Correlation between afferent rearrangements and behavioral deficits after local excitotoxic insult in the mammalian vestibule: an animal model of vertigo symptoms?

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SUMMARY STATEMENT

Early sequence of afferent injury and repair in vestibular sensory epithelium that correlates with balance disorders and functional restoration is detailed in a rodent model of excitotoxicity.
ABSTRACT

Damage to inner ear afferent terminals is believed to result in many auditory and vestibular dysfunctions. The sequence of afferent injuries and repair, as well as their correlation with vertigo symptoms remains poorly documented. In particular, information on the changes that take place at the primary vestibular endings during the first hours following a selective insult is lacking. In the present study we combined histological analysis with behavioral assessments of vestibular function in a rat model of unilateral vestibular excitotoxic insult. Excitotoxicity resulted in an immediate but transient alteration of the balance function that was resolved within a week. Concomitantly, vestibular primary afferents underwent a sequence of structural changes followed by spontaneous repair. Within the first two hours after the insult, a first phase of pronounced vestibular dysfunction coincided with extensive swelling of afferent terminals. In the next 24 hours, a second phase of significant but incomplete reduction of the vestibular dysfunction was accompanied by a resorption of swollen terminals and fiber retraction. Eventually, within one week, a third phase of complete balance restoration occurred. The slow and progressive withdrawal of the balance dysfunction correlated with full reconstitution of nerve terminals. Competitive re-innervation by afferent and efferent terminals that mimicked developmental synaptogenesis resulted in full re-afferentation of the sensory epithelia. By deciphering the sequence of structural alterations that occur in the vestibule during selective excitotoxic impairment, this study offers new understandings on how a vestibular insult develops in the vestibule and how it governs the heterogeneity of vertigo symptoms.
INTRODUCTION

Vestibular disorder symptoms include vertigo, dizziness, postural imbalance and lack of gaze fixation during head movements. Although they may have a central origin, it is commonly believed that they predominantly result from transient or permanent impairments of vestibular function in the inner ear (Brandt et al. 2009; Chabbert 2013; Soto and Vega 2010). The static deficits commonly encountered following a unilateral vestibular insult, consisting in ocular nystagmus and postural instability are generally attributed to a functional imbalance between bilateral vestibular nuclei (Curthoys and Halmagyi 1995; Dieringer 1995). However, the mechanisms that support the vestibular dysfunction or lack of function have never been fully elucidated so far. This lack of knowledge impairs the development of pharmacological approaches aiming to efficiently protect the vestibule under pathological conditions.

Different aetiologies, including alterations of endolymph properties, sensory cells insult or death and uncoupling of vestibular afferents resulting from diverse pathogenic situations have been proposed to explain sudden vestibular dysfunctions. Among these pathogenic conditions including ototoxicity, infections, ischemia, trauma, inflammation or aging, the impact of the excitotoxicity remains unclear. Often associated with ischemic conditions, its clinical confirmation generally relies on stroke or thrombosis pictures in MRI (Iadecola and Anrather 2011). However, excitotoxic damage can be far subtler, remaining imperceptible at the MRI observation. Excitotoxicity is primarily a pathophysiological phenomenon that affects specifically glutamatergic synapses (Olney 1969). This process results from the excessive release of glutamate in the vicinity of presynaptic elements and its spillover within the synaptic cleft, and from the sustained activation of glutamate receptors located at postsynaptic terminals. Under these conditions, the prolonged afflux of cations in the...
postsynaptic terminal is accompanied by an influx of water that in turn induces swelling of dendrites and may lead to complete synaptic uncoupling.

The sensory structures of the inner ear including hair cells and primary neurons are excitable cells. As well as neurons from the central nervous system, they are particularly vulnerable to excitotoxicity. When exposed to ischemic/reperfusion conditions (Puel et al. 1995; Tabuchi et al. 2002), or acoustic trauma (Kujawa and Liberman 2009; Puel et al. 1998) the cochlea exhibits characteristic patterns of excitotoxic damages. Synaptic damage and its associated functional impairments are significantly reduced in presence of selective blockers of the glutamate receptors (Puel et al. 1994; Puel et al. 1998). Based on these pre-clinical proofs of concept, clinical studies using similar types of protective approach for auditory synapses have demonstrated significant reduction of the tinnitus associated with acoustic trauma (van de Heyning et al. 2014). At the vestibular level, partial or complete degeneration of vestibular endorgans was demonstrated in human, in conditions of vertebrobasilar arteries occlusion (Kitamura and Berreby 1983; Oas and Baloh 1992). This damage was implicated in the acute vestibular disorders experienced by the patients. Excitotoxic insult of vestibular primary afferent was proposed as a possible cause of vestibular neuritis (Dyhrfjeld-Johnsen et al. 2013). More recently, the postulate of repeated excitotoxic insults in the vestibular sensory epithelia, as the result of blood perfusion alteration in the inner ear was also proposed to explain the iterative and progressive symptomology of the Menière’s syndrome (Foster and Breeze 2013).

