, the submandibular gland was reflected caudally and laterally, and the ventral surface of bulla was exposed and opened widely with a fine dental burr. The basal cochlea was opened and picked to expose the vestibule. Neuroepithelium and membranous labyrinth were ablated by curettage and aspiration. A small Gelfoam pledget was placed in the bulla and the skin was closed with suture. In the present study, we chose the frequency of horizontal spontaneous nystagmus as a marker. The frequency of spontaneous nystagmus was measured as the number of quick phase beats occurring over the period of 30 seconds. The eye movements were counted three times for each animal and means were obtained.

Drug treatment

Animals were given with drinking water containing L-NAME (5 mg/100 ml) for 4 weeks before UL and this treatment was continued until the seventh day after the operation. Daily ingested amounts of L-NAME were cal culated as milligrams per 100 g body weight of the animal. Systolic blood pressure was indirectly measured by means of a tail cuff method in a conscious state to assess the effects of L-NAME. On day 28, animals with systolic blood pressure higher than 180 mmHg were selected and UL was performed. Control animals were given with normal tap water. Single dose (2 mg/kg) of SNP was intraperitoneally injected 30 minutes prior to the UL.

Preparation of cells

Horizontal slices of the brain stem of Sprague-Dawley rats, aged 14 to 17 days, were prepared, as described previously (Kay & Wong, 1986). Briefly, the animals were anesthetized with ether and decapitated. The brain stem was rapidly removed and placed in cold artificial cerebrospinal fluid. The coronal slices (400 μm thick) of the brain stem were made with a sliding microtome (Vibroslice, WPI, USA). These slices were incubated in artificial cerebrospinal fluid saturated fully with 95% O_2/5% CO_2 at room temperature for 1 hour. The slices were treated with pronase (0.2 mg/ml) for 40~60 min and subsequently exposed to thermolysin (0.2 mg/ml) at 32°C. After this treatment, the portion of medial vestibular nuclear neuron was removed by micropunching and the digested slice was then gently agitated. Then, the dissociated neurons were transferred into a recording chamber mounted on an inverted microscope (IX 70, Olympus, Japan).

Electrophysiological recordings

Whole-cell membrane current and potential were recorded at room temperature by using standard patch-clamp techniques. Patch pipette had a resistance of 3~6 MΩ when filled with a pipette solution. Membrane currents were measured with an Axopatch 200B patch-clamp amplifier (Axon instruments, USA). Command pulses were applied using IBM-compatible computer and pCLAMP 7 software (Axon instruments, USA). The data were filtered at 5 kHz and displayed on an oscilloscope (Tektronix, USA) with a computer monitor, and a pen recorder (Polygraph, Grass, USA).

Solutions

The experimental bath with a volume of 0.5 ml was continuously perfused at 0.6 ml/min with artificial cere brospinal fluid, and the drugs were added to the perfusate. Artificial cerebrospinal fluid had following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$, 1.3, $CaCl_2$ 2.4, D-Glucose 10, $NaHCO_3$ 24. Whole cell cur rent-clamp re cording were made using borosilicate patch pipettes with the solution containing following composition (mM): K-glu conate 122.5, KCl 17.5, NaCl 8, HEPES 10, EGTA 0.2, Mg-ATP 4; pH 7.3 with Tris. Voltage-dependent potassium currents were obtained in bath solution that had the following composition (mM): KCl 3, Choline-Cl 140, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, Glucose 30; pH 7.4 with KOH. The patch pipette solution had the following composition (mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 0.1, HEPES 10, EGTA 0.5, Mg-ATP 2; pH 7.3 with KOH. In order to decrease the intracellular free Ca^{2+} concentration less than 10 nM in some experiments, EGTA in the pipette solution was increased from 0.5 to 11 mM. Sodium nitroprusside (SNP), 4-aminopyridine (4-AP), and 1H-[1,2,4] oxadiazolo [4,3-a] quinozalin-1-one (ODQ) were products of Sigma Chemical Co (St Louis, MO, USA).

Statistics

All values are expressed as mean±stand errors (SE) of means. Differences between two groups were determined by Student's t test and were considered to be significant when P values are less than 0.05.

RESULTS

Effect of SNP and L-NAME on the frequency of spontaneous nystagmus following UL

After UL, spontaneous nystagmus, whose quick phase

was toward the intact side, appeared. The frequency of spontaneous nystagmus was decreased gradually and disappeared by 72 h post-operation in control animals. In L-NAME treated animals, the average amount of L-NAME ingested during 4 weeks was 0.72±0.02 mg/100 g of body weight/day. The frequency of spontaneous nystagmus was maintained higher than that in control animals, and spontaneous nystagmus did not disappear by 7 day after UL. In contrast, pre-UL treatment with SNP resulted in a significant decrease in spontaneous nystagmus frequency, especially during the early phase of rehabilitation ($P<0.05$, n=6) (Fig. 1).

