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ABSTRACT

In the rat utricle, synaptic contacts between hair cells and the nerve fibers arising from the vestibular primary neurons form during the first week after birth. During that period, the sodium-based excitability that characterizes neonate utricle sensory cells is switched off. To investigate whether the establishment of synaptic contacts was responsible for the modulation of the hair cell excitability, we used an organotypic culture of rat utricle in which the setting of synapses was prevented. Under this condition, the voltage-gated sodium current and the underlying action potentials persisted in a large proportion of non-afferented hair cells. We

then studied whether impairment of nerve terminals in utricle of adult rats may also affect hair cell excitability. We induced selective and transient damages of afferent terminals using glutamate excitotoxicity *in vivo*. The efficiency of the excitotoxic injury was attested by selective swellings of the terminals and underlying altered vestibular behavior. Under this condition, the sodium-based excitability transiently recovered in hair cells. These results indicate that the modulation of hair cells excitability depends on the state of the afferent terminals. In adult utricle hair cells this property may be essential to set the conditions required for restoration of the sensory network after damage. This is achieved via re-expression of a biological process that occurs during synaptogenesis.

INTRODUCTION

The developmental sequence of innervation of vestibular organs has been well described in rodents, based on morphological and functional studies (for review see Eatock and Hurley, 2003). Nerve fibers arising from Scarpa's ganglion enter the sensory epithelia a few days before birth and first immature synaptic contacts can already be identified at birth. Synapses acquire their mature phenotype by the end of the first postnatal week (Desmadryl and Sans, 1990, Dechesne et al., 1986), giving rise to the first recordable synaptic activities (Curthoys, 1982; Desmadryl et al., 1986). During that period, hair cells gradually acquire the biophysical properties that bring them their mature character (Rush et al., 1998).

We have previously shown that, at birth (P0), rat utricle hair cells transiently express a neuronal-like tetrodotoxin (TTX)-sensitive voltage-gated Na⁺ current (I_{Na}), enabling these cells to generate sodium-driven action potentials (AP) (Chabbert et al., 2003). I_{Na} is downregulated during the first postnatal week, decreasing the sodium based excitability in mature hair cells. This developmental excitability, also observed in the mouse utricle (Geleoc et al., 2004), has also been reported in other sensory organs of the higher vertebrates, such as the cochlea (Evans and Fuchs, 1987; Kros et al., 1998) and the retina (Pan and Hu, 2000; Kawai et al., 2001). Although in most cases, the physiological relevance of the I_{Na} remains unknown, we first reported that I_{Na} was involved in the activity-dependent secretion of brain-derived neurotrophic factor (BDNF) in the neonate rat utricle (Chabbert et al., 2003). Regarding the major role of BDNF in the establishment and the stabilization of synaptic contacts (Ernfors et al., 1995; Shimmang et al., 1995), we proposed that the transient hair cell excitability may contribute to the synaptogenesis process in the vestibular organs. A direct demonstration of this hypothesis remains to be provided.

In this study, we tested the hypothesis that the modulation of hair cell excitability is controlled by the vestibular nerve fibers. We first prevented the establishment of synaptic

contacts in neonate rat utricles cultured *in vitro* before synaptic stabilization. Under this condition, the membrane expression of I_{Na} and the ability to fire AP persisted in the non-afferented hair cells. We then selectively damaged afferent terminals in adult utricles using glutamate excitotoxicity *in vivo*. In this condition, a large proportion of hair cells transiently expressed I_{Na} and recovered the ability to fire AP. These observations demonstrate that the state of the afferent terminals directly affects hair cell excitability. The physiological relevance of this biological process on post-injury repair processes is discussed.

METHODS

Animals: Experiments were performed on newborn and adult (40 days old) female Wistar rats in accordance with French Ministry of Agriculture regulations and European Community Council Directive no. 86/609/EEC, OJL 358, 18 December 1986. Neonate rats were decapitated, and adult animals were anesthetized with pentobarbital solution injection (0.1 ml/100 g) before decapitation. All efforts were made to minimize the number of animals used and their suffering.

Denervated utricle organotypic culture: Utricle cultures were prepared as previously described (Gaboyard et al., 2005). Briefly, utricles were explanted aseptically from P0 rats. The otolith membrane was removed and utricles were excised. Nerve fibers were trimmed close to the epithelium. Epithelia were embedded in 10 μ l of extracellular matrix extracted from Engelbreth-Holm-Swarm tumors (Harbor Bio-products, Norwood, MA) on laminin-coated coverslips, and covered with 2 ml of feeding medium, a 1:1 mixture of Dulbecco's modified Eagle Medium (DMEM) and Ham's F-12 nutrient (Gibco BRL, Invitrogen, Gaithersburg, MD, USA) medium supplemented with 10% fetal bovine serum, glucose (5 g/L), glutamine (1.5 mM), sodium bicarbonate (1.1 g/l), and HEPES buffer (15 mM, pH 7.4). Cultures were maintained for 10 days in a humidified 5% CO_2 atmosphere before electrophysiology and immunocytochemical experiments. The feeding medium was renewed every three days.

Excitotoxic impairment of vestibular nerve terminals: Experiments were performed on 78 adult female rats (body weight 180-250 g). Pharmacological agents were applied to the inner ear as previously described by Guitton et al. (2003). Surgery was carried out under general anesthesia induced with isoflurane (Baxter S.A., Lessignes, Belgium), using a Minerve apparatus (induction 10 min at 2.5 %, maintenance at 2% under 0.8 L O_2 /min). Under aseptic condition, the right otic bulla was exposed through a retro-auricular incision and a hole was created with a micro-drill, revealing the round window. Gelfoam (Gelita Tampon, B. Braun Medical Inc., Melsungen, Germany) filled with 2.5 μ l of artificial perilymph solution (APS) (see below), containing 5 mM kainate, a glutamate receptor agonist (Sigma, St Louis, USA), was applied in the round window niche. The tympanic bulla was closed with dental cement (Unifast Trad, GC Corporation, Tokyo, Japan) and the surgical incision was sutured. We

treated 42 rats with kainate alone (17 for electron microscopy, 12 for immunochemistry and 13 for electrophysiological investigations), and 15 rats with kainate plus 200 μ M DNQX (6, 7-dinitroquinoxaline-2, 3 (1H, 4H)-dione, Sigma), a competitive kainate/quisqualate (non-N-methyl-D-Aspartate, NMDA) receptor antagonist (6 for electron microscopy, 6 for immunochemistry and 3 for electrophysiology). 21 animals were used as control with gelfoam filled with 2.5 μ l APS alone. After surgery, animals were returned to their cages and maintained at $22 \pm 2^\circ\text{C}$.

