Neuropharmacology 144 (2019) 133–142

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

# Apamin treatment accelerates equilibrium recovery and gaze stabilization in unilateral vestibular neurectomized cats: Cellular and behavioral aspects

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## HIGHLIGHTS

- The study focuses on an animal model of unilateral permanent vestibular loss, the induced vestibular syndrome and the effects of pharmacological treatment with apamin.
- The originality of this approach relies on the use for the first time, of SK channels as pharmacological targets for antivertigo treatment.
- We show for the first time a substantial effect of apamin treatment at behavioral level, with significant attenuation of the acute vestibular syndrome and faster posturo locomotor recovery in vestibulo lesioned animals.
- We also demonstrate a regulation of SK channels that supports their direct involvement in both the alleviation of the vestibular syndrome and the functional recovery.

## ARTICLE INFO

*Keywords:*
Vestibular compensation
SK channels
Apamin
Vertigo
Cat model
Vestibular function recovery
Unilateral vestibular neurectomy

## ABSTRACT

Sudden and complete unilateral loss of peripheral vestibular inputs evokes characteristic vestibular syndrome comprised of posturo-locomotor, oculomotor, vegetative and cognitive symptoms. Subsequently to the vestibular insult, a neurophysiological process called central vestibular compensation promotes the progressive restoration of the posture and balance. The modulation of the excitability of vestibular secondary neurons has been demonstrated to be a key process of this mechanism. However, the molecular mechanisms that support this modulatory process have thus far not been fully identified. The present study used a combination of a radiolabeled apamin binding experiment and a functional assessment of the vestibular function to demonstrate that unilateral vestibular neurectomy (UVN) induces both ipsi- and contralateral up-regulation of the apamin-sensitive calcium-activated small conductance $K^+$ (SK) channels, within the first days following the insult. We also demonstrate that apamin administration during the acute phase of the vestibular syndrome significantly reduces both the posturo-locomotor and vestibulo-ocular deficits induced by the UVN. This is illustrated by the reduction of both the spontaneous nystagmus and the static and dynamic balance unsteadiness. These data suggest that the regulation of SK channel expression may be part of the vestibular compensation process. It is also indicated that the pharmacological modulation of SK channels may be a potential way to alleviate the vestibular syndrome.

## 1. Introduction

Balanced sensory inputs arising from the vestibular end organs located in the two inner ears are essential for achieving high fidelity signaling of any head accelerations. Central integration of these vestibular inputs with those of vision and proprioception allows the vestibular system, to permanently react to accelerations of our head by triggering appropriate motor responses to maintain our posture and balance (Asher, 1984; Luxon, 1984; Jones et al., 2009).

Sudden alteration of the sensory inputs arising from peripheral vestibular receptors evokes characteristic vestibular syndrome that is characterized by a cascade of functional disorders that includes postural imbalance at rest and during movement, spontaneous nystagmus and oscillopsia, associated with cognitive and neurovegetative disorders. These vestibular disorders result from alteration of the vestibulo spinal and vestibulo oculomotor reflexes as well as modulations along the

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https://doi.org/10.1016/j.neuropharm.2018.10.029
Received 1 April 2018; Received in revised form 17 October 2018; Accepted 21 October 2018
Available online 24 October 2018

vestibulo cerebellar and cortical pathways (Angelaki and Cullen, 2008; Jones et al., 2009; Lacour and Tighilet, 2010). Vestibular syndrome may be especially pronounced in humans, in cases of vestibular deafferentation syndrome (Halmagyi et al., 2010) and, more largely, under unilateral vestibular impairments such as labyrinthine fistula, vestibular neuritis or Ménière disease. In humans, as in animal models of vestibular disorders of peripheral origin, the vestibular syndrome is generally composed of several phases, the amplitude of which depends on the type, stage and severity of the peripheral damage (for review see Lacour et al., 2009). The “acute” phase characterizes the period in which static disorders (posturo-locomotor symptoms and spontaneous nystagmus at rest) are the most prominent. This phase generally lasts several hours but may extend to days. Subsequently, spontaneous decline of the vestibular symptom's amplitude occurs through a phenomenon referred to “vestibular compensation”. The early phase of vestibular compensation occurs within days following the vestibular insult and consists of a cascade of complex biological changes that occur in the brain stem vestibular nuclei in order to counteract the alteration of the functional homeostasis (Smith and Curthoys, 1989; Darlington and Smith, 2000; Lacour and Tighilet, 2010). Over a longer period, which may last several months, the remaining vestibular disorders progressively disappear, giving rise to a “compensated” state. Both the early and late compensation processes act to restore posture and balance, though in most cases, dynamic deficits never fully disappear.

It is assumed that the different phases of vestibular syndrome are supported by major changes in the excitability of the vestibular secondary neurons (VSNs) within the brain stem vestibular nuclei (VN). Sudden, unilateral vestibular lesions abruptly depress the spontaneous resting activity of the VSNs on the deafferented side and, conversely, increase the excitability of those located in the VN contralateral to the lesion. These opposite effects rely on the removal of the excitatory glutamatergic inputs from the vestibular primary neurons (VPNs) on the VSNs of the deafferented side. These effects also result from the runaway of the enhanced excitability of the VSNs on the side opposite to that of the insult, due to the decreased weight of the commissural inhibition exerted by the ipsilateral VSNs. It is important to note that the depression of the ipsilateral VSNs spontaneous discharge is furthermore accentuated by the increased weight of the commissural inhibition exerted by the contralateral VSNs (Ris et al., 1995, 1997). This situation results in an imbalance of the activity between opposite VNs (Xerri et al., 1983; Ris et al., 1995; Ris and Godaux, 1998; Vidal et al., 1998). Subsequently, the discharge activity of the VSNs on the deafferented side spontaneously recovers. This phenomenon was first observed after several weeks following the insult in cat models of unilateral labyrinthectomy (UL; Precht et al., 1966) or unilateral vestibular neurectomy (UVN; Xerri et al., 1983) and was later demonstrated to already be present in the first days following the peripheral damage in slices preparations of the VN in rat models of UL (Him and Dutia, 2001). Changes in the electrical properties of VSNs were characterized by a significant increase in their input resistance, spike amplitude and frequency, and they were shown to be restricted to the type B VSNs of the medial VN. Type A neurons are characterized in vitro by a deep monophasic afterhyperpolarization at rest, have a stable spontaneous activity and respond linearly when probed with dynamic stimuli such as ramps, sine waves, or white noise. Type A would correspond to the most “regular” tonic neurons identified in vivo. Type B neurons which are characterized by a biphasic after hyperpolarization, show more nonlinear responses and are more sensitive to current injection. A subset of type B neurons display slow-threshold spikes, which suggest that type B neurons constitute a heterogeneous population. Overall, the type B neurons likely correspond to irregular phasic neurons identified in vivo (For a review see Beraneck and Idoux, 2012).

We recently demonstrated in a cat model of UVN, that significant BDNF-dependent remodeling of excitability markers occurs in the brainstem VNs during an early time window after UVN (Dutheil et al., 2016). Over the first three days following the unilateral loss of the vestibular inputs, the membrane expression of the type-2 cation-chloride co-transporter (KCC2) is significantly down regulated in the VSNs of the deafferented side. Meanwhile, the expression of the type-a gamma-aminobutyric acid (GABAa) receptors increases. It is assumed that in these conditions, GABA acquires a transient depolarizing action on the deafferented VSNs, which may support the observed increase in the VSNs excitability.