Effects of SNP on the spontaneous action potential firing of the medial vestibular nuclear neuron

To study the effect of nitric oxide on a medial vestibular nuclear neuron, we used current-clamp technique and recorded the spontaneous action potential firing in the neuron. Medial vestibular nuclear cells quickly isolated from rat brain stem appeared to have round or pyramidal shaped cell bodies. In I=O nA mode, action potential firing appeared spontaneously, and 40 μM SNP were then applied. The most obvious effect of SNP on neuronal excitability was an increase in the firing frequency from 9.8±1.51 to 18.5±3.27 spikes/sec ($P<0.05$, n=6) (Fig. 2). At the same time, SNP reduced the after-hyperpolarization of the action potential from 13.2±1.2 mV to 6.7±0.8 mV ($P<0.05$) and decreased the action potential amplitude by about 25%. Exogenous application of ODQ (4 μM) abolished these effects of SNP on the action potential firing (n=6).

Effects of SNP on the voltage dependent potassium currents

It is well known that nitric oxide affects blood vessel smooth muscle by opening potassium channels. But, Zsombok et al (2000) reported that nitric oxide increases excitability by depressing a calcium activated potassium currents in snail neurons, and Behrend et al (1997) observed that cGMP inhibits potassium currents in vestibular hair cells. The increase in excitability could in principle arise by decreasing the activity of potassium channels. To investigate these possibilities, we used voltage clamp technique to analyze potassium currents. Voltage-dependent potassium currents of the medial vestibular nuclear neuron were activated by a 400 ms test pulses from −60 to +40 mV in 10 mV increments from a holding potential of −70 mV. The threshold for activation of the whole potassium current was about −30 mV. Larger depolarizing voltage steps elicited faster and larger outward currents that were sustained during depolarizations. SNP reduced the amplitude of the currents from 4498±87.5 m to 2638±67.8 mV (n=6). In order to determine whether SNP directly inhibited outward potassium currents or not, we explored the effects of 1H-[1,2,4] oxadiazolo [4,3-a] quinozalin-1-one (ODQ), a specific inhibitor of soluble guanylyl cyclase, on the SNP-inhibited currents. One μM ODQ itself did not affect the basal outward currents, however, as shown in Fig. 3 (n=6), application of 1 μM ODQ blocked the inhibitory effects of 10 μM SNP on the voltage-dependent potassium currents, indicating that SNP acts through activation of soluble guanylyl cyclase in medial vestibular nuclear neurons.

Effect of SNP on the 4-aminopyridine-depressed potassium currents

It has been reported that the medial vestibular nuclear neuron possesses three types of potassium channel; delayed rectifier, A-type and calcium-dependent. To investigate which potassium current is affected by nitric oxide, we recorded SNP-induced potassium currents after application of 4-AP, delayed rectifier and A-type potassium channel blokcer. Application of 5 mM 4-AP reduced the amplitude of the currents from 4938±47.1 m to 3112±152.10 mV (n=6). After blockade of the currents with 4-AP, the currents were further decreased by 10 μM SNP (2251±38.8 mV) (Fig. 4).

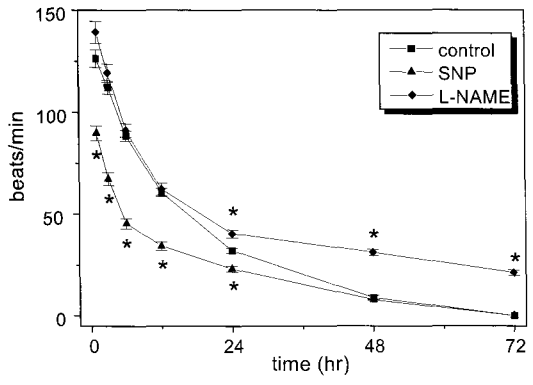

Fig. 1. Effects of sodium nitroprusside and L-NAME, a NOS inhibitor, on the frequency of spontaneous nystagmus after unilateral labyrinthectomy (means±SE, n=6, *Significantly different at $P<0.05$). ■: Control, ▲: SNP treated group, ◆: L-NAME treated group.