Thin sections and transmission electron microscopy

Utricles were processed as previously reported (Seoane et al., 2001). Briefly, sensory epithelia were rapidly removed and fixed by incubation for two hours in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS. They were then kept overnight in 0.1 M PBS at 4°C . Classical techniques were used to prepare the samples for transmission electron microscopy: post-fixation for 1 hour in 1% OsO_4 in cacodylate buffer (pH 7.2), subsequent dehydration with increasing concentrations of ethanol (70° , 95° , 100°) and embedding in epoxy resin. Utricles were longitudinally sectioned in $1\mu\text{m}$ sections with a diamond knife on an ultra-microtome (Reichert OMU3 Vienna, Austria). Sections were stained with toluidine blue (Rhône Poulenc, France). Ultrathin transverse sections were cut, stained with 2% uranyl acetate and lead citrate and examined using a JEOL 6300F electron microscope.

Morphological evaluation of nerve terminal damages. The degree of nerve terminal swelling was estimated by measuring the calices thickness at half its height (corresponding to the level of the type I hair cell nucleus) and the diameter of the boutons innervating the type II hair cells under each experimental condition using the METAMORPH software (Universal Imaging). Swelling was measured from semithin sections of three utricles for each experimental condition. The proportion of nerve terminals damaged by the kainate treatment in each epithelium was estimated on entire semi-thin sections cut through the longitudinal axis of treated ($n=5$) and control utricles ($n=5$). Nerve terminals displaying widths larger than $1\mu\text{m}$ were considered as swollen. We counted the total number of hair cells present in each semithin section and determined the percent of hair cells that displayed swollen terminals. The evaluation was made on single sections chosen randomly over series of consecutive sections of each utricle to mimic the conditions encountered in the electrophysiological recordings. Student t-Test ($p<0.05$) was used to characterised the significant relevance.

Evaluation of vestibular dysfunction: Vestibular dysfunction was evaluated by observation of spontaneous motor behavior as previously described (Boadas-Vaello et al., 2005). Briefly, rats were placed for one minute on a table top and the experimenter rated the animals from 0 to 4 for circling, retropulsion and abnormal head movements. Circling was defined as stereotyped circling movement. Retropulsion consisted of backward displacement of the animal. The head bobbing consisted of intermittent extreme backward extension of the neck. The rats were also rated 0 to 4 for the tail-hang reflex, contact inhibition of the righting reflex and air righting reflex tests. When lifted by the tail, normal rats exhibit a “landing” response consisting of forelimb extension. Rats with impaired vestibular

function bent ventrally, sometimes “crawling” up towards their tails, thus tending to occipital landing. For the contact inhibition of the righting reflex, rats were placed supine on a horizontal surface and a metal bar grid was lightly placed in contact with the soles of the animals’ feet. Healthy rats quickly right themselves, whereas the vestibular-deficient rats lie on their back, with their feet up and “walk” with respect to the ventral surface. For the air righting reflex, the animals were held supine and dropped from a height of 40 cm onto a foam cushion. Normal rats are successful in righting themselves in the air whereas vestibular-deficient rats are not. The results of all tests were combined and expressed as a percentage (versus a maximal score of 24). To provide a positive control condition for the behavioral assessment of vestibular dysfunction, four animals were injected intraperitoneally with 1000 mg/kg of 3,3'-iminodipropionitrile (IDPN) 2 days before behavioral assessment. This treatment causes a complete loss of vestibular hair cells and hence of vestibular function (Llorens et al., 1993).

Electrophysiological recordings: Whole-cell voltage and current clamp recordings were made using an Axopatch 200B amplifier (Axon Instruments; Molecular Devices Corp. Sunnyvale, CA, USA) in hair cells from cultured or acutely isolated rat utricles as previously described (Chabbert et al., 2003). Epithelia were placed in an experimental chamber containing 2 ml of artificial perilymph solution (in mM): 137 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 5.6 D-glucose, 10 HEPES-NaOH, pH 7.4, adjusted to 305 mOsm/l. Amino acids and vitamins for Eagle's MEM were added from concentrates (Gibco BRL). The pipette solution contained (in mM): 135 KCl, 0.1 CaCl₂, 5 EGTA, 3 MgATP, 1 NaGTP, 5 HEPES-NaOH, pH 7.3, adjusted to 300 mOsm/l. After seal formation (>10 GΩ) onto the basolateral membrane of hair cells and membrane disruption, the membrane capacitance (C_m, 5.4±1.4 pF, n=16) and series resistance (R_s, 4 to 10 MΩ) were estimated from the decay of the capacitive transient induced by a ± 10 mV pulse from a holding potential of -100 mV. R_s was compensated for up to 85% after cancellation of the capacitive transients. Voltage errors resulting from uncompensated R_s did not exceed 5 mV and were not corrected. No linear leakage compensation was performed and the liquid junction potential was not corrected (-2 mV). Data were collected using pClamp 9.0 software (Axon Instruments) and analyzed with Origin 4.1 software (Microcal Software, Northampton, MA). Data were sampled at 5 or 10 kHz and filtered at half sampling rate (8-pole Bessel filter). The mean chord conductances of I_{Na} were calculated from the currents adjusted for driving force, assuming a Na⁺ reversal potential of +56 mV. This value was estimated by applying 1 ms voltage steps to -30 mV from holding potential -110 mV to fully activate I_{Na} followed by 20 ms depolarizations from -80 to +70 mV in 10 mV increments. Protocols for voltage activation and inactivation of I_{Na} and current injections are described in the legends of the Figure 1. Relative activation curves for I_{Na} were best fitted with a single Boltzmann function of the form: $G_{Na}/G_{Na,max} = 1/(1+\exp((V_{1/2}-V_m)/k))$ for activation where G_{Na} is the voltage-dependent sodium conductance, G_{Na,max} is the maximal sodium conductance, V_{1/2} is the potential at which activation is half-maximal, V_m is the membrane potential, and k the slope factor. Relative inactivation curves of I_{Na} were fitted using the equation: $I_{Na}/I_{Na,max} = 1/(1+\exp((V_m-V_{1/2})/k))$ where I_{Na,max} is the peak sodium current elicited after the more hyperpolarized prepulse, V_m is the preconditioning pulse potential and V_{1/2} is the half-inactivation potential.

We checked for the presence of $g_{K,L}$ a negative activating delayed rectifier current that distinguishes between type I and type II hair cells, using a protocol described in detail elsewhere (Gaboyard et al., 2005). Hair cells displaying I_{Na} with a half inactivation more hyperpolarized than -85 mV, characteristic of the tetrodotoxin-insensitive sodium current, carried by the $Na_v1.5$ subunit and mostly found in the striolar region of the rat utricle (Wooltorton et al., 2005), were excluded from the analysis. The chamber was continuously superfused at a rate of 1 ml/min, using a peristaltic pump. All experiments were conducted at room temperature (22-24°C). Results are presented as mean \pm standard deviation (SD). n values represents the total number of hair cells studied in each experimental condition.