The unilateral enhancement of the excitability of the VSNs on the deafferented side may by itself, promote a significant reduction of the activity imbalance between opposite VNs (that would be reinforced through the commissural inhibition of the contralateral VSNs); furthermore, it cannot be excluded that a decrease in the contralateral VSN's excitability may also occur. Such an action could occur either extrinsically, through the action of inhibitory fibers arising from the cerebellum (Kitahara et al., 1998), or intrinsically, by increasing the expression (Dutheil et al., 2016) or the efficacy (Yamanaka et al., 2000) of GABAa receptors. In a previous study, we focused on specific excitability markers: the cation-chloride co-transporter KCC2, which determines the hyperpolarizing action of GABA, and the GABAa receptors (Dutheil et al., 2016). Large conductance calcium-activated (BK) channels in the vestibular nucleus and their role in plasticity through a CAMKII-dependent mechanism has been reported (Gittis et al., 2010; van Welie and du Lac, 2011). A critical role for BK channels following unilateral vestibular lesioning has recently been confirmed using a mouse model lacking BK channels. BK channels were downregulated resulting in enhanced excitability in MVN and were shown to be required for adaptive plasticity following deafferentation (Nelson et al., 2017). In the present study, we investigated whether the expression of another specific excitability marker, the calcium-activated small conductance $K^+$ small conductance (SK) channel, is modulated in the vestibular nuclei after UVN. The presence of apamin-sensitive channel subtypes was previously reported in mammal VN (Mourre et al., 1986; de Waele et al., 1993; Johnston et al., 1994), and their selective blocking using apamin both in vitro and in vivo was shown to trigger rhythmic bursts in type B MVN neurons (de Waele et al., 1993; Johnston et al., 1994; Saito et al., 2008). Further, it is also well known that these channels exert a hyperpolarizing influence when activated or up-regulated (for review see Bond et al., 2005; Mourre et al., 2017).

With the aim to monitor the expression of the SK subunit proteins subsequent to sudden, unilateral and permanent deprivation of vestibular inputs, we performed binding experiments using radiolabeled apamin, a selective SK channel blocker, in association with functional assessment of vestibular functional recovery in the cat model of unilateral vestibular neurectomy (UVN) previously developed in our laboratory (Xerri and Lacour, 1980; Dutheil et al., 2016). We herein provide a first demonstration that the expression of SK channels is modulated both in ipsi- and contra-lateral VNs subsequent to the UVN. Selective pharmacological modulation of the SK channel apamin-sensitive subunits also significantly modulates the cognate vestibular syndrome. This result provides a potential new avenue for the development of the antivertigo medications.

## 2. Material and methods

### 2.1. Animals

Experiments were performed on 18 adult male European cats (3–5 kg) obtained from the “Centre d’élevage du Contigné” (Contigné, France). All experiments were carried out in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication n° 80–23) revised 1996 for the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the Policy on Ethics approved by the Society for Neuroscience in November 1989 and amended in November 1993. Cats were housed in our animal housing facility (Fédération 3C, Centre Saint-Charles, Aix-Marseille

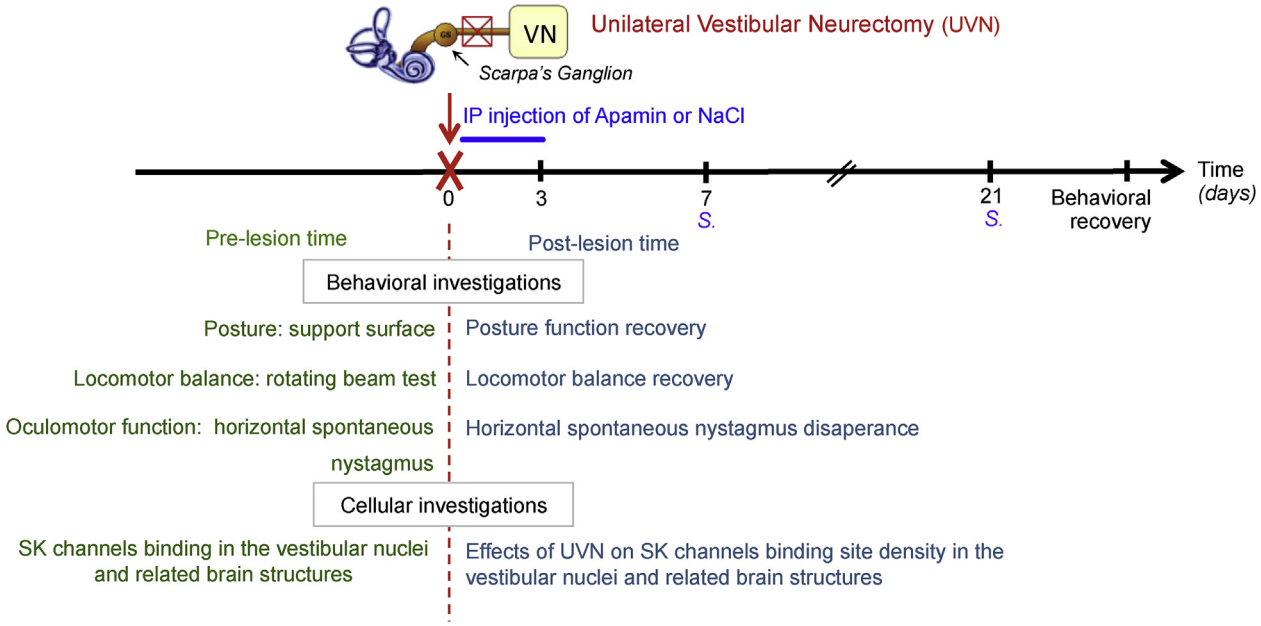


**Fig. 1.** Study design. Experimental protocol for studying the effects of UVN on the SK channels expression in the VN and related structures (n = 6 animals per group) and the consequences of apamin intraperitoneal injection on the time course of oculomotor and posturo-locomotor function recovery (n = 4 animals per group).

University) under the veterinary and National Ethical Committee supervision (French Agriculture Ministry Authorization: B13-055-25). Animals were housed in a large confined space with normal diurnal light variations and free access to toys, water and food. Every attempt was made to minimize both the number and the suffering of animals used in this experiment. Eighteen animals were used for SK channels binding study in the vestibular nuclei and related brain stem structures. A group of intact animals (n = 6) was used as control group. The remaining 12 cats were subjected to left UVN and killed at 2 survival times: 1 week (n = 6) and 3 weeks (n = 6). Survival times were selected from our previous behavioral and electrophysiological investigations in the cat, which had showed major postural deficits in acute cats (1 week) and nearly complete recovery in compensated animals (3 weeks) (see Lacour et al., 1989). To determine the effects of apamin treatment on the time-course of the cats' recovery at a behavioral level, 8 additional UVN cats were used for this study, they received during the three post UVN days, an intraperitoneal injection of NaCl (n = 4) or Apamin (n = 4) (see study design in Fig. 1).