The recent development of animal and *in vitro* models of excitotoxically-induced vestibular insults provided the opportunity to confirm the central role of glutamate in the excitotoxic cascade, while identifying the cellular effectors involved (Brugeaud et al. 2007; Liu 1999; Shimogori and Yamashita 2004). Interestingly, some studies
reported the capacity of the neuronal vestibular network to spontaneously repair following selective destruction of the vestibular primary synapses (Travo et al. 2012) and to support functional restoration (Brugaud et al. 2007). Together, these studies have highlighted the cellular events that occur in the first days after the induction of the vestibular insult and that support excitotoxically-induced vestibular dysfunctions. What is primarily lacking today is to acquire information on what happens in the first hours after the onset of the insult. This information is essential for the development of early protection strategies to counteract the excitotoxically-induced vestibular insult.

The present study was designed to directly address this question. Using an excitotoxic paradigm to mimic vestibular pathological condition, we detailed the time course of induced balance disorders using behavior testing, together with cellular changes that occur at the contact between vestibular hair cells and their nerve afferent terminals using immunohistochemistry, light, electron and confocal microscopy. We report a direct correlation between the peak crisis of vestibular disorder symptoms and severe damage to afferent terminals in the very early stage of the insult. This study also confirms the remarkable plastic ability of the vestibular afferent terminals to engage a repair process following excitotoxic injury.
RESULTS

Altered vestibular behavior following transtympanic injection of kainic acid

Single and unilateral (right ear) transtympanic injection of kainic acid (STTK, 12.5 mM, 100 µl) rapidly elicited stereotypical vestibular dysfunction that was characterized by circling behavior, walking backward and head bobbing when exploring. The rat also displayed body torsion during tail hanging and failed to rectify full supine position in the air righting or contact inhibition reflex tests. Vestibular dysfunction scores were collected according to the method previously described (Brugeaud et al. 2007; Desmadryl et al. 2012; Llorens et al. 1993; Llorens and Rodriguez-Farre 1997). The time course of the onset and progress of the vestibular dysfunction was studied at different time points from 1 hour to 1 week following the STTK (Fig. 1).

Following the STTK, pronounced altered vestibular behavior was evident as soon as the rats awakened from anesthesia. Altered vestibular behavior developed in three distinct phases. An initial phase was identifiable within the 2 first hours following the insult induction (Fig.1 insert). One hour following the insult induction, the mean vestibular dysfunction score already exceeded 7 (7.34 ± 0.77, n = 29) and peaked at 9.90 ± 0.62 (n = 20) 1.5 h after the kainate administration. The mean peak score statistically differed from the scores at 1 h (p = 0.018) after the induction. A second phase with quite stable though highly pronounced vestibular dysfunction lasted between 2 and 7 h (8.08 ± 0.84, n = 23, and 6.15 ± 0.58, n = 26, respectively) after the initial insult. Subsequently, a third phase of progressive recovery occurred with slowly reduced vestibular dysfunction. At 96 h post-induction, this phase ended with a complete withdrawal of vestibular dysfunction. At this time, mean score values of
1.14 ± 0.55 (n = 7) did not statistically differ from those obtained in normal pre-lesioned animals (p = 0.125).

In summary, following unilateral excitotoxic insult, three different phases of altered behavior were observed: 1) a fast and acute crisis within 2 hours following the insult, 2) a stable phase of pronounced vestibular dysfunction lasting 7 hours after the induction and, 3) a slower recovery period that lasted 4 days before normal functional behavior was restored.

Evidence of excitotoxic damage at vestibular afferent terminals in the first hours following transtympanic injection of kainic acid