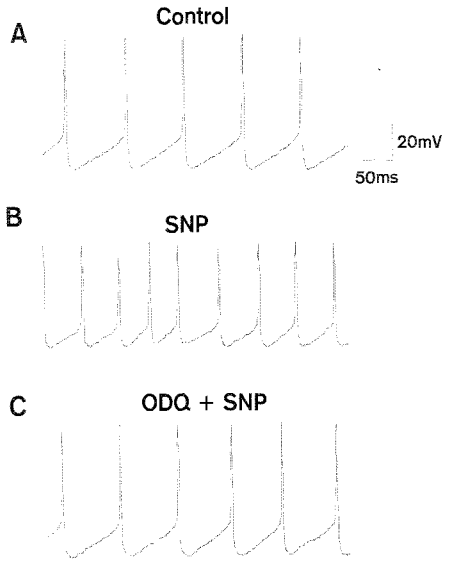

Fig. 2. Effects of SNP on spontaneous action potential firing in medial vestibular nuclear neurons. (A) for normal firing, (B) for treatment of 40 μM SNP, and (C) for cotreatment of 40 μM SNP and 4 μM ODQ, a soluble guanylyl cyclase inhibitor.

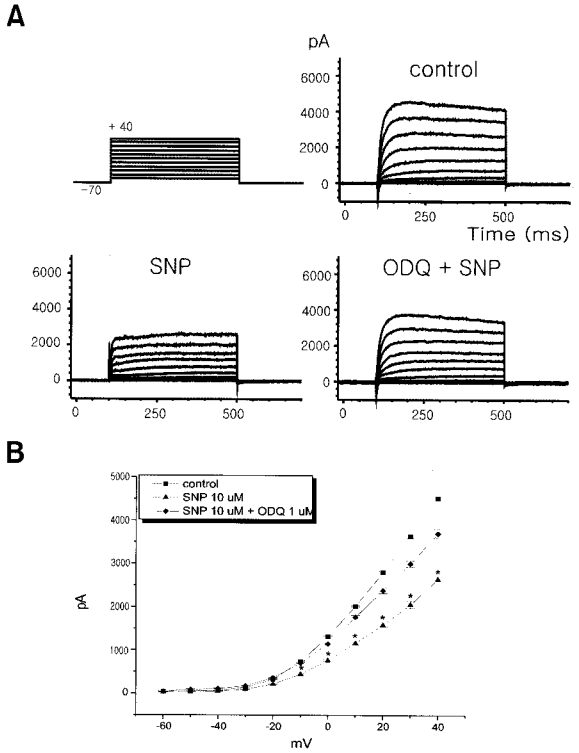

Fig. 3. Effects of ODQ on the SNP-induced decrease of outward potassium currents in medial vestibular nuclear neurons. In each panel, the cell was held at −70 mV and was tested for depolarization while 400 ms were applied from −60 mV to+40 mV in 10 mV increments. (A) application of 1 μM ODQ blocked the inhibitory effects of 10 μM SNP on the voltage-dependent potassium currents. (B) current-voltage relationships (means ± SE, n=6, * Significantly different at $P<0.05$). ■: Control, ▲: 10 μM SNP, ◆: cotreatment of 10 μM SNP and 1 μM ODQ.

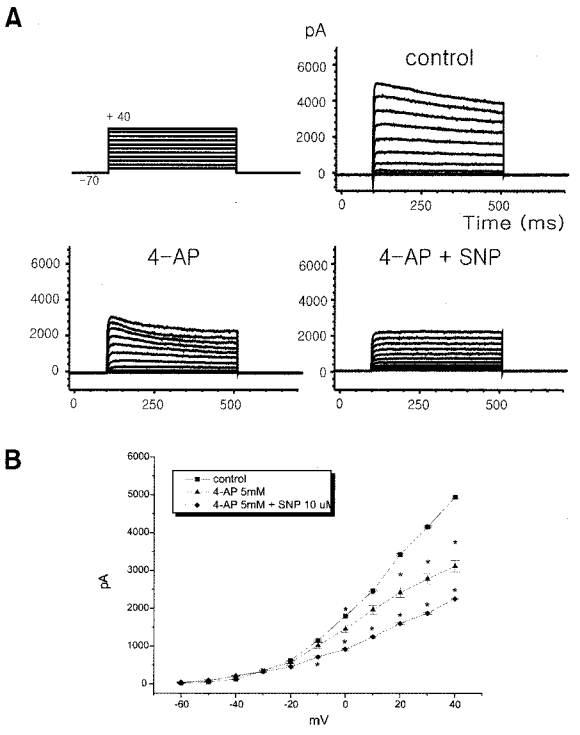

Fig. 4. Effects of SNP on the 4-AP evoked reduction of outward potassium currents in medial vestibular nuclear neurons. (A) application of 10 μM SNP further decreases the amplitude of 4-AP (5 mM) evoked potassium currents. (B) current-voltage relationships (means±SE, n=6, *$P<0.05$) ■: Control, ▲: 5 mM 4-AP, ◆: cotreatment of 5 mM 4-AP and 10 μM SNP.