### 2.2. Vestibular neurectomy

A left side vestibular nerve section was performed under aseptic conditions through a dissecting microscope. Animals were first anaesthetized with Ketamine (20 mg/kg, i.m.; Rhône Poulenc, Mérieux, France), received analgesic (tolfenamic acid 4 mg/kg i.m.; Vetoquinol, Lure, France), maintained under fluothane anesthesia (2%) and were kept at physiological body temperature using a blanket. The vestibular nerve was sectioned on the left side at a post-ganglion level in order to leave the auditory division intact after mastoidectomy, partial destruction of the bony labyrinth, and surgical exposure of the internal auditory canal (see Xerri and Lacour, 1980 for more details). All animals were treated post-operatively with antibiotics (ampicillin 20 mg/kg, i.m.; Panpharma, Luitré, France) for 7 days and with analgesics (tolfenamic acid 4 mg/kg i.m.; Vetoquinol, Lure, France) for 3 days. Classical postural, locomotor, and oculomotor deficits displayed by the animals in the days following nerve transection were used as criteria indicating the effectiveness of the vestibular nerve lesion. Completeness of vestibular nerve section had already been assessed by histological procedures in previous studies (Lacour et al., 1976).

### 2.3. Tissue preparation

Cats of each group were deeply anesthetized with ketamine dihydrochoride (20 mg/kg, IM, Merial, Lyon, France) and killed by decapitation; after removal from the skull, their brains were cut into several blocks containing the brainstem structures (VN and IO), and the blocks were rapidly frozen with $CO_2$ gas. Coronal sections (10-μm-thick) were cut in a cryostat (Leica, Reuil-Malmaison, France), thawed onto Superfrost + + glass slides (Fisher Scientific, Elancourt, France), and stored at −80 °C until radio autography. Experiments were carried out blind; the group that the cats belonged to was unknown to the person conducting binding.

### 2.4. Apamin binding experiment

To carry out this binding experiment using radioactivity, we had an authorization to carry out a nuclear activity granted by the Nuclear Safety Authority (asn) under the number T130663. For binding experiments, tissue sections were incubated with highly radioactive apamin labeled with [$^{125}$I] (PerkinElmer) as previously described (Mourre et al., 1986). Brain sections were incubated with 25 pM [$^{125}$I]-apamin, at 4 °C in 100 mM Tris-Cl buffer, containing 0.5% bovine serum albumin (BSA), pH 7.4. Non-specific binding was assessed by adding a large excess of native apamin (Sigma, 0.1 μM) before adding [$^{125}$I]-apamin. Sections were incubated for 60 min and rinsed three times, each for 20 s, in the same buffer. The sections were rinsed a fourth time, for 20 s, in water. Dried sections were placed on Kodak BioMax MR films. Serial sections of one naive cat were added with experimental sections, serving as internal standards for labeling on the different films. Autoradiograms were exposed for 12 days to obtain unsaturated labeling and thus to allow the detection of increases or decreases in labeling. Films were then processed in a Kodak Industrex developer. Autoradiograms were analyzed, and radioactivity quantified with NIH Image software. Plastic standards (Amersham) were used to calibrate [$^{125}$I] concentrations. Mean apamin-sensitive SK channels protein density was calculated for each unilateral nucleus, using two to three measurements in each stereotaxic level for each animal. Non-specific binding was detected on autoradiograms of sections incubated with unlabeled 0.1 μM apamin, corresponding to around 15% of total binding. Specific binding was calculated as the difference between total

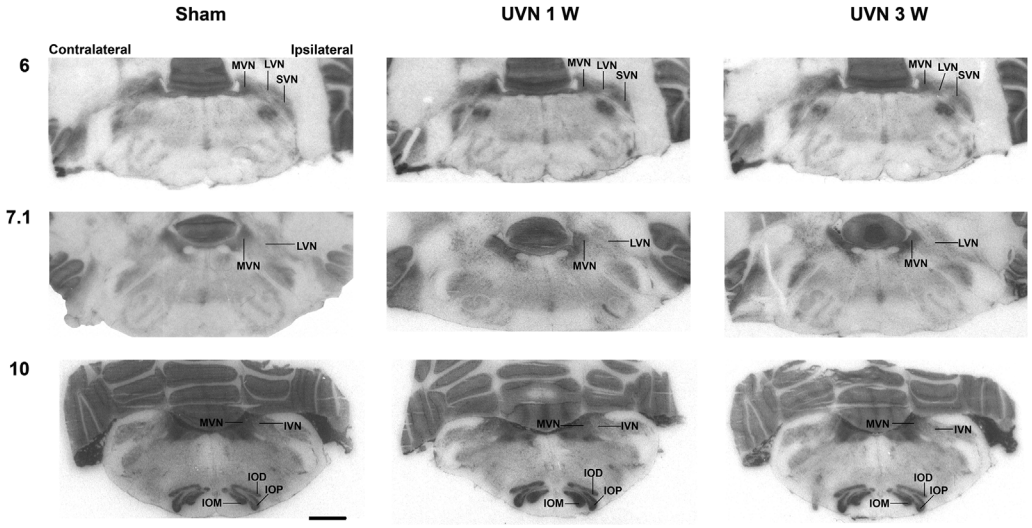


**Fig. 2.** [$^{125}$I] apamin binding sites in the cat brainstem. Coronal sections from representative sham and unilateral vestibular neurectomized cats showing increases in SK protein binding sensitive to apamin in the different structures of the brainstem on the contralateral and ipsilateral sides of lesion, 1 (1 W) or 3 (3 W) weeks after unilateral vestibular neurectomy, compared to the Sham (A). Illustrations are given for serial sections collected from the rostral (6) to the caudal (10) parts of the brainstem. IVN, inferior vestibular nucleus; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; SVN, superior vestibular nucleus; IOD, IOM, and OIP, dorsal accessory, medial accessory, and principal nucleus of the inferior olive, respectively. Bar: 1 mm.

and non-specific binding for a given area. Azur II stained sections were used for reference. Cat brainstem structures including each of the four main vestibular nuclei (medial, inferior, superior and lateral) and the three subdivisions of the inferior olive (the principal nucleus (IOP), medial accessory (IOM), and dorsal accessory (IOD) of the inferior olive) were identified and named using a cat brain atlas Berman's stereotaxic atlas (Berman, 1968).

### 2.5. Apamin administration

Apamin (0.3 mg/kg, dissolved in NaCl 0.9%, Genepep, France) was injected intraperitoneally (i.p.) 30 min before each behavioral test. Systemic administration of apamin was chosen because it was found to cross the blood–brain barrier, although weakly (Habermann and Cheng-Raude, 1975). The animals were allocated to two different subgroups (vehicle-lesioned and apamin-lesioned (0.3 mg/kg). For each subgroup, we determined the effects of these drug treatments on the recovery of posturo-locomotor and oculomotor functions through adapted behavioral tests. The behavioral evaluation of the effects of apamin administration was conducted in a blind condition.

### 2.6. Behavioral investigations

#### 2.6.1. Spontaneous nystagmus recovery

Spontaneous horizontal vestibular nystagmus induced by the UVN was recorded by video tracking of the eyes movements as described (Tighilet et al., 2006). The frequency of horizontal spontaneous nystagmus was measured in the light as the number of quick phase beats recorded in 10 s towards the contralateral side relative to the UVN (five repeated measures per animal per sampling time). Each recording session (duration = 15 min) was conducted during the same period of the day (in the morning) to counteract possible variations due to the alertness state of the animals. Full recovery was achieved when the vestibular nystagmus completely disappeared in the light.