To verify whether the observed kainic acid-induced vestibular dysfunctions stem from direct damage to vestibular endorgans, the tissue was processed for electron microscopy. The qualitative aspect of the lesion and/or repair was assessed in the utricle on both semi- and ultra-thin sections to investigate the tissue condition both at cellular and subcellular levels. Figures 2 to 4 illustrate, respectively, the early damage observed at the subcellular level 2h after the STTK, the patterns of change in tissue conditions from 2 h to 24 h after the STTK and the quantification on these morphological changes from 1 h to 1 week after the STTK. This morphometric analysis was performed on ultra-thin sections. Tissue damage observed in the injured ear was always compared to the intact contralateral ear that did not receive transtympanic kainate injection. In controlateral tissue, the typical morphology of sensory epithelia containing type I and type II hair cells and supporting cells was observed (Fig. 2A-B; Fig. 3A).
Using electron microscopy, we observed the vestibular sensory epithelia at the cellular and subcellular level (Fig. 2). Two hours after the STTK, typical features of glutamate-elicited excitotoxicity were observed in the injured epithelia (Fig. 2C-F). Some hair cells with their typical afferent terminals were still present: type I hair cells exhibited a pear shape connected by surrounding calyx terminals, while type II hair cells displayed a more cylindrical shape and were innervated by classical bouton terminal; however most of the afferent terminals were highly swollen, leaving large vacuoles all along the sensory epithelia (Fig. 2C-D). Membrane disruptions of damaged afferent terminals were often observed (Fig. 2E-F). Vacuoles resulting from the swelling of afferent terminals were so extensive that hair cells were distorted and generally no longer identifiable as type I or type II hair cells with regard to their morphology (Fig. 2D-F). Supporting cells were also squeezed while their nuclei remained in the right position (Fig. 2D-E). No obvious morphological change of efferent terminals was observed at this time (data not shown). Changes that may occur presynaptically were not investigated in the present study.

At the tissue level, the large swellings and induced-distortion of the sensory and supporting cells within the epithelium were observed on semi-thin sections (Figure 3). The quantification of hair cells based on their morphology clearly demonstrated an increase in the number of hair cells for which the specific type I or type II classification was no more identified because of these morphological changes 2 h after the STTK.

On the basis of histological analysis of vestibular sensory epithelia 2 h after the STTK, it can be concluded that the acute peak of altered vestibular behavioral crisis coincided with stereotypical features of excitotoxic damage to afferent terminals within the vestibular epithelia.
Resorption of swollen afferent terminals in the first 24 hours following excitotoxic damage

The utricle, a sensory graviceptor, was observed at the tissue level and quantified at the cellular level on semi-thin sections covering the entire period from 1 h to 1 week after the STTK. At the tissue level, the epithelial damage and its progressive resorption were obvious from 2 hrs to 24 hrs after the STTK (Fig. 3). The time course analysis of the vestibular damage in the utricle at several time points between 2 and 24 h following the STTK demonstrated a progressive reduction in the size and number of the swollen afferent terminals (Fig. 3B-F). During that period, despite extensive swelling inside the sensory epithelia, afferent terminals were seen beneath the epithelia in the conjunctive tissue. At the level of semi-thin section observations, the kainic acid-induced damage had almost completely vanished 24 h after the STTK (Fig. 3F). The general organization of the sensory epithelia appeared normal when compared to the contralateral, non-injured epithelia (Fig. 3A). Histological analysis of crista ampullaris gross morphology following STTK provided results similar to those obtained in utricles (Fig. S1). Altogether, histological observations over the first 24h following the STTK clearly demonstrated a spontaneous resorption of the excitotoxically-induced afferent terminal swellings in the mammal vestibular sensory epithelia.

In order to specify the time-course of the swelling resorption, we performed a morphometric analysis of utricle epithelia over the 24 h period following the excitotoxic insult induction. The different cell types were identified and quantified using defined morphological criteria to discriminate between supporting cells and hair cells: type I, type II or undetermined type. These criteria were based on the localization of nuclei within the sensory epithelium and the hair cell shape. Results
are shown in figure 4. Over time, no significant loss of supporting or hair cells was quantified in the damaged ear when compared to the contralateral intact ear (Fig. 4A, data only shown for 2 h and 24 h post STTK). The number of identified type II hair cells in the injured ear did not change between 2 h and 24 h after STTK, but, it was significantly reduced in comparison with the quantification in the contralateral ear. Conversely, the number of identified type I hair cells was significantly reduced in injured utricles 2 h after STTK, although it did not significantly differ from intact tissue 24 h after the injury. The number of hair cells with undetermined type increased 2 h after STTK and was still significantly different from the contralateral ear 24 h after the injury, consistent with the significant reduction of identifiable type I or II hair cells. Figure 4B details the time course of the changes in the number of undetermined, type I and type II hair cells between 1 and 24 h after the STTK. Quantification at 168 h is also provided for comparison. As early as 1 h after the STTK, some hair cells were no longer identifiable as type I/II hair cells. At this time, the number of type II hair cells had already significantly decreased to its minimum and the number of type I hair cells started to decline while the number of undetermined hair cell type conversely increased. From 2 to 12 h, a maximal and steady quantity of undetermined type of hair cell was reached and paralleled a minimal number of identifiable type I hair cells. Twelve hours (12 h) after the STTK, the number of identified type I hair cells drastically and statistically increased. Conversely, the number of hair cells with undetermined type started to decline. After 24 h post-STTK, exclusively the identified type I hair cells were quantitatively similar to counts in the contralateral non injured ear. Both the numbers of type II and hair cells with undetermined type remained statistically different. One week (168 h) after the STTK, all numbers of identified type I, type II and undetermined type of hair cells were
similar to the contralateral ear. Taken together, these results demonstrated that spontaneous resorption of swelled afferent terminals occurred in the injured vestibular epithelia after kainic acid-induced excitotoxic damage following a specific time course. The resorption process resulted in a general restoration of sensory and supporting hair cell organization. However, to determine the degree of damage and repair of terminal afferents at the sub-cellular level, a more precise analysis by electron microscopy was required.