Effect of high EGTA on the SNP-depressed potassium currents

To block the calcium dependent potassium currents, we used a pipette containing 11 mM EGTA. Currents recorded with pipette solution in different cells of the same population (2987±70.8 mV) were smaller than those recorded with pipette solution containing 0.5 mM EGTA. After blockade of the currents, SNP did not inhibit the outward potassium currents (n=6) (Fig. 5).

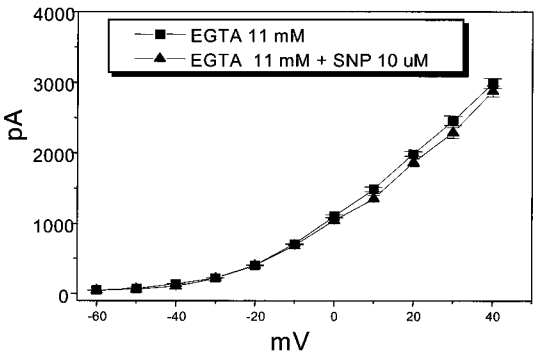

Fig. 5. Effects of SNP on outward potassium currents in the presence of intracellular high EGTA. Currents were recorded with a pipette solution containing 11 mM EGTA (means±SE, n=6, *$P<0.05$). ■: Without SNP treatment, ▲: 10 μM applied to the bathing medium.

DISCUSSION

The results of the present study demonstrated that pre-UL administration of SNP resulted in a marked increase in the rate of spontaneous nystagmus compensation. In contrast, L-NAME ingestion for 4 weeks inhibited the vestibular compensation. Although there was a time difference in drug effects, it might mostly likely be due mostly to the difference of methods of administration between single injection of SNP (2 mg/kg i.p.) and 4 weeks ingestion of L-NAME (0.72±0.02 mg/100 g of body weight/day). The effects of L-NAME may be caused by inhibition of nitric oxide synthase (NOS) in the ipsilateral vestibular medial nucleus, which is activated by increased intracellular calcium immediately after UL.

In the present study, SNP increased the neuronal excitability by decreasing action potential latency and increasing the firing frequency. In addition, SNP reduced the after-hyperpolarization of the action potential. SNP also

inhibited whole potassium currents in the medial vestibular nuclear neuron. However, application of ODQ blocked effects of SNP on both the action potential firing and potassium current. After blockade of delayed rectifier and A-type potassium currents by 4-AP (5 mM), SNP still inhibited the outward potassium current. However, after blockade of calcium dependent potassium currents by high EGTA pipette solution (11 mM), SNP did not inhibit the outward potassium currents. These results suggest that nitric oxide inhibits potassium currents by activation of intracellular guanylyl cyclase, and the site of nitric oxide action is the calcium dependent potassium current.

Potassium channels play an important role in regulating membrane potential and cell excitability by modifying action potential and firing rates (Peusner et al, 1998). Generally, the closure of potassium channels causes depolarization, which increases a cell excitability, and the opening of potassium channels produces hyperpolarization, which decreases the excitability. Calcium dependent potassium current has been reported to contribute to the repolarizing phase of an action potential and to control the repetitive discharge of spikes (Adams et al, 1982; Pineda et al, 1992; Viana et al, 1993).

Nitric oxide has many different intracellular action sites including G protein, cycloxygenase, protein kinase C and guanylyl cyclase and it is functionally coupled with a glutaminergic receptor (Dawson et al, 1992; Meller et al, 1992; Blute et al, 1999; Di Giovanni et al, 2003). Stimulation of the glutaminergic receptor increases cellular calcium concentration, which in turn activates nitric oxide synthase (NOS). Darlington and Smith (1996) reported that upregulation of glutaminergic receptors in vestibular nuclei ipsilateral to the labyrinthectomy increases cellular calcium concentration which is responsible for vestibular symptoms. Our results, taken together, suggest that nitric oxide production in medial vestibular nuclear neurons may be changed following UL. Furthermore, the nitric oxide would help facilitate vestibular compensation by inhibiting calcium-dependent potassium currents, thereby increasing excitability in ipsilateral vestibular nuclear neurons.