#### 2.6.2. Posture recovery

The support surface measure serves to evaluate the postural stability of the animal. Posture deficits and recovery were evaluated by measuring the surface delimited by the four legs of the cat while standing erect at rest (without walking). The support surface is considered a good estimate of postural control since it reflects the cat's behavioral adaptation in compensating the static vestibulospinal deficits induced by the vestibular lesion (Tighilet et al., 1995). As a rule, the surface was very small in the normal cat (approximately 50–100 $cm^2$) and was largely increased in the days following the UVN. To quantify the support surface, the cats were placed in a device with a graduated transparent floor that allowed them to be photographed from underneath. Five repeated measurements were conducted for each cat tested at each postoperative time, and an average was calculated for each experimental session. The support surface was measured as the surface delimited by the four legs by an image analysis system (Canvas, 9™, Deneba software, Miami, FL). Data recorded after the vestibular lesion were compared to the pre-lesion values.

#### 2.6.3. Equilibrium functional recovery

Locomotor balance function was quantified using an adapted rotating beam experimental device (Xerri and Lacour, 1980). Two compartments (0.5 × 0.6 × 0.5 m) were connected by a horizontal beam (length: 2 m; diameter: 0.12 m). The beam was placed 1.2 m off the ground and could be rotated along its longitudinal axis, with a constant angular velocity ranging from 0° to 588.4°/s (approximately 1.5 turn/s). Behavioral training on the rotating beam consisted of depriving the animals of food overnight before the first training session. The animals were conditioned to cross the beam and were rewarded by a small piece of fish (or meat) placed in a small bowl in the target compartment. The first crossings were made on the immobile beam, and thereafter, crossings were made on the rotating beam. As a rule, the rotation velocity of the beam was progressively increased after four consecutive trials without a fall. Equilibrium function was thus quantified by measuring the highest speed of beam rotation that did not induce a fall. This maximal rotation speed determined the maximal locomotor balance performance (Max P.). Preoperative training on the rotating beam necessitated 6 to 10 periods depending on the cats. Training was stopped when the cats' Max P. was reached and was stabilized at its highest level, which was found to be remarkably similar from one cat to another in each group. The animals were then subjected to a unilateral vestibular neurectomy on the left side, after which postoperative locomotor balance was measured beginning on the second postoperative day and continuing until complete recovery. Data recorded (Max. P.) after the vestibular lesion were compared to the pre-lesion values (for details see Tighilet et al., 2006).

### 2.7. Statistical analysis

Using Graphpad Prism6 software, the effects of the lesion and of time course on apamin binding sites densities of the different groups were tested by means of a two-way ANOVA, followed by adapted post hoc tests between groups (Tukey's test), where $P < 0.05$. All the data were expressed as the mean ± standard error of the mean (S.E.M.), and a P value of $< 0.05$ was taken as the minimum level of significance.

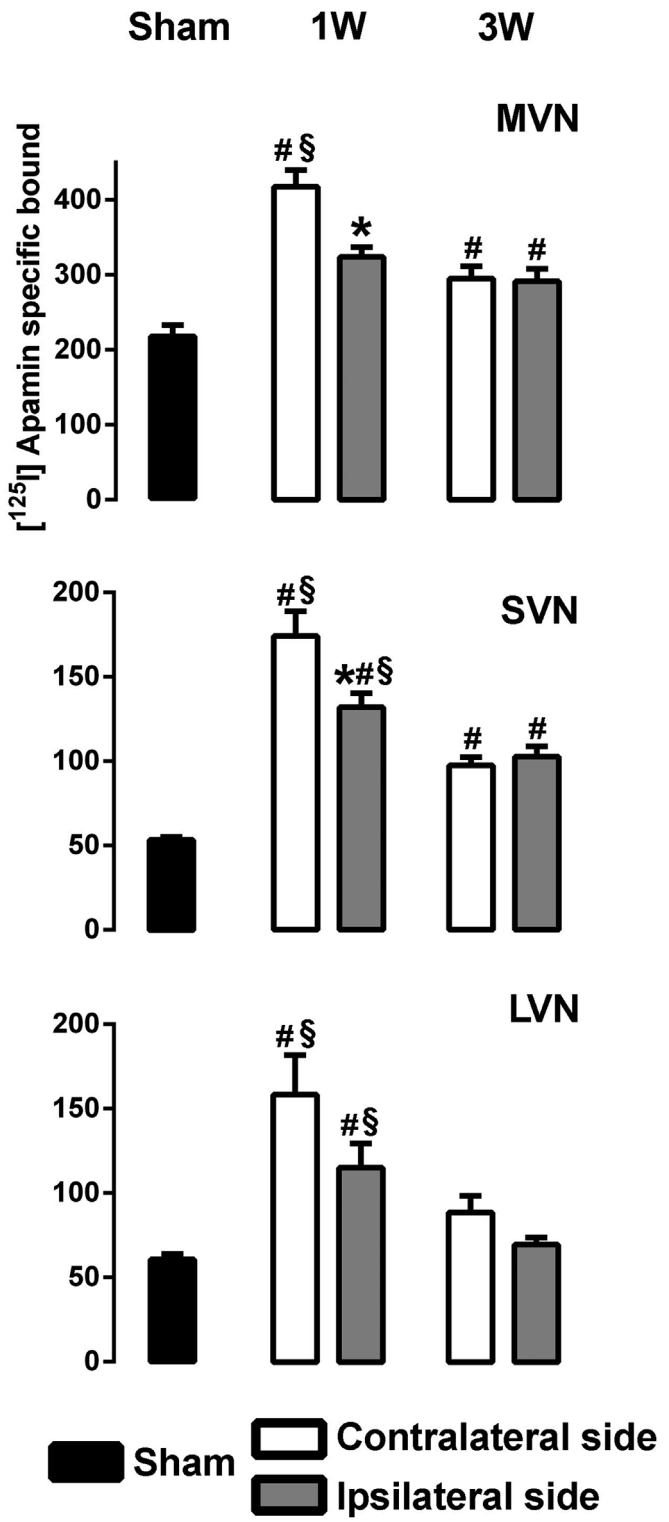


**Fig. 3.** Effects of a unilateral vestibular neurectomy on the density of [$^{125}$I] apamin binding sites in the vestibular nuclei: medial (MVN), superior (SVN), and lateral (LVN) nuclei. Variations of binding level in the sham cat group (n = 6) and in cat groups 1 (1 W) and 3 (3 W) weeks after unilateral vestibular neurectomy (n = 6 per group). Data are given as a value of binding on the ipsilateral and contralateral side of the UVN in the structures. The results are expressed as the mean values of femtomole of [$^{125}$I] apamin specifically bound per milligram of protein from autoradiograms. *, $P < 0.05$ (lesioned versus intact side in each group); #, $P < 0.05$ (1 W or 3 W group versus sham group for each intact and lesioned side respectively), and §, $P < 0.05$ (1 W group versus 3 W group for each intact and lesioned side, respectively).

Since there were four cats in each group, the effects of the lesion and the time course on behavioral performances were tested by means of the Kruskal-Wallis nonparametric test, followed by adapted post hoc tests between groups (Dunn's test), where $P < 0.05$.

## 3. Results

### 3.1. SK channel binding site density increases after unilateral vestibular neurectomy

To investigate whether UVN alters apamin-sensitive SK channels in the cat VN, we used binding experiments. Fig. 2 illustrates the spatial distribution of the apamin binding site density in representative serial frontal sections collected from the rostral (6) to the caudal (10) parts of the brainstem in a control cat (Sham) and in two representative cats sacrified one (1 W) or three (3 W) weeks after UVN. In the control cat, the pattern of apamin binding was heterogeneous: intermediate to low levels of binding sites were found in the vestibular complex while higher levels were found in the inferior olive complex. In particular, the medial and inferior vestibular nuclei contained apamin binding site densities higher than those of the superior and lateral vestibular nuclei. This distribution of SK2 and SK3 channel proteins was similar to that of the rat brainstem as previously reported (Mourre et al., 1986; Mpari et al., 2008).