Immunocytochemistry: Utricles were fixed by incubation overnight at 4°C with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). They were embedded in 4% agarose (Invitrogen Ltd, Palsley, UK) in PBS and cut into 60 μ m sections with a vibrating blade microtome in ice-cold PBS. Free-floating sections were incubated for 1 h at 4°C in PBS supplemented with 10% normal donkey serum and 0.3% Triton X-100. The sections were incubated overnight at 4°C with a rabbit polyclonal antibody recognizing the α -subunit of the voltage-gated sodium channel ($Na_v1.2$) (brain type II, 1/100, Sigma), together with a mouse monoclonal anti-neurofilament 200 antibody (NF) (clone N52, 1/500, Sigma), in PBS supplemented with 5 % normal donkey serum and 0.1% Triton X-100. Sample sections were rinsed with PBS, and incubated for 2 h at room temperature with biotinylated anti-rabbit IgGs (1:300; Jackson ImmunoResearch Labs, West Grove, PA, USA), and then with streptavidin Alexa-Fluor 546 conjugate (1:500; Molecular Probes, Invitrogen) and fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgGs (1/300, Jackson Labs). Sections were mounted in Fluorsave Reagent (Calbiochem, Meudon, France) and observed with a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with 40x and 63x oil immersion lenses. For all ages of rat studied, negative controls, for which the primary antibody was omitted, showed no specific fluorescence (data not shown).

Statistical tests: Statistical analysis were performed using Khi^2 test (significant at least $p<0.05$) for the percentage of I_{Na} expression and Student's t-Test ($p<0.05$) to compare I_{Na} densities. ANOVA ($p<0.05$) followed by Duncan's test ($p<0.05$) was used for behavioral scores. Statistically significant differences were indicated by asterisks (*) in figures.

RESULTS

Persistence of electrical excitability in non-afferented utricle hair cells

At birth in the rat utricle, most hair cells (78.9 %; 45/57) transiently express a voltage-gated sodium current (I_{Na}) that brings them the ability to fire sodium-based action potentials (Chabbert et al., 2003). During the first postnatal week, I_{Na} is dramatically down regulated and

remains in a small population of hair cells from P10 until adulthood (Fig.1A). Since the first postnatal week, is a period of intense synaptogenesis in the rodent vestibular organs (Curthoys, 1982; Desmadryl et al., 1986), we hypothesized that the down regulation of I_{Na} in hair cells resulted from the establishment of synaptic contacts with the nerve fibers arising from the vestibular ganglion. To test this hypothesis, we checked whether preventing the setting of synaptic contacts may affect the developmental expression of I_{Na} in hair cells. Utricles were acutely excised at P0 and maintained in culture for 10 days (P0+10DIV) without vestibular ganglion. In this condition, whole-cell patch-clamp recordings revealed that a large percentage of P0+10DIV hair cells still expressed I_{Na} (64.0 %, 16/25) (Fig.1A), conversely to those after 10 days of normal development, P10 (9.6 %, 3/31). At P0+10DIV, both hair cell types could be distinguished (40% type I, 60% type II; $n=20$) based on the presence of the low-voltage activating outwardly rectifying conductance ($g_{K,L}$) (Correia and Lang, 1990, Chen and Eatock, 2000). I_{Na} was present in half of the type I hair cells (4/8) and in two third of the type II hair cells (8/12). The density of I_{Na} obtained by dividing the maximal sodium conductance ($G_{Na\ max}$) by the hair cell membrane capacitance, did not differ between hair cells after 10 days in culture (3.6 ± 1.4 nS/pF, $n = 6$) and P0 hair cells (4.1 ± 1.2 nS/pF, $n = 8$), but was significantly higher ($p<0.05$) than those of P10 hair cells (1.3 ± 0.5 nS/pF, $n = 3$) (Fig. 1B). The pharmacological properties (complete block by 100 nM tetrodotoxin-TTX, not shown) and biophysical properties of I_{Na} in P0+10DIV hair cells did not differ from those of P0 hair cells (Fig. 1C and Table 1). Figure 1D (bottom) shows representative I_{Na} in P0, P10 and P0+10DIV hair cells respectively. Similar to what is observed at P0, TTX-sensitive action potentials (AP) could be evoked in hair cells at P0+10DIV by the injection of depolarizing currents in the range of those that would be carried by mechanosensitive transduction channels (Fig. 1D). No such APs were elicited in acutely excised P10 hair cells. All together these observations reveal that the down regulation of I_{Na} in the utricle hair cells does not occur in absence of ongoing innervation.

Recovery of excitability in hair cells following nerve terminals impairment

To examine whether a similar property may explain the lack of excitability in adult hair cells, we investigated whether impairment of afferent nerve terminals in adult utricles (subsequently to synapse stabilization) led to the recovery of hair cell excitability. Initially, we aimed to record I_{Na} in utricles explanted from adult rats and maintained for several days in culture. The nerve terminals present in the epithelia degenerated in one day. Unfortunately, we could not achieve satisfactory culture conditions for recording hair cell currents. We overcame this problem by inducing selective damage of nerve terminals *in vivo*, in adult rats. We adapted to the rat vestibule, a method previously developed in the mammalian cochlea to induce transient excitotoxic injuries to the first auditory synapse (Puel et al., 1994; Guitton et al.,

2003). We applied a gelfoam containing 5 mM of kainate into the round window niche of adult P40 rats. Indeed, kainate has been shown to induce excitotoxic damages to vestibular synapses (Liu, 1999). Figure 2 illustrates the consequences of kainate treatment on the nerve terminals in utricles. Swellings of calyx and bouton nerve terminals were clearly identifiable on the entire utricle 48 hours after kainate application (K+48h; Fig. 2A). These swellings were particularly marked in the striolar region, in which type I hair cells were often expelled from their calyx anchorage. Swellings were less pronounced in the peristriolar region, but both calyx and bouton nerve terminals were destructured with respect to those present in control ears (Fig. 2B-C and Fig. 3A-D). Quantitative assessment revealed that about 75 % (75.1 ± 5.82 %, over 5 epithelia) of hair cells displayed swollen afferent terminals in treated utricles versus less than 6 % (5.8 ± 2.7 %, over 5 epithelia) in control utricles (Fig. 2D). In our experimental conditions, kainate-induced impairments were transient. No morphological trace of swelling was observed one week after kainate application ($n = 3$) (Fig. 3E, F). The specificity of the excitotoxic injury was confirmed by the absence of morphological damage when DNQX ($n = 3$), a competitive non-NMDA receptor antagonist, was applied simultaneously with kainate (Fig. 3G, H). Estimation of the nerve terminals size in each condition is given in Table 2. Morphological examination of the synapses structure at higher magnification did not reveal significant alteration of the active zone organization both in hair cells and in afferent nerve terminals (Fig. 4). Ribbons were always found at the contact with the basal hair cell membrane and surrounded by numerous synaptic vesicles as reported in control preparations. Postsynaptic densifications were also identifiable at the afferent terminals membrane facing the ribbons, although disorganization of their internal ultrastructure was clearly identifiable in K+48h condition. Efferent terminals never displayed any sign of swelling (Fig. 4I-K).