**Repair of afferent terminals throughout the first week following excitotoxic insult**

As previously stated, the overall organization of the sensory epithelium appeared to be properly restored within the first 24 hours following the kainate injury when observed using light microscopy on semi-thin section preparations (see Fig. 3). However, this level of observation did not provide enough detail to be able to assess the precise stage of the repair at the subcellular level, particularly when substantial vestibular dysfunction persisted (Fig. 1). To address this point, we used electron microscopy to examine the contacts between hair cells and their associated afferent terminals in rat utricles 24 h after STTK (Fig. 5). Compared to non-damaged tissue, most type I hair cells were easily identified according to their pear shaped morphology, constricted neck and the basal localization of their nuclei (Fig. 5A). Type II hair cells were often contacted by bouton afferent terminals normally facing ribbons and by efferent terminals filled with synaptic vesicles facing synaptic cisterns (Fig. 5B). No further swelling was observed. However, close examination of type I hair cells and their associated calyx terminals revealed unusual features of altered
contacts (Fig. 5C-G): some terminals were simply absent, while other terminals
displayed features of immature terminals (Desmadryl and Sans 1990). Some type I
hair cells were surrounded by fragmented calyx terminals (Fig. 5C). Single (Fig. 5E)
or multiple (Fig. 5F) ribbons were found to face remnant membranes of calyx
terminals with no organelle left. The most striking feature was the presence of
efferent terminals, full of vesicles with small mitochondria that directly contacted the
type I hair cells (Fig. 5 D, 5 G). In some cases, efferent terminals appeared to
compete with afferent terminals in front of presynaptic ribbons (Fig. 5G). It is
noteworthy that a similar innervation pattern is usually observed during development
(Desmadryl et al. 1992). Quantification of type I hair cells (n=117) and their calyx
terminal afferents revealed that only 26.33 ± 0.85 % of type I hair cells were
contacted by fully formed calyces 24 h after the excitotoxic injury. The others were
contacted by either a partial calyx terminals (32.60 ± 7.44 %) or not contacted at all
(41.07 ± 6.63 %).

One week after the kainic acid induction of excitotoxic damage, vestibular tissue was
analyzed to assess the degree of completion of the repair process. On semi-thin
sections (Fig. 6A), the gross morphology of sensory epithelia did not significantly
differ from contralateral non-injured ear tissue (for cell quantification, see Fig. 4B). At
the ultra-structural level, the detailed morphology also appeared normal. Type I and
type II hair cells were clearly identifiable (Fig. 6B-C) with proper calyx (Fig. 6D) or
bouton (Fig. 6E) afferent terminals connected.
Replenishment of afferent terminals with synaptic vesicles

To further investigate the process of hair cell re-innervation following excitotoxic damage to vestibular afferents, we monitored the expression of synaptophysin (Fig. 7) and synapsin (Fig. 8), two proteins associated with synaptic vesicles (Brachya et al. 2006), in cristae at 2 h, 7 h, 12 h and 24 h after the STTK. In contralateral non-injured sensory epithelia (Fig. 7A), immunolabeling against synaptophysin revealed dot-like structures restricted to the base of the sensory cells (Fig. 7A arrows) and the apex of the epithelia (Fig. 7A: arrow heads). This labeling matched previously described expression of synaptophysin in both the efferent and afferent bouton terminals, as well as at the upper portion of the calyx afferent terminals reported in the adult rodent vestibule (Dechesne et al. 1997; Sage et al. 2000; Scarfone et al. 1988; Scarfone et al. 1991). Two hours after the excitotoxic insult, synaptophysin expression did not differ from the contralateral ear, except for a slight disorganization at the base of hair cells and at the apex of calyces (Fig. 7B). The lack of synaptophysin expression in the conjunctive tissue underlining the cristae up to 7 h after the STTK is noteworthy. Seven hours after the excitotoxic injury synaptophysin immunoreactivity started to appear in some afferent fibers entering the sensory epithelia as well as in hair cells. Subsequently, immunolabelling increased during the next hours in both the afferent terminals and in hair cells (Fig. 7D-E). Of note are entering fibers intensively expressing synaptophysin 24 h after the STTK injection (Fig. 7E, open arrows). This alteration of the synaptophysin expression confirmed the re-organization of nerve terminals mimicking the processes observed during the developmental synaptogenesis period (Dechesne et al. 1997; Gaboyard et al. 2003; Sage et al. 2000; Scarfone et al. 1991). Synapsin expression was more specific to efferent terminals only in the adult vestibule, as we observed in the contralateral ear.
(Fig. 8A-B). Synapsin expression was mostly restricted to dot-like structures at the base of sensory cells that lined but clearly did not overlap or co-express with the neurofilament staining (Fig. 8B, arrows), corresponding to mature localization of efferent terminals (Favre et al. 1986; Holstein et al. 2005). Twelve hours after the excitotoxic damage, synapsin immunoreactivity increased in the cristae, with a reorganization of efferent terminals, and increasing expression in fibers entering the epithelia and in hair cells (Fig. 8C-F, arrow-heads). A similar pattern of expression of these proteins associated with synaptic vesicles was described during the process of perinatal synaptogenesis in the vestibule (Scarfone et al. 1991).
DISCUSSION