### 3.2. Vestibular complex

In the medial vestibular nucleus (MVN, Fig. 3, Table 1), a two-way ANOVA revealed an interaction ($F_{2,238} = 4.20$, $P < 0.05$) between the lesion and the postlesion time, as well as an effect of postlesion time ($F_{2,238} = 31.59$, $P < 0.01$), and lesion ($F_{1,\ 238} = 5.23$, $P < 0.05$) on the apamin binding site level. One week after UVN, the apamin binding site density was significantly increased on the ipsilateral side compared to the control (Tukey's, + 52%, $P < 0.01$). This increased apamin binding site density persisted 3 weeks after UVN. In the MVN contralateral to the lesion, the increase of apamin binding sites density in the 1-week post-lesion group was significantly stronger than that of the ipsilateral side compared to both the control and the 3 weeks post-lesion group (+90% and +37% respectively, $P < 0.01$). Moreover, at the one-week post-lesion delay, the apamin binding site density was significantly higher on the contralateral side than on the ipsilateral side (+29%, $P < 0.01$). Three weeks post-lesion, the binding level increased bilaterally on both sides in comparison with the control group (Tukey's, + 40%, $P < 0.05$).

In term of the superior vestibular nucleus (SVN, Fig. 3, Table 1), a two-way ANOVA showed an interaction ($F_{2,174} = 14.46$, $P < 0.001$) and an effect of post lesion time ($F_{2,174} = 153.1$, $P < 0.001$), as well as a significant effect of the lesion ($F_{1,\ 174} = 4.95$, $P < 0.003$), on the apamin binding site level. One week after UVN, the apamin binding site density significantly increased on the ipsilateral side and even more on the contralateral lesion side compared to the control (+129 and + 249% respectively, $P < 0.001$). The binding level was significantly higher on the contralateral side than on the ipsilateral side ($P < 0.01$). Moreover, three weeks after UVN, apamin binding significantly increased on both sides compared to the control but significantly decreased compared to that observed at the one-week post-lesion ($P < 0.01$ and $P < 0.05$ respectively). No binding variation was observed at this time point between the two sides.

In the lateral vestibular nucleus (LVN, Fig. 3, Table 1), data analysis revealed an effect of lesion ($F_{1,196} = 5.64$, $P < 0.05$) and of post lesion time ($F_{2,196} = 19.23$, $P < 0.01$), but no interaction between the two factors ($F_{2,196} = 0.86$, NS) was found on the apamin binding. Tukey's test showed that one week after UVN, the binding level was significantly increased on the contralateral (+140%, $P < 0.01$) and ipsilateral (+108%, $P < 0.05$) sides of the lesion compared to the control, similar to the finding for the SVN and MVN. No significant

**Table 1**
Levels of Apamin binding sites in the vestibular complex and related nuclei.

| | | Sham | | 1 W | | 3 W | |
|---|---|---|---|---|---|---|---|
| | | Controlateral | Unilateral | Controlateral | Unilateral | Controlateral | Unilateral |
| Medial vestibular nucleus | MVN | 218.80 ± 22.24 | 213.20 ± 20.55 | 417.90 ± 22.04 | 324.10 ± 12.61 | 295.40 ± 16.05 | 291.30 ± 16.86 |
| Superior vestibular nucleus | SVN | 48.82 ± 1.52 | 58.76 ± 2.56 | 180.30 ± 10.20 | 133.10 ± 5.92 | 97.51 ± 4.75 | 102.60 ± 6.13 |
| Lateral vestibular nucleus | LVN | 55.46 ± 3.59 | 65.94 ± 4.82 | 115.40 ± 14.33 | 158.50 ± 23.62 | 69.65 ± 4.34 | 88.75 ± 9.83 |
| Inferior vestibular nucleus | VIN | 309.90 ± 21.69 | 325.10 ± 29.16 | 363.50 ± 19.28 | 351.20 ± 15.98 | 338.00 ± 24.36 | 373.00 ± 26.25 |
| Principal accessory inferior olive | IOP | 554.00 ± 33.15 | 512.80 ± 37.93 | 461.40 ± 23.38 | 437.90 ± 21.51 | 400.20 ± 44.70 | 422.50 ± 51.51 |
| Dorsal accessory inferior olive | IOD | 411.20 ± 29.82 | 434.40 ± 30.06 | 282.10 ± 10.37 | 257.40 ± 12.24 | 287.30 ± 30.71 | 323.30 ± 25.47 |
| Medial accessory inferior olive | IOM | 667.90 ± 42.92 | 700.50 ± 50.11 | 423.90 ± 21.59 | 445.90 ± 33.58 | 462.90 ± 52.33 | 478.60 ± 46.98 |
| Medial nucleus of the solitary tract | SM | 372.80 ± 36.08 | 407.90 ± 37.46 | 366.40 ± 32.09 | 365.70 ± 59.56 | 349.40 ± 54.91 | 291.80 ± 54.06 |
| Dorsal motor nucleus of the vagus | DMV | 458.60 ± 36.00 | 468.80 ± 46.38 | 527.30 ± 74.47 | 510.00 ± 83.87 | 441.30 ± 58.38 | 461.60 ± 36.53 |

Data expressed in mean ± S.E.M. in fmol/mg of protein. Contralateral and ipsilateral: sides related to UVN, 1 W and 3 W: 1 and 3 weeks after UVN.

binding variation was found between the two sides at this time point, even if the binding level on the contralateral side was higher than that on the ipsilateral side. Moreover, at three weeks post-UVN, the binding level was similar to that of the control. In contrast, in the inferior vestibular nucleus, no variation of apamin binding levels was found following UVN compared to the control whatever the post lesion time-points (Table 1).

### 3.3. Nuclei associated to the vestibular complex

In the inferior olive complex (Fig. 4, Table 1), the UVN induced a decrease in apamin binding site level in the principal nucleus (IOP), medial accessory (IOM), and dorsal accessory (IOD) of the inferior olive. In all the subdivisions, a two-way ANOVA revealed an effect of post-lesion time ($F_{2,92} \geq 5.32$, $P < 0.01$), but no significant interaction ($F_{2.92} \leq 0.74$, NS) and no effect of the lesion ($F_{1.92} \leq 0.42$, NS) was found on the apamin binding site level. Tukey's test showed that the apamin binding site density in the IOM and IOD was significantly reduced 1 and 3 weeks after UVN ($P < 0.05$), regardless of the lesion side that was studied. In the principal nucleus of the inferior olive, only a tendency of a bilateral reduction of the binding level was observed after 1 and 3 weeks post-UVN. In the medial nucleus of the solitary tract and in the dorsal motor nucleus of the vagus (Table 1), the data analysis indicated that the UVN caused no difference in the levels of apamin binding sites, regardless of the post-lesion time and the lesion sides (interaction and time, $F_{2,44} \leq 0.58$, NS; lesion $F_{1,44} \leq 1.32$, NS).

### 3.4. Functional alterations following unilateral vestibular neurectomy

It is well known that unilateral vestibular damage leads to a vestibular syndrome, which is characterized by spontaneous horizontal nystagmus, with its slow phase directed toward the lesioned side, postural imbalance, yaw and head tilt, circling and rolling toward the damaged side, and extension of the contralateral forelimb. Over time, these symptoms decrease and even disappear.