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REFERENCES

Adams PR, Constanti A, and Brown DA, Clark RB. Intracellular $Ca2^+$ activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurons. *Nature* 296: 746−749, 1982

Behrend O, Schwark C, Kunihiro T and Strupp M. Cyclic GMP inhibits and shifts the activation curve of the delayed-rectifier (IIK1I) of type I mammalian vestibular hair cells. *Neuroreport* 18: 2687−2690, 1997

Blute TA, De Grenier J, Eldred WD. Stimulation with N-methyl-D-aspartate or kainic acid increases cyclic guanosine monophosphate-like immunoreactivity in turtle retina: in volvement of nitric oxide synthase. *J Comp Neurol* 404: 75−85, 1999

Darlington CL, Smith PF. The recovery of static vestibular function following peripheral vestibular lesions in mammals: the intrinsic mechanism hypothesis. *J Vestib Res* 6: 185−201, 1996

Dawson TM, Dawson VL, Snyder SH. A novel neuronal messenger molecule in the brain: the free radical, nitric oxide. *Ann Neurol* 32: 297−311, 1992

De Waele C, Vibert N, Beaudrimont M, Vidal PP. NMDA receptors contribute to the resting discharge of vestibular neurons in the normal and hemilabyrinthectomized guinea pig. *Exp Brain Res* 81: 125−133, 1990

Di Giovanni G, Ferraro G, Sardo P, Galati S, Esposito E, La Grutta V. Nitric oxide modulates striatal neuronal activity via soluble guanylyl cyclase: An in vivo microiontophoretic study in rats. *Synapse* 48: 100−107, 2003

Dieringer N. 'Vestibular compensation': neural plasticity and its relations to functional recovery after labyrinthine lesions in frogs and other vertebrates. *Prog Neurobiol* 46: 97−129, 1995

Flugel G, Holm S, Flohr H. Chronic inhibition of nitric oxide synthase prevents functional recovery following vestibular lesions, in: Moncada S, Feelisch M, Busse R, Higgs EA ed, *The biology of nitric oxide: Physiology and clinical aspects*, Portland press, London, p 381−387, 1994

Kay AR, Wong RK. Isolation of neurons suitable for patch-clamping from adult mammalian central nervous system. *J Neurosci Methods* 16: 227−238, 1986

Kitahara T, Takeda N, Kubo T, Kiyama H. Nitric oxide in the flocculus works the inhibitory neural circuits after unilateral labyrinthectomy. *Brain Res* 815: 405−409, 1999

Meller ST, Dykstra C, Gebhart GF. Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex. *Eur J Pharmacol* 214: 93−96, 1992

Paterson S, Zheng Y, Smith PF, Darlington CL. The effects of L-NAME on vestibular compensation and NOS activity in the vestibular nucleus, cerebellum and cortex of the guinea pig. *Brain Res* 879: 148−155, 2000

Peusner KD, Gamkrelidze G, Giaume C. Potassium currents and excitability in second-order auditory and vestibular neurons. *J Neuroscience Res* 53: 511−520, 1998

Pineda JC, Ggalarraga E, Bargas J, Cristancho M, Aceves J. Charybdotoxin and apamin sensitivity of the calcium-dependent repolarization and after hyperpolarization in neostriatal neurons. *J Neurophysiol* 68: 287−294, 1992

Ris L, Godaux E. Neuronal activity in the vestibular nuclei after contralateral or bilateral labyrinthectomy in the alert guinea pig. *J Neurophysiol* 80: 2352−2367, 1998

Sansom AJ, Darlington CL, Smith PF. Pretreatment with MK-801 reduces spontaneous nystagmus following unilateral labyrinthectomy. *Eur J Pharmacol* 220: 123−129, 1992

Smith PF, Curthoys IS. Neuronal activity in the ipsilateral medial vestibular nucleus of the guinea pig following unilateral labyrinthectomy. *Brain Res* 444: 308−319, 1988

Smith PF, Darlington CL. Neurochemical mechanisms of recovery from peripheral vestibular lesions (vestibular compensation). *Brain Res Rev* 16: 117−133, 1991

Viana F, Bayliss DA, Berger AJ. Multiple potassium conductance and their role in action potential repolarization and repetitive firing behavior of neonatal rat hypoglossal motoneurons. *J Neurophysiol* 69: 2150−2163, 1993

Zsombok A, Schrofner S, Hermann A, Kerschbaum HH. Nitric oxide increases excitability by depressing a calcium activated potassium current in snail neurons. *Neurosci Lett* 295: 85−88, 2000