Consequences of the afferent terminals impairment on the vestibular function were evaluated using a battery of tests that included observation of spontaneous motor behavior previously described (Boadas-Vaello et al., 2005). Rats treated with kainate transiently displayed signs of vestibular dysfunction. This was attested by significant increase in the rating scores of vestibular dysfunction at K+48h relative to control rats. Signs of vestibular dysfunction were no longer detected one week after kainate application or under neuroprotection conditions (Fig. 5).

During afferent terminals impairment (K+48h), the proportion of utricle hair cells expressing I_{Na} increased dramatically with respect to control hair cells still afferented at P40 (55.2 %, 16/29 versus 5.8 %, 1/17; $p < 0.01$; Fig. 6A). 40 % of the tested cells (6/15) were identified as type I hair cells. I_{Na} was present in one third (2/6) of these latter cells. The density of I_{Na} in hair cells at K+48h was in the same order to that estimated in P0 cells (3.1 ± 0.1 nS/pF, $n = 4$,

versus 4.1 ± 1.2 nS/pF, $n = 8$). The pharmacological (not shown) and biophysical properties of I_{Na} in hair cells at K+48h did not differ significantly from those of hair cells in utricles acutely isolated at P0 (Table 1). Hair cells displaying I_{Na} density ≥ 3 nS/pF displayed TTX-sensitive AP upon injection of depolarizing currents (Fig. 6B). One week after kainate application, hair cells were no longer able to express high I_{Na} density and to generate sodium-based AP ($n = 11$). These properties were also not observed if DNQX was applied simultaneously with kainate ($n = 14$).

We previously reported that $Na_v1.2$, one of the commonest voltage-gated sodium channel α -subunits in rat utricle hair cells, is down regulated during synaptogenesis (Chabbert et al. 2003). To study whether the regulation of $Na_v1.2$ expression during synaptic impairment may support the observed re-expression of I_{Na} , we checked its membrane expression using immunohistochemistry. $Na_v1.2$ was not detected in utricle hair cells from adult control rats (Fig. 7A). However, 48 hours after kainate application, $Na_v1.2$ immunostaining dramatically increased in hair cells. The labeling was prominent at the hair cells basal membrane (Fig. 7B). Recovery of $Na_v1.2$ staining did not persist beyond one week after kainate treatment in most studied utricles (4 of 5) (Fig. 7C). $Na_v1.2$ membrane re-expression was not observed when DNQX was applied with kainate (all 3 rats tested) (Fig. 7D). These observations reveal that impairment of the afferent terminals in adult rat utricle triggers the re-expression of $Na_v1.2$ at the hair cell membrane.

DISCUSSION

This study shows that the modulation of the sodium-based excitability of the sensory cells is dependent on the state of the afferent terminals in the mammal utricle. The voltage-gated sodium current expression and underlying hair cell excitability are down regulated in neonate utricles as the ongoing nerve fibers contact the hair cells. Conversely, when mature afferent terminals are selectively impaired *in vivo*, hair cells re-express I_{Na} and recover the ability to fire sodium-based action potentials. These observations provide new data which help to understand of the cellular interactions involved in synaptogenesis and post-injury repair of the vestibular sensory organs.

Persistence of electrical excitability in non afferented utricle hair cells

Denervated organotypic culture has been previously used to test whether morphological or electrophysiological differentiation depended on ongoing innervation (Rush et al., 1998). Results obtained on mouse utricles demonstrated that neither calyx formation nor sustained postnatal innervation was required for morphological differentiation of the two types of hair

cells, nor for the developmental acquisition of voltage-gated potassium currents. Using similar preparation in the rat, we confirmed that the developmental expression of $g_{K,L}$ is independent on innervation (Gaboyard et al., 2005). Conversely, present study shows that the developmental expression of I_{Na} depends on the ongoing innervation, since the down regulation of voltage-gated sodium currents does not occur in the absence of synaptogenesis. This applies to both hair cell types. At P0+10DIV the density of I_{Na} in hair cells remained relatively high, allowing hair cells to fire sodium-based action potentials as in neonate period. This could be interpreted as a consequence of a bloc of hair cell differentiation under the culture conditions, since the large density of I_{Na} is usually restricted to P0-P3 hair cells in the rat utricle (Chabbert et al., 2003). However, since other voltage-gated ionic currents, such as $g_{K,L}$ that mark utricle hair cell maturity (Rusch et al., 1998) were observed at P0+10DIV (see also Gaboyard et al., 2005), it is unlikely that only intrinsic factors that would affect I_{Na} expression in vivo are lost in culture. A study recently published by our group, strongly support the idea of a control of I_{Na} expression by the ongoing innervation. We studied the expression I_{Na} in utricle hair cells from rats developed under enhanced gravity (Brugeaud et al., 2006). This experimental condition was reported to delay synaptogenesis (specifically the synaptic stabilization) by four days (Gaboyard et al., 2003; Bouet et al., 2004). In this model, I_{Na} down regulation was also delayed by four days, whereas its biophysical properties were unaffected. This additional observation confirms the relationships between the innervation and the down regulation of I_{Na} , and strengthens the idea of a control of the developmental expression I_{Na} by the innervation. In type II hair cells that develop and become innervated earlier than type I, it can be assume that the down regulation of I_{Na} would occur earlier. This remains to be demonstrated. We can question whether the influence of the nerve fibers on the hair cell electrical phenotype results from the cell-to-cell contact of the nerve terminals with the hair cell membrane or whether it results from synaptic stabilization. Since at birth, when immature synaptic contacts are identifiable, most hair cells express I_{Na} , the latter hypothesis appears more likely.

Recovery of excitability in adult utricle hair cells following nerve terminals impairment *in vivo*

The use of the gelfoam method to produce selective excitotoxic impairment of the nerve terminals is to our knowledge unique in the vestibule. Such a technical approach appears appropriate since we observed morphological damage similar to that reported after intralabyrinthine application of glutamatergic agonists in mammals (Liu 1999; Shimogori and Yamashita, 2004). The transient occurrence of the selective damages and the underlying vestibular disorders confirms the efficiency of the method. The glutamatergic nature of the excitotoxic impairment was attested first by the selective swelling of afferent terminals bearing

glutamate receptors conversely to the efferents, and second, by the neuroprotective effect of DNQX. This result is in agreement with our recent demonstration that the non-NMDA receptors play a major role at the primary vestibular synapse (Bonsacquet et al., 2006). The fact that the biophysical and pharmacological properties of I_{Na} in the kainate-treated utricles are similar to those in neonate utricles, suggests that the same sodium channel is re-expressed after excitotoxicity. This is supported by the re-expression of the Nav1.2 subunit. The dependency between hair cell excitability and the state of the nerve terminals was confirmed by the down-regulation of I_{Na} as the nerve fibers re-contacted the hair cells one week after the kainate application. The lack of reafferentation after kainate treatment in 20% of the tested rats may result from the toxic effect of kainate previously reported on auditory ganglion neurons (Juiz et al., 1989). The persistence of I_{Na} in a small population of adult hair cells under control conditions remains unexplained. It may result from hair cells undergoing re-afferentation after hair cell regeneration, which has been reported to occur in adult mammal utricles (Warchol et al., 1993). However, the small degree of turnover would not account for the estimated proportion of persistent I_{Na} . Another possibility is that the persistent I_{Na} might involve the $Na_v1.5$ subunit, the expression of which is restricted to striolar hair cells and is not developmentally regulated (Wooltorton et al., 2005). Based on the observations reported here, we suggest that the recovery of hair cell excitability results from the release of cellular mechanisms that normally prevent membrane expression of I_{Na} after synaptogenesis is complete.