#### 3.4.1. Nystagmus

At the first post-UVN day, the frequency of spontaneous nystagmus was 15 beats/10 s in the UVN-NaCl and 11 beats/10 s in the UVN-apamin groups. The number of eye beats decreased significantly in these two experimental groups to reach control values at D5 in the UVN-apamin group and D8 in the UVN-NaCl group ($p < 0.0001$) (Fig. 5A).

#### 3.4.2. Posture function recovery

In four-footed animals standing erect, vestibular syndrome leads to an increased support surface delimited by the four paw pads. This parameter provides a good estimation of postural stability and recovery. Further, the support surface displays the tonic asymmetries of extensor and flexor muscles of the anterior and posterior paws that are induced by the vestibular deafferentation. The return to preoperative control values was faster for the UVN-apamin group (26 days) than for the UVN-NaCl group (42 days) ($p < 0.0001$) (Fig. 5B).

#### 3.4.3. Locomotor balance recovery

In line with the data regarding posture function and the nystagmus, animals of the UVN-apamin group more quickly recovered their dynamic locomotor balance and crossed the rotating beam at their maximal performance (Max P.) on the 26th day after deafferentation. The cats of the UVN-NaCl group reached their Max P. 42 days after deafferentation ($P < 0.0001$; Fig. 5C).

## 4. Discussion

### 4.1. Up-regulation of the apamin-sensitive SK channel subunits in the vestibular nuclei after unilateral vestibular neurectomy

This is the first demonstration of a direct link between alterations of the sensory information arising from peripheral vestibular sensors and changes in the expression of calcium-activated $K^+$ small conductance (SK) channels in the brain stem vestibular nuclei (VN). The methodological approach we used in the present study indicates that these changes are exerted directly at the protein level. The binding method allows for the presence of a protein in its functional configuration to be followed after it is addressed to the cell membrane. In the present case, we selectively followed the membrane expression of the SK channel subunits that were capable of binding the apamin ligand. The SK channels consist of an assembly of three subunits (SK1-SK3), and only subunits SK2 and SK3 bind apamin (Bond et al., 2005). These are the two subunits whose membrane expression was selectively monitored in our study.

In the present case, the increased expression of the apamin-sensitive SK channels was observed following a situation of total and irreversible unilateral loss of the vestibular inputs. UVN, which was performed for many years in our laboratory (Xerri and Lacour, 1980), consists in sectioning the proximal part of the 8th cranial nerve (between the Scarpa ganglion and the entry of the nerve fibers into the brainstem), which results in a complete and definitive deafferentation of the VSNs on the lesioned side. In contrast, unilateral labyrinthectomy (UL), while eliminating the peripheral vestibular end organs, preserves Scarpa's ganglion neurons, as well as their connection with the brain stem VSNs. Under UVN conditions, characteristic vestibular syndrome is systematically observed, which consists of static (postural alterations at rest and spontaneous nystagmus) and dynamic (impairment of locomotion) deficits (Tighilet et al., 2006). Quantitative analysis of various components of vestibular syndrome was carried out using behavioral testing previously described (Dutheil et al., 2013, 2016; Tighilet et al., 2015).

The membrane expression of apamin-sensitive SK channels subunits is differently modulated according to the brainstem region and stage considered. One week after UVN, a significant increase in the

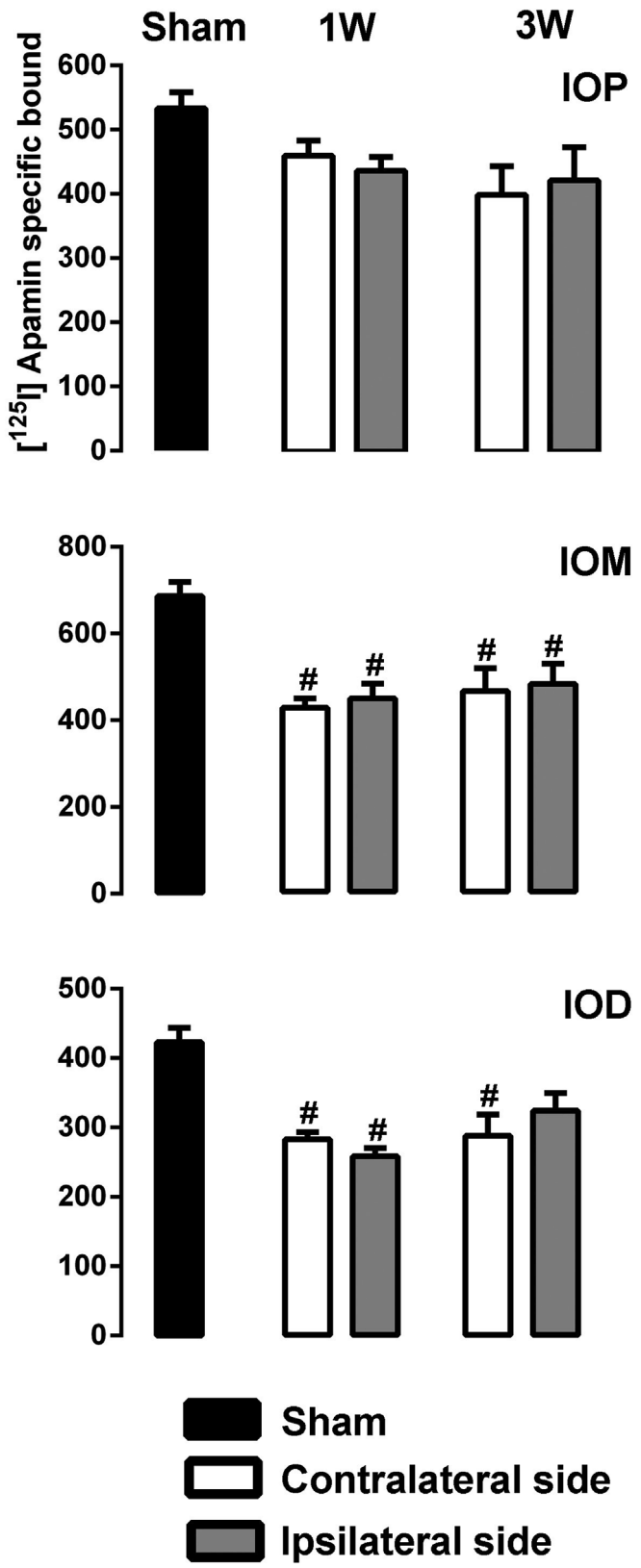


**Fig. 4.** Effects of a unilateral vestibular neurectomy on the density of [$^{125}$I] apamin binding sites in the three parts of the inferior olive: IOD, IOM, and IOP; dorsal accessory, medial accessory, and principal nucleus of the inferior olive, respectively. Variations of binding level in the sham cat group (n = 6) and in cat groups 1 (1 W) and 3 (3 W) weeks after unilateral vestibular neurectomy (n = 6 per group). Data are given as a value of binding on the ipsilateral and contralateral side of the UVN in the structures. The results are expressed as the mean values of femtomole of [$^{125}$I] apamin specifically bound per milligram of protein from autoradiograms. #, P < 0.05 (1 W or 3 W group versus sham group for each intact and lesioned side, respectively).