The present study addresses the question of the different stages and time course of damage and repair processes of inner ear nerve terminals following excitotoxic damage. Adapted to vestibular afferents, this question is crucial to understand how physical damage at the contact between sensory hair cells and nerve terminals arising from the primary vestibular neurons governs the heterogeneity of vestibular disorder symptoms.

Inducing and assessing excitotoxic damages in the mammal vestibule

The term ototoxicity relies to specific damages induced by different drugs to auditory or vestibular sensory cells. Vestibular hair cell loss is a typical pathophysiology induced by aminoglycosides, such as gentamicin, or nitriles such as 3,3’-iminodipropionitrle (IDPN). Recent studies demonstrated that additional to hair cell impairment, the calyx innervation of type I hair cells was also altered by gentamicin or IDPN administration, suggesting that excitotoxicity and damage to the afferents might also been involved in the pathophysiology of drug-induced ototoxicity (Hirvonen et al. 2005; Sedo-Cabezon et al. 2015). Finally the sole correlation between structural excitotoxic damages within the vestibule and altered vestibular behavior has rarely been studied. Excitotoxic injury of vestibular afferents was previously undertaken using different delivery methods. Surgical approaches, syringe pumps and gelfoam containing glutamate agonists were used to deliver excitotoxic drugs in the immediate proximity of the vestibule (Brugeaud et al. 2007; Liu 1999; Shimogori and Yamashita 2004). In comparison to the currently used transtympanic injection these delivery methods were supposed to reduce the dosage and exposure time necessary for the excitotoxic agents. However, none of the extensive
histological damage we observed in the present study (i.e. large vacuoles in place of afferent terminals with distortion of hair cells) was described in the previous studies. Reasons for the variable severity of excitotoxic damages may include, firstly, a difference in the effective dose of drug that reaches the inner ear. Liu (1999) used a direct application but of only small drops with a concentration of 10 to 50 nM kainic acid. Brugeaud et al. (2007) delivered a higher dose of 5 mM kainic acid but with extended diffusion over time from a gelfoam placed against the round window, probably resulting in longer exposure but a lower peak dose. The time points of histological exploration may also play a role in the observed degree of damage severity. Through their early analysis of excitotoxic damages performed 15 min following the kainic acid application, Liu and colleagues (1999) may have missed the maximum damage that we observed in the present study 1.5 h after the kainic acid injection. Shimogori and Yamashita (2004) investigated vestibular epithelia as late as 1 week after the injury (when we generally observed complete macroscopic recovery), and the histological method used was only light microscopy without the resolution necessary to precisely observe synaptic injury. One recent study using transtympanic injections of kainic acid (Dyhrfjeld-Johnsen et al. 2013) resulted in vestibular dysfunction recorded with both behavioral testing and electronystamography. Damaged afferents were clearly shown 24 h and 48 h after the insult, but initial tissue lesion following the excitotoxic induction during the early stage was not described.

Regarding the behavioral assessments, vestibular deficit induced by Brugeaud et al. (2007) was quite similar to those we observed 48 h after induction. However, they did not observe a pronounced behavioral peak crisis as we did in the present study (personal communication), suggesting that the delivery method may not have
induced an immediate, strong lesion similar to the one obtained through transtympanic injection. Conversely, Shimogori and Yamashita (2004) observed AMPA-induced vestibular disorders immediately following the excitotoxic induction. They reported strong spontaneous nystagmus during the first 12 h post-injury that could correspond to our initial 12 h of peak injury. In summary, these different studies compared to present results strongly suggest that 1) selective damage to the vestibular afferents directly supports the altered behavior characteristic of vestibular deficit; 2) the degree of structural damage (severity and duration) is directly reflected by the severity of vestibular deficit; and 3) following vestibular damage and deficit, structural repair and functional recovery occur.