Plasticity in voltage-gated sodium channel expression

The present results strongly suggest that in utricular hair cells, the expression of the sodium current is a dynamic process. Such plasticity has been widely studied in spinal sensory neurons in which the expression of various sodium channels isoforms is regulated during development (Roy and Narahashi 1992) or following nerve injury (Waxman et al., 1994, Black et al., 1999; Dib-Hajj et al., 1999; Kim et al., 2001). Using immunocytochemistry, we demonstrated that $Na_v1.2$ subunit is down-regulated in hair cells during synaptogenesis (Chabbert et al., 2003). This result is consistent with other observations in mammalian retinal ganglion cell axons in which $Na_v1.2$, first clustered at immature nodes of Ranvier, is down-regulated and replaced by $Na_v1.6$ as myelination proceeds. In contrast, $Na_v1.2$ continues to be expressed throughout adult axons of retinal ganglion cells in shiverer mice (Boiko et al., 2001), which lack compact myelin, and also on adult photoreceptors in the human retina (Kawai et al., 2005), which lacks myelin. In the present study, we showed that $Na_v1.2$ membrane expression is up-regulated following nerve terminal impairments. Such a phenomenon has also been reported to occur in rat hippocampal neurons following kainate-induced seizure (Gastaldi et al., 1997). Together,

these observations suggest that $Na_v1.2$ may be specifically involved in a wide variety of developmental processes in sensory neurons. However, since various Na_v isoforms are expressed in utricular hair cells (Chabbert et al., 2003; Mechaly et al., 2005) it would be of interest to test whether other Na_v isoforms are also regulated after nerve terminals impairment. Several neurotrophic factors have been shown to regulate sodium channel transcription in spinal sensory neurons. For example, exposure to nerve growth factor (NGF) and/or glial cell neurotrophic factor (GDNF) differentially promotes the expression of sodium currents (Leffler et al., 2002; Fjell et al., 1999). In addition, in PC12 cells expressing trkB receptor, BDNF increases type II sodium channel mRNA expression (Fanger et al., 1995). We previously reported that an activity-dependent secretion of BDNF occurs in the rat utricle during synaptogenesis (Chabbert et al., 2003). Therefore, it would certainly be worthwhile studying whether this is reactivated following nerve terminals impairment and to what extent it controls the expression of the voltage-gated sodium current.

Physiological relevance and perspectives

Ischemic damage to the inner ear constitutes a major cause of hearing loss and vertigo in man (Puel, 1995). It has now been clearly established that such damage is mediated by the massive release of glutamate by hair cells, resulting in excitotoxic damage to auditory (Puel et al., 1994; Puel et al., 1995; Hakuba et al., 2003) and vestibular (Liu, 1999; Shimogori and Yamashita, 2004) synapses. The transient nature of such damage, which is well documented in the mammalian cochlea, indicates that local repair processes may occur under specific conditions (Puel et al., 1995). We provide here the first evidence that such processes also exist in the vestibular endorgans and that they occur through the modulation of hair cell sodium excitability. The involvement of the efferents in that repair process remains to be elucidated. Further experiments using selective bloc of the efferent synaptic transmission would allow to asses their involvement in the restoration of the vestibular function following excitotoxicity. The data presented here are thus of particular importance since they help to understand how synaptic contacts between hair cells and their cognate afferents are formed and repaired in mammalian vestibular organs. Studies of this type are a prerequisite for the development of strategies to protect the vestibular synapse against local ischemia and repairing damage caused by this condition.

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LEGENDS

Figure 1. Persistence of electrical excitability in non afferented utricle hair cells in culture. (A) Diagram showing the percentage of utricle hair cells expressing I_{Na} as a function of rat age. Data in white columns were taken from Chabbert et al. (2003). Data in black columns were recorded in hair cells from utricle acutely isolated at P0, P1, and P10 and from cultured utricle (P0+10DIV) ($p < 0.01$). (B) Comparison of I_{Na} density (mean \pm SD) at indicated stages ($*p < 0.05$, $**p < 0.01$). Mean membrane capacitances (C_m) did not differ significantly between stages: 4.3 ± 1.4 pF at P0, 3.7 ± 0.9 pF at P10 and 4.0 ± 1.1 pF at P0+10DIV. In A and B, the total number of cells studied at each stage of development is reported at the top of each column. (C) Plot of relative activation (\bullet ; between -70 and +20 mV from a V_{hold} of -110 mV) and inactivation (\blacksquare ; test potentials: 5 ms at -30 mV; conditioning prepulses 10 ms between -120 and -30 mV from at V_{hold} of -110 mV) of I_{Na} at indicated stages, expressed as G/G_{max} and I/I_{max} as a function of test potentials and conditioning prepulses respectively. (D) Representative voltage responses (upper traces) in the current-clamp mode of the whole-cell patch-clamp technique, following given protocol and at indicated stages of development. Underlying whole-cell currents recorded in voltage-clamp mode are given below. Capacitive transients have been truncated for clarity. Depolarizing current injections (100 pA steps) were applied to cells held under hyperpolarizing holding current. Traces shown in the figure are single traces.

Table 1. Electrophysiological properties of I_{Na} . Mean potentials at which half of the cell's sodium channels are activated ($V_{1/2 act}$) or inactivated ($V_{1/2 inact}$) in given experimental conditions. No significant difference in $V_{1/2 act}$ and $V_{1/2 inact}$ was observed between the studied conditions. Data are given as mean \pm SD.

Figure 2. Morphological evaluation of excitotoxic damages. (A) Longitudinal sections through the entire utricle revealing numerous swollen nerve terminals below hair cells at K+48h. (B, C) High magnification observations in control and K+48h conditions. (D) Diagram

showing the mean percent of hair cells with swollen nerve terminals over the total number of hair cells studied (59/1015 in control (C) and 540/728 in kainate-treated rats) in semithin sections of five epithelia in given conditions ($***p < 0.001$). Scale Bars = 30 μm in A, 10 μm in B, C.

Figure 3. Morphological consequences of transient nerve terminals impairment. Electron microscopy observations of vestibular nerve terminals at type I (hcI, top) and type II (hcII, bottom) hair cells in the peristriolar region of utricles from adult rats in given conditions. (A, B) Illustration of intact nerve calyx (nc), afferent (a) and efferent (e) bouton terminals in control utricles. (C, D) 48 hours after kainate application, both calyx and bouton terminals displayed characteristic swellings. The calyx membrane often came off from the hair cells membrane (arrow). Bouton swellings resulted in indentations of type II hair cells basal membrane. No damage persisted in the sensory epithelium one week after kainate application (E, F) or if DNQX was applied with kainate (G, H). (sc) supporting cells. Scale bars indicate 1 μm in all panels.