expression of apamin-sensitive SK was observed bilaterally in the three VNs studied: medial NV (MVN), superior NV (SVN) and lateral NV (LVN). At this stage, up regulation of SK expression was always higher on the opposite side of the lesion with respect to the injured side. In contrast, three weeks after the unilateral loss of vestibular inputs, the up regulation of SK channels expression only persisted in the MVN and SVN, albeit to a lesser extent and with no difference between the opposite VNs. Conversely, in the inferior olive complex, SK channel expression was significantly reduced at 1 and 3 weeks after the UVN, in the medial accessory and dorsal accessory (IOM and IOD) but not in the principal nucleus of the inferior olive. No difference in expression between the intact side and the injured side was observed at this level. A previous study reported that there was no evidence of any change in SK channel gene expression in the VNs following surgical unilateral labyrinthectomy in the rat (Patkó et al., 2003). This result contradicts the present observations and may be explained by either a lack of specificity of the method used or by the fact that the peripheral insult could selectively impact the post transcriptional processes, such as the translational process or the membrane addressing of the SK subunit proteins. Another likely hypothesis based on the results of Patko's study is that SK channels modulation is dependent on the nerve section. Indeed, these authors observed that a section of the facial nerve caused a strong modulation of the SK channels mRNAs in the deafferented facial motoneurons, while no change was observed in the vestibular nuclei following surgical unilateral labyrinthectomy. Preservation of Scarpa's ganglion in the surgical labyrinthectomy procedure may prevent Wallerian degeneration that may, in turn, trigger the SK channels up regulation process, as has been observed with other changes previously described in VNs after UVN (Dutheil et al., 2016).

### 4.2. Expected functional relevance of the SK channel up regulation in VNs

SK channels are members of the voltage insensitive calcium-activated potassium channel family. Upon elevation of the cytosolic calcium concentration, the channels open, allowing $K^+$ ions to leave the cell as a function of the $K^+$ equilibrium potential. Consequently, their activation leads to the cell hyperpolarization (for a review see Bond et al., 2005). More specifically, SK channels are thought to regulate neuronal excitability by contributing to the slow component of synaptic after hyperpolarization (AHP) (Köhler et al., 1996; Pedarzani et al., 2001). Activation or up regulation of SK channels is expected to limit the firing frequency of repetitive action potentials. In regard to these specific gating properties, one can elaborate on the expected functional consequences of the up regulation of SK channels in the VNs depending on the cell type (excitatory or inhibitory neurons) and the side considered (intact versus deafferented).

The expression of apamin-sensitive SK channels has been previously reported in mammal VSNs (Mourre et al., 1986; de Waele et al., 1993; Johnston et al., 1994). Given the diversity of the neuronal populations and the complexity of the neural network present in the vestibular nuclei, one can consider two different scenarios. Let first consider a simplistic representation of the vestibular commissure pathway (Fig. 6) involving on one hand (Fig. 6A), type I excitatory neurons on one side, acting on type II inhibitory neurons on the opposite side (Kasahara et al., 1968; Mano et al., 1968; Precht et al., 1973), and on the other hand, type I inhibitory neurons acting on type I excitatory neurons on the opposite side (Shimazu and Precht, 1966; Mano et al., 1968; Kasahara and Uchino, 1971). The first scenario considers the increase of the SK expression in the neurons located on the deafferented side

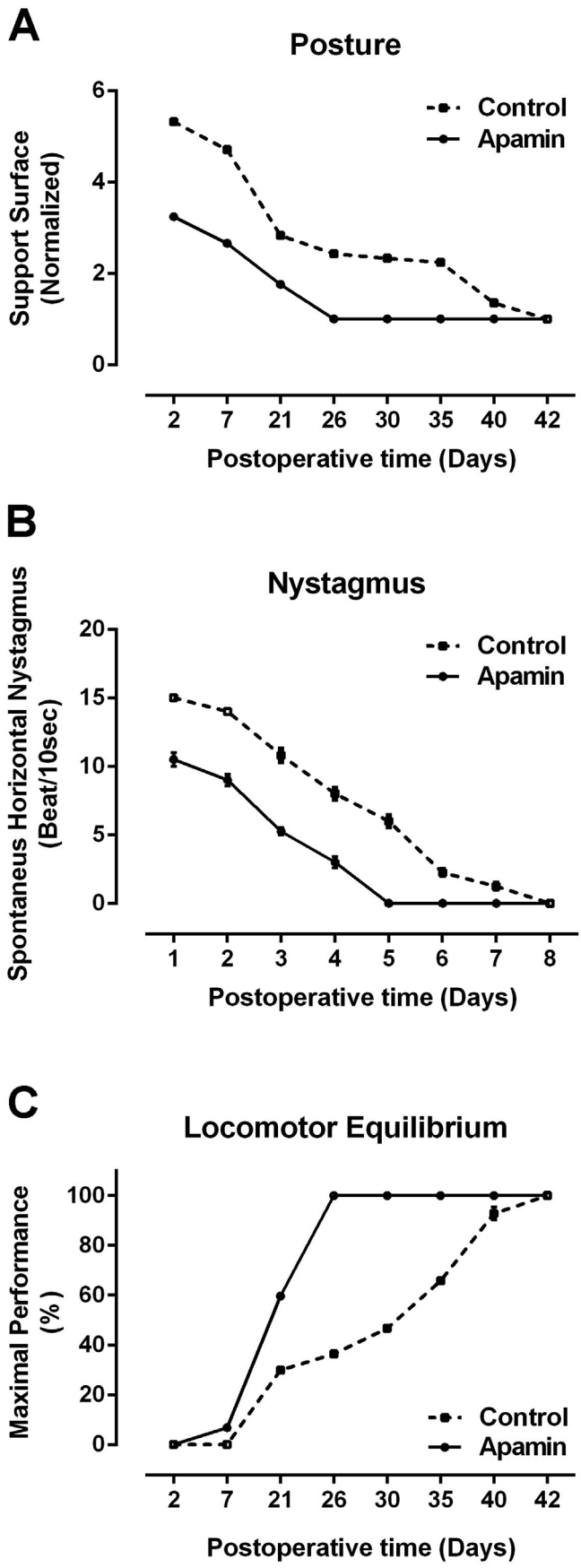


**Fig. 5.** Behavioral recovery time-course can be accelerated according to the apamin treatment after unilateral vestibular neurectomy. ***A***, Curves indicating the mean postoperative recovery of the support surface in the two experimental groups of cats (UVN−NaCl and UVN-apamin**)**. The support surface evaluated in $cm^2$ and normalized with respect to the preoperative values referred to unity (1 being close to 50 $cm^2$) is reported on the ordinate as a function of the postoperative time in days on the abscissae. Each point represents the mean value (n = 5 measurements per cat) calculated in the UVN-apamin group (N = 4) and the UVN-NaCl group (N = 4). Standard errors of the mean are reported as vertical lines. Note the strong increase in support surface in the days following unilateral vestibular neurectomy and the significant time of recovery for this static balance parameter in the UVN-apamin group compared with the UVN-NaCl group: 26 days vs 42 days. ***B***, Curves illustrating the time-course (abscissae) of disappearance of horizontal spontaneous nystagmus (HSN) frequency (ordinates) for each group of vestibular deafferented cats at different postoperative days. Each data point represents the mean number of HSN quick phase movements in 10 s for 4 animals (five repeated measures per animal per sampling). ***C***, The maximal performance (Max P.) is defined as the highest beam rotation speed that did not lead to a fall on four consecutive crossings. The curves are expressed in percent of the preoperative maximal performance (ordinates) as a function of the postoperative time in days (abscissae). Each point represents the mean value calculated in the UVN-apamin group (N = 4) and the UVN-NaCl group (N = 4). Note the significant time of recovery for this dynamic balance parameter in the UVN-apamin group compared with the UVN-NaCl group: 26 days vs 42 days.