**Excitotoxic features at vestibular afferents during acute peak crisis**

Transtympanic injection of kainic acid induced vestibular dysfunctions corresponding to moderate behavioral disorder score (Brugeaud et al. 2007; Desmadryl et al. 2012; Dyhrfjeld-Johnsen et al. 2013; Vignaux et al. 2011). We report 3 sequential stages starting with a pronounced acute crisis during the two first hours after the insult induction, followed by a relatively high and stable vestibular dysfunction period lasting several hours, and a subsequent slow functional recovery period that is completed within several days. The acute crisis coincides with histological damage stereotypical of excitotoxicity within the vestibular end organs. Large swelling of afferent terminals already observed 1 h after the insult induction persisted during 7 h, correlating with the duration of the acute crisis. Such extensive swelling of afferent terminals with hair cell distortion was previously described in the cochlea, following noise induced trauma or models of excitotoxicity with glutamate receptors agonists.
(Pujol and Puel 1999; Ruel et al. 2007) and in both cochlea and vestibule following ischemia (Puel et al. 1994; Sasaki et al. 2012). In the central nervous system as well, ischemia, local or systemic injection of glutamate receptors agonists induce swelling of afferent terminals (Nikonenko et al. 2009; Schwob et al. 1980; Sperk et al. 1983). This trauma has been referred to an early stage of excitotoxicity. *In vivo* and *in vitro* experiments were used to demonstrate that this mechanism mainly depends on non-NMDA receptors and results from massive influx of Na\(^+\), Cl\(^-\) and water into the afferent terminals (Choi 1987; Hoyt et al. 1998). Depending on the cause and duration of the insult, subsequent death of the injured neurons may occur through apoptotic and necrotic pathways (Gwag et al. 1997; Koh et al. 1990; Munir et al. 1995). The balance between neuronal repair and neuronal death after excitotoxicity was well described in the cochlea and basilar papilla of birds using sustained and/or higher doses of glutamate receptor agonists (Sun et al. 2001).

In the present study, we show that the slow and partial functional recovery of balance function coincides with a subsequent stage of retraction of the afferent terminals occurring within the first 24h after the excitotoxic insult induction. The retraction terminates either with partial loss of the calyx apposition or with a complete "stripping" of the hair cell basolateral membrane. Features of the retraction phase remained visible within the epithelia up to 24 h under the present paradigm. This retraction mechanism seems to mainly affect the distal, unmyelinated part of the nerve terminals of Scarpa’s ganglion neurons. Working on the severity of this excitotoxic model will help deciphering some fundamental questions concerning the degree of damage and the capacity for repair (Tos et al. 2013).
Repair of vestibular afferent terminals mimics developmental process

In the present study, swelling process and resorption of afferent terminals correspond to the acute episode of the vestibular disorder with pronounced behavioral dysfunction. Subsequently, we observed the retraction of nerve terminals followed by the spontaneous repair of afferent terminals that starts 12 h after the excitotoxic insult and coincides with a slow functional recovery of the balance function. Interestingly, the step of spontaneous repair displays features previously observed in mammal vestibule during the perinatal synaptogenesis period (Dememes et al. 2001; Desmadryl et al. 1992; Desmadryl and Sans 1990). Competition between afferent and efferent terminals for innervation of type I hair cells, with direct apposition of efferent terminals on type I hair cells, were often observed. Synaptophysin and synapsin are both required for neurotransmission and involved in synaptogenesis and neuronal plasticity (Brachya et al. 2006; Cesca et al. 2010). During maturation of vestibular endorgans, synaptophysin and synapsin switch from a pre-synaptic and diffuse expression in hair cells with post-synaptic expression along entering fibers, to a precise location at the apex of calyces and in afferent and efferent terminals in adults (Dechesne et al. 1997; Favre et al. 1986; Gaboyard et al. 2003; Holstein et al. 2005; Scarfone et al. 1988; Scarfone et al. 1991). In the present study, expression of synaptophysin and synapsin increased 12 h after the excitotoxic insult and their expression appeared to mimic what was previously described during the developmental process with increasing labeling of afferent fibers and terminals. Our observation confirmed that afferent repair that occurred after excitotoxic damage appeared to elicit mechanisms observed during the development of this sensory organ (Brugeaud et al. 2007). As supposed for other system during repair plasticity (Hou and Dahlstrom 2000; Marti et al. 1999), the increased expression of
synaptophysin and synapsin could reflect a process of replenishment of small synaptic vesicles to re-establish lost terminal contacts. Another likely role for the increased expression of these proteins in calyx terminals could be their participation in the process of membrane resealing. This mechanism previously described for synaptobrevin or SNAP25 (Steinhardt et al. 1994), would indeed be a good counter to the destruction of the membrane during the swelling process.

Taken together, these results confirm and strengthen previous observations that spontaneous repair of vestibular afferent terminals occurs in mammal vestibular sensory epithelia following excitotoxic damage (Brugéaud et al. 2007; Dyhrfjeld-Johnsen 2013). The repair process follows a precise time-course that has already started 24 h after the excitotoxic injury and is completed within 1 week. Its sequence follows successive and identified steps: swelling, resorption and retraction of nerve terminals followed by competitive re-innervation and de novo formation of afferent terminals (Figure 9). The re-innervation process mimics the process encountered during developmental synaptogenesis.

**Capacities and limits for functional recovery**

The structural repair was accompanied by functional recovery of the vestibular behavior. As previously stated, various studies have explored over the last decades the mechanisms of inner ear damage and repair using different paradigms to mimic pathological conditions in the auditory organ. Animal models of excitotoxic lesions through local application of glutamate receptors agonists, noise trauma and ischemia enabled deciphering of the correlation between damage to the auditory synapses and hearing loss (for review see Pujol and Puel 1999; Ruel et al. 2007). Spontaneous
repair of afferents was reported in the cochlea and this synaptic plasticity was accompanied by recovery of the auditory function. Recently, the repair capacities were challenged by strong auditory trauma that induces synaptopathy or neuronal death, resulting in partial but irreversible loss of auditory function (Kujawa and Liberman 2009; Liberman and Liberman 2015; Lin et al. 2011).

In the vestibule, no deleterious effect of over-stimulation has ever been reported, but ischemia appears as a potential and comparable traumatic insult (Jiang and Umemura 1993; Kitamura and Berreby 1983; Nario et al. 1997; Oas and Baloh 1992; Ritter and Veit 1977; Rubinstein et al. 1988; Tange 1998). Better protection and repair might also occur in the vestibular endorgans since major differences in the branching pattern of connections exist between the sensory organs. Primary vestibular neurons display multiple connections to both type I and type II hair cells (for recent review see Eatock and Songer 2011), while cochlear primary neurons display a single connection to only one inner hair cell (for recent review, see Kujawa and Liberman 2009). The redundancy of connections in the vestibule might promote higher protection than in the single-branched cochlear neurons. The severity of damage and the degree of fiber retraction beneath the sensory epithelium might condition the capacity of nerve fibers for regrowth and reconnection to occur (Tos et al. 2013). In the present study and previous ones using the kainic acid paradigm (Brugeaud et al. 2007; Desmadryl et al. 2012; Dyhrfjeld-Johnsen et al. 2013), we demonstrated that the severity and duration of the acute vertigo crisis correlate with the extent of the afferent terminal insult. It can be assumed that too much damage and retraction of the afferent terminals would compromise the repair process capacity. A massive release of glutamate during sustained ischemia or prolonged excitotoxic conditions would cause massive synapse uncoupling and pronounced
retraction that would further delay and ultimately prevent any possibility of regrowth and reconnection. Such a situation may reflect the clinical tableau sometimes encountered in humans in cases of complete vestibular areflexia as a result of conditions diagnosed as labyrinthitis or vestibular neuritis, or in cases of cophosis encountered in sudden sensory hearing loss conditions (Moalla et al. 2007). Further quantitative analysis will be needed to precisely establish the plastic capacities of vestibular synapses exposed to different traumatic conditions. The present model of kainic acid-induced excitotoxic might be a good starting tool for further investigation of such questions by performing some experiments involving dose-response or modified duration of exposure to the glutamate agonists. Also quantitative assessment of the presynaptic proteins should bring information on the state of the presynapse during the excitotoxic process.

In birds, destruction of hair cells by ototoxic agents induces a complete regeneration of the sensory epithelia with a full set of sensory cells and complete reinnervation (Zakir and Dickman 2006). However, the branching pattern of the regenerated synaptic connections is modified and correlates with alteration in gaze responses (Haque et al. 2009). In response to peripheral impairment of the vestibule, such as rebranching, reinnervation or destruction, a central compensatory mechanism restores balance function (Yu et al. 2014). One question raised by this work concerns the mechanism of central compensation. This process has to deal with, and balance both the early stages of the insult, inducing a strong alteration of the peripheral information, and the subsequent and progressive spontaneous restoration of the peripheral vestibular afferents re-introducing a modified sensory signal. A better understanding of what happens in terms of reacational recomposition within the brain
stem vestibular nuclei, depending on the type of peripheral damage will probably provide answers to this question (Lacour et al. 2009).

In conclusion, this study provides, for the first time, precise information on the sequence of the early events that occur following an excitotoxic insult within the vestibular endorgans. We have detailed the structural changes that occur at vestibular afferent terminals and their correlation with vestibular disorder symptoms. Going further will require identifying the molecular actors involved and determining the correlation between the severity of the insult and spontaneous repair capacities. This information is a prerequisite for further development of targeted pharmacological approaches to efficiently protect vestibular afferent terminals and/or promote their regeneration.
MATERIAL AND METHODS

Animals

Experiments utilized adult female Wistar rats (2 months of age, 200-224g; CERJ, Le Genest, France) in accordance with the French Ministry of Agriculture regulations and European Community Council Directive no. 86/609/EEC, OJL 358.

Unilateral vestibular excitotoxic insult

Using a surgical microscope, single trans-tympanic injection of the glutamate receptor agonist, kainic acid (referred as STTK for Single Trans-Tympanic Kainic acid injection, 12.5 mM, 100 µl; Ascent Scientific, Bristol, UK; n = 29) was performed under isoflurane anesthesia according to the procedure previously detailed (Dyhrfjeld-Johnsen et al. 2013). In summary, rats were anesthetized with isoflurane and kept in lateral position. Applying traction to the auricle, the posterior portion of the tympanic membrane was penetrated with a sterile insulin syringe (0.33 x 12.7mm or 0.5 x 16 mm). kainic acid, dissolved in 100 µl of saline, was slowly injected into the middle ear cavity. After injection, the animal was kept in lateral position for 10-15 min under isoflurane, and observed until recovered from the anesthesia.

Control animals (n=8) were also subjected to transtympanic injection into the right middle ear of only saline solution.

Behavioral experiments

Vestibular dysfunction score was determined as previously described (Boadas-Vaello et al. 2005; Brugeaud et al. 2007). Altered vestibular behaviors were scored on a scale from 0 to 4, respectively ranging from normal behavior (rating 0) to maximal identified vestibular impairment (rating 4). The table 1 describes the observed
behavior and associated criteria used to score the vestibular dysfunction (normal = 0; mild deficit = 2; maximal and severe dysfunction = 4). The six different tests were sequentially scored and summed to rate the vestibular dysfunction: 1- head bobbing (abnormal intermittent backward extension of the neck); 2- circling (stereotyped movement ranging from none to compulsive circles around the hips of the animal); 3- retro-pulsion (typical backward walk reflecting vestibular disturbance); 4- tail-hanging reflex (normally induces a forelimb extension to reach the ground, unilateral dysfunction results in axial rotation of the body); 5- contact-inhibition reflex (normally leads animal to release hold grip on metal grid in a supine position when their back touch the ground; in case of vestibular dysfunction with lack of full body orientation reference, animal retains grip on the grid in a supine position); 6- air-righting reflex (necessary for rats to land on all four feet when falling from a supine position; vestibular dysfunction impairs normal body repositioning with a maximal dysfunction leading the animal to land on its back when dropped from a height of 40 cm onto a foam cushion). Rats were scored for vestibular dysfunctions just before the STTK and evaluated 1, 1.5, 2, 3, 5, 7, 24, 48, 72, 96 and 168 h after the injection (Fig. 1). Scores were expressed as mean ± sem.

**Tissue preparation for Histology**

Animals were deeply anesthetized with Pentobarbital (100 mg/kg), then perfused transcardially with heparin PBS (0.01 M) followed by a fixative solution (2% paraformaldehyde, 2.5% glutaraldehyde, 1% picric acid, with 5% sucrose for light and electron microscopy; 4% paraformaldehyde, 1% picric acid, with 5% sucrose for immunolabelling). Temporal bones were postfixed in the same solution before
vestibular epithelia were dissected in PBS. Tissue from right injected ear and control naive left ear were carefully identified and segregated.

**Light and Electron microscopy**

Whole fixed vestibular organs from animals perfused at 2, 5, 7, 12, 24 or 168 h after the STTK were postfixed in 0.5% OsO$_4$, dehydrated, and embedded in Araldite. For light microscopy, semi-thin (1 µm) sections were systematically cut with an ultramicrotome (Ultracut, Reichert-Jung). To quantify hair cells within utricles and ensure comparative unbiased counts between the different time points, we determined a pre-establish pattern for sectioning and sampling. Sensory epithelia were embedded in resin with a longitudinal alignment to obtain transverse sections from the first third part of the utricle following an antero-posterior orientation (Desai et al. 2005b). Transverse semi-thin sections were then cut within each sample. When hair cells were first observed in sections, 100 µm of epithelium was cut before collection for quantification. Then, 5 sets of 3 serial sections were collected, each set was separated by 25 µm. Semi-thin sections were toluidin-blue stained, mounted on slides and scanned using the Nanozoomer Slide Scanner (Obj. 40X; Hamamatsu, Japan). For electron microscopy, ultra-thin sections (silver-gold, 80-90 nm) were cut at 2, 24 and 168 h post STTK. Five sections were cut and collected after each set of semi-thin sections. They were stained with uranyl