Table 2. Size analyze of nerve terminals. Sizes (in μm) of calyx and bouton terminals measured in given experimental conditions. Nerve terminals size is significantly larger in K+48h condition relative to other given conditions ($***p < 0.001$). Values are given as mean \pm SD in each condition and total number of cells studied is given between parentheses.

Figure 4. Synapse ultrastructure after kainate treatment. (A-D) Synaptic ribbons in type I hair cells in given conditions. (A, B) Synaptic ribbon (arrow) in type I hair cell (hcI) surrounded by a swollen nerve calyx (nc) at K+48h. Synaptic vesicles (arrow head) are close to the hair cell basal membrane facing the postsynaptic density (asterisk). (C) Two synaptic ribbons in a single type I hair cell at K+48h. (D) Ribbon structure in control condition. (E-H) Synaptic ribbons in type II hair cells (hcII). (E, G) Postsynaptic densities in swollen afferent boutons contacting type II hair cells at K+48h. Ribbon is closed to basal membrane and surrounded by synaptic vesicles. (H) Ribbon and postsynaptic density in control type II hair cell. (I-K) No damage of efferent terminals (e) was observed at K+48h.

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Figure 6. Transient recovery of excitability in adult utricle hair cells following nerve terminals impairment. (A) Diagram showing the percentage of utricle hair cells expressing I_{Na} in given conditions. The total number of recorded cells is reported at the top of each column. (** $p < 0.01$) (B) Representative voltage responses (upper traces) for the given protocol, with the underlying whole-cell currents (bottom traces). Capacitive transients have been truncated for clarity. All traces are single traces. A high-density I_{Na} supporting a sodium-based AP was observed only 48 hours after kainate application (K+48h). Voltage and current traces shown under neuroprotection (K+DNQX+48h) and recovery (K+1week) conditions are those of the only cells expressing I_{Na} in these two sets of conditions (mean C_m : 3.3 and 3.9 pF, respectively).

Figure 7. Transient recovery of $Na_v1.2$ expression in adult utricle hair cells following nerve terminals impairment. Immunolocalisation of neurofilament-N52 (red) and $Na_v1.2$ α subunits (green) in adult rat utricles in given conditions. $Na_v1.2$ labelling is not detected in utricle hair cells or in supporting cells in control (A). 48h after kainate application, a strong $Na_v1.2$ immunoreactivity is observed at the basal hair cells membrane of (arrows) (B). $Na_v1.2$ immunoreactivity do not persist one week after kainate application (C) and is absent in K+DNQX+48h experimental conditions (D). Scale bars indicate 10 μ m in all panels.

Table 1

conditions	$V_{1/2}$ act, mV	$V_{1/2}$ inact, mV
P0	-40.1 ± 2.3 (10)	-77.6 ± 2.9 (6)
P0+10DIV	-43.1 ± 4.0 (6)	-74.1 ± 4.8 (4)
K+48h	-43.4 ± 6.6 (10)	-72.6 ± 4.6 (3)

Table 2

conditions	calices	boutons
control	$0,61 \pm 0,03$ (49)	$0,84 \pm 0,03$ (36)
KA+48h	$2,09 \pm 0,14$ (39)	$2,32 \pm 0,26$ (25)
KA+1week	$0,65 \pm 0,02$ (12)	$0,75 \pm 0,03$ (32)
KA+DNQX+48h	$0,54 \pm 0,02$ (23)	$0,86 \pm 0,10$ (11)

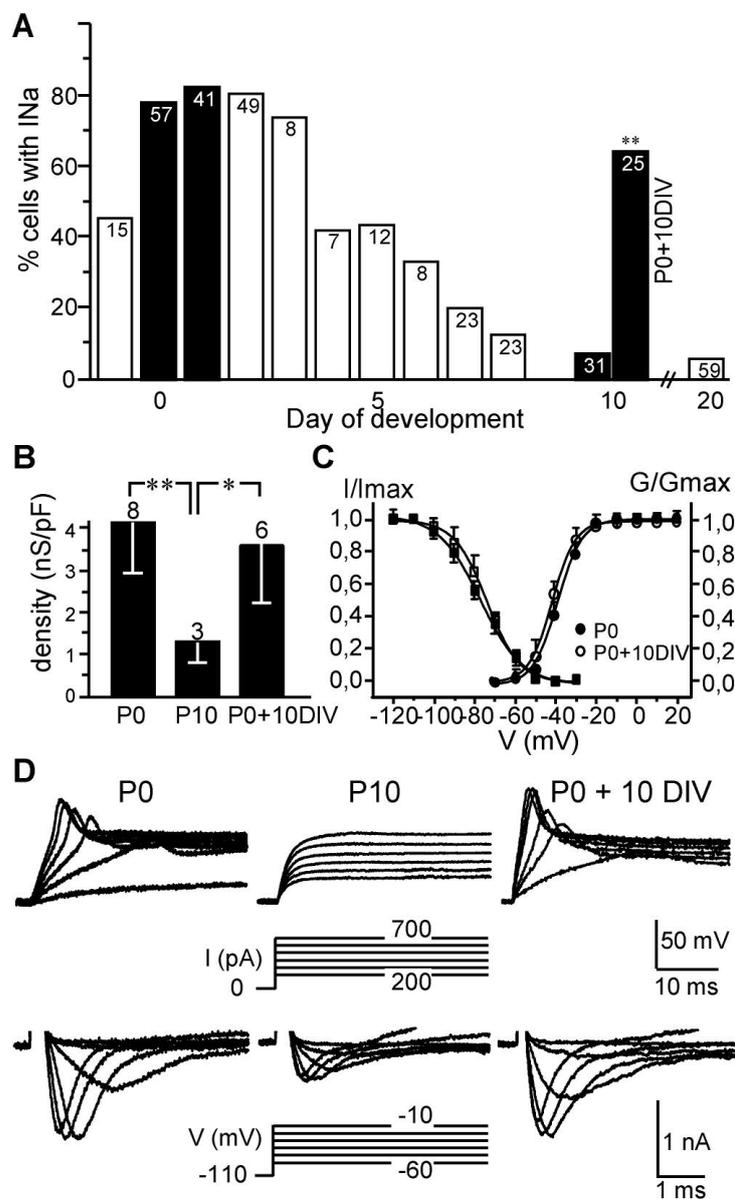


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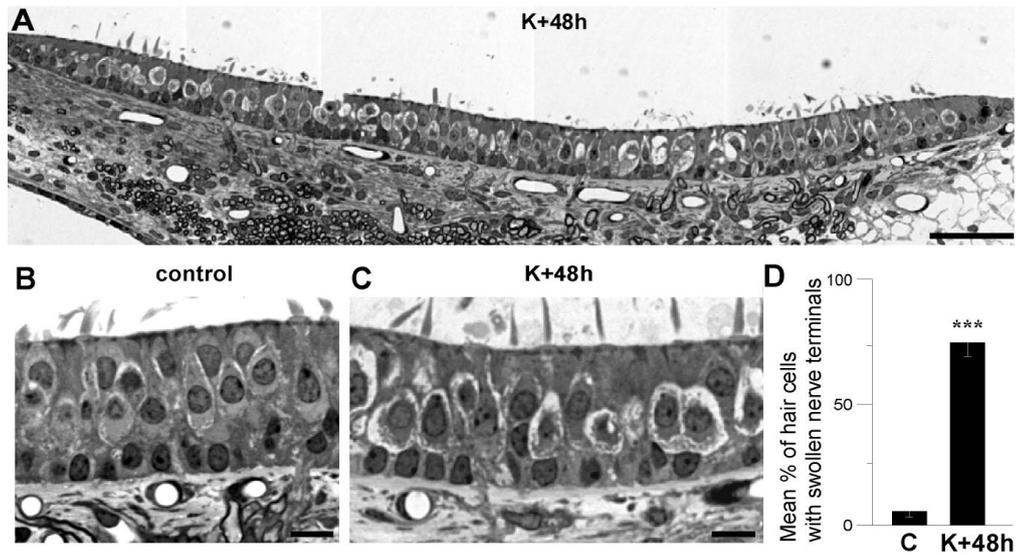


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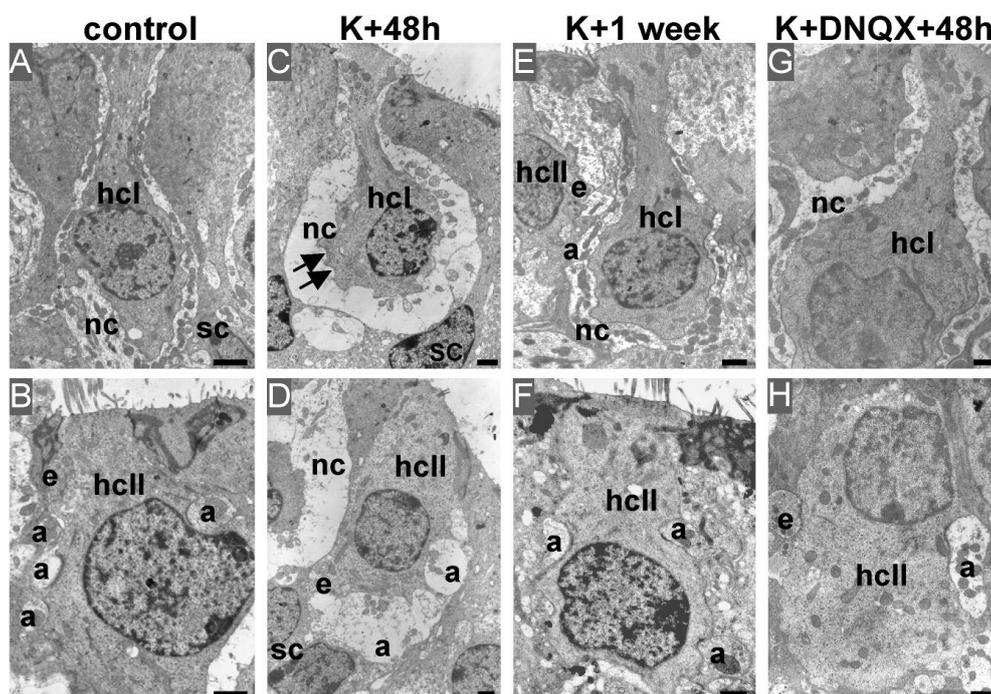


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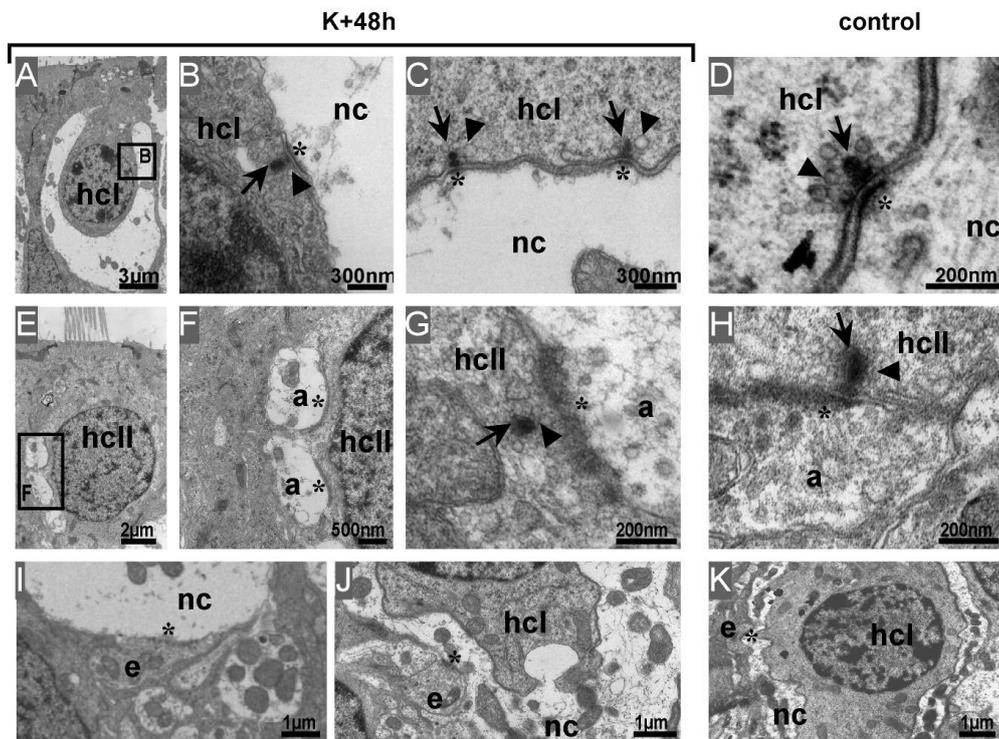


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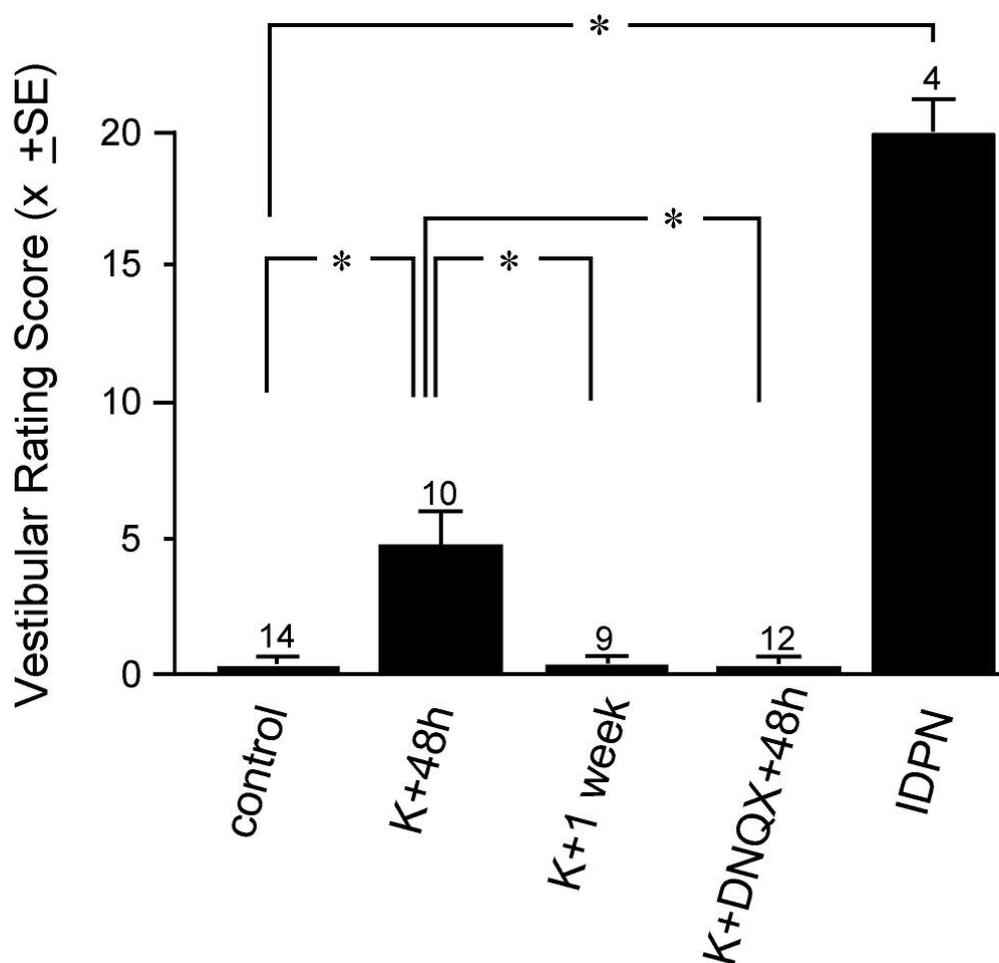


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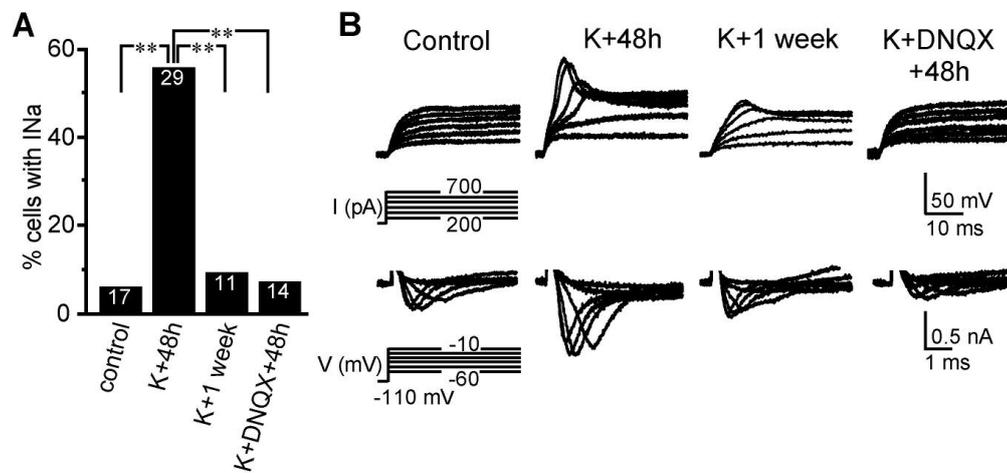


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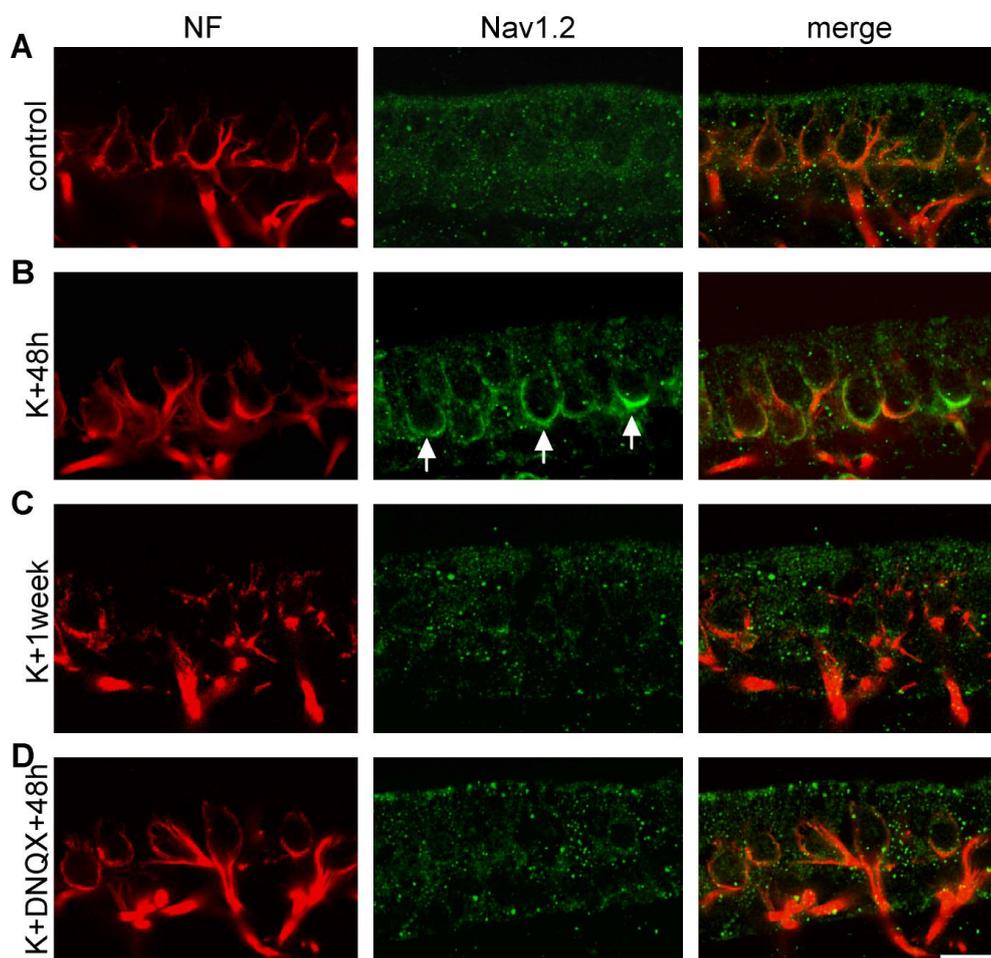


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