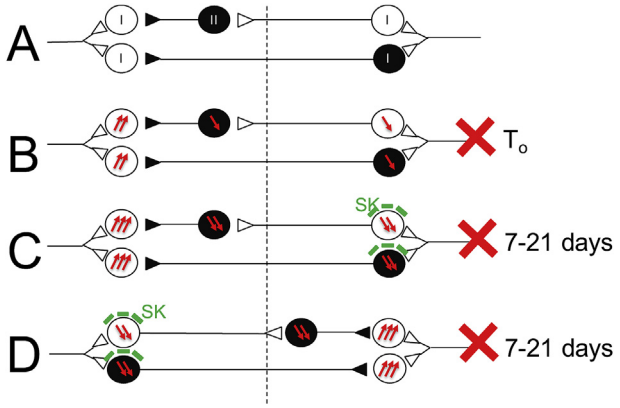


**Fig. 6.** Schematic representation of the vestibular commissural pathway in VN, with putative consequences on the excitability of vestibular neurons of the UVN and SK channel up-regulation. A. Simplistic representation of the vestibular commissural pathway involving Type I (empty circles) and Type II neurons (filled circles) in homologous VN in control condition. B. Immediate consequences of UVN on the excitability of the VN neurons. Arrows represents neural activity. Cross illustrates the loss of peripheral vestibular inputs upon UVN. Green squares represent SK channels expression level at the membrane of the VN neurons. C. Functional consequences of SK channels up-regulation in deafferented VN (scenario 1, Discussion) observed between 7 and 21 days following UVN. D. Functional consequences of SK channels up-regulation in intact VN (scenario 2, Discussion) observed between 7 and 21 days following UVN. Dashed line represents the midline of the brainstem.

(Fig. 6C). The SK up-regulation in the type I excitatory or inhibitory neurons will further increase the disinhibition already started at the onset of the UVN (Fig. 6B) in the type I excitatory neurons on the intact side. This will maintain and strengthen the electrical imbalance between intact (high activity) and deafferented (low activity) side. Such a situation would maintain and even increase the vestibular syndrome. In the second scenario, increased SK expression in the same neuronal populations on the intact side will lead to mirror effects: reduced excitability in the intact VN and subsequent increased excitability in deafferented VN (Fig. 6D). This process would reduce the imbalance between opposite VN contributing to functional recovery. Regarding the recovery rate in both posturo-locomotor and vestibulo-ocular functions observed at 7 and 21 days after UVN, the second scenario

should be privileged. Regarding the projection neurons, it is known that type-B VSNs neurons (in which SK channels are expressed; de Waele et al., 1993; Johnston et al., 1994; Saito et al., 2008) represent the majority of the descending fibers constituting the vestibulo-spinal pathway. From a functional point of view, the higher expression of SK channels in the intact vs deafferented VN observed at 1-week post UVN could support the initial compensatory stage in the UVN animals (Tighilet et al., 2015; Dutheil et al., 2016). The symmetrization of the SK channel expression in the VN on both the intact and deafferented sides observed at 3-weeks post UVN may be responsible for later postural adjustments.

Our group has previously published a number of papers showing changes in expression of histamine receptors, membrane transporters (KCC2), GABA receptors and BDNF in the UVN model (Tighilet et al., 2006, 2007; Dutheil et al., 2013, 2016). All these reactive phenomena could restore the excitability level in the deafferented VN, which is crucial for functional recovery. One can ask whether the SK channels upregulation observed in present study is solely a phenomenon concomitant to pre-cited molecular changes, or whether a link exists between these different reactive changes? It can be assumed that the SK channels upregulation is another strategy at the service of the excitability homeostasis. The post-UVN increased calcium concentration within activated VN neurons could be a common effector of these different reactive processes. Other more direct common denominators may also link the increase in excitability, neurotrophins and histamine expression, neuroinflammation and SK expression. However further investigations are needed to explore this possibility.

### 4.3. Functional consequences of the apamin administration

Administration of apamin during the acute phase of the UVN-induced vestibular syndrome was initially designed to decipher the functional consequences of the SK channel blockade. Antagonization of the apamin-sensitive SK channels significantly alters the time course of the vestibular syndrome induced by the UVN. Reduction of the oculomotor and posturo-locomotor deficits are noticed 24 h after the first apamin administration, while the effect persists well beyond the period of drug application. Indeed, a significant reduction of the horizontal spontaneous nystagmus was noticed from the first administration of apamin and this effect significantly reduced the period of expression of the spontaneous nystagmus. One can thus distinguish an immediate effect of the presence of apamin on the static (posture surface and horizontal nystagmus parameters) and dynamic (locomotor balance function) vestibular deficits, from a persistent effect. The immediate modulatory effect of apamin could combine a stimulatory action on the excitatory type I VSNs on the injured side, whose excitability is greatly reduced after UVN, with a simultaneous action on type I inhibitory VSNs of the opposite side, whose excitability is already strongly stimulated by the removal of the inhibitory control of the ipsilateral VN. The antivertigo action of apamin could result from a rebalancing of the spontaneous activity between opposite VNs. This hypothesis is interesting because, in contrast to a vestibulo-depressant action, that aims at reducing the imbalance between opposite VNs by simultaneous inhibitory actions (Chabbert, 2016), the excitatory action of apamin would reach a similar result by exacerbating neuronal hyperexcitability. Acceleration of the vestibular compensation under apamin administration, which is already observed in the acute phase of the syndrome and extends to its late phase could be an illustration of such a phenomenon. Beyond apamin, other molecules which block SK channels may also be efficient in alleviating the vestibular syndrome. Given the diversity and complexity of the neuronal populations present in the VN (see above), it cannot be excluded that agonization of SK channels may also promote these benefits through rebalancing the spontaneous activity between opposite VN. Considering the action of apamin on the VNs, it cannot be ruled out that the observed behavioral effects may result from an action on neural structures belonging to the vestibular network but located outside of the VNs. For example, an apamin-sensitive current has been demonstrated in the rat VPNs (Limón et al., 2005). However, the low contribution of this current to the electrical discharge of the cultured VPNs seems to exclude an efficient modulating action at this level. Finally, an extravestibular action of apamin is also conceivable as the expression of SK channels is ubiquitous. However, the modulatory effect on the spontaneous nystagmus we describe here circumscribes this action to a modulation targeted to the vestibular pathway.

## 5. Conclusion

This work demonstrates, for the first time, that sudden and unilateral loss of peripheral vestibular inputs alters the expression of apamin-sensitive SK-type channels in the brainstem vestibular nuclei. This process may participate in the acute vestibular syndrome as well as the compensatory mechanisms. The administration of apamin produces significant antivertigo effect, the mechanisms of which remain to be elucidated.

## Conflicts of interest

The authors declare no competing financial interests.

## Acknowledgments

This research was supported by grants from the "Ministère de l'Enseignement Supérieur et de la Recherche" and "CNRS" (UMR 7260 Aix-Marseille Université). The authors thank V Gilbert and E Mansour for taking care of the animals and and A El Ahmadi for expertise in statistical analysis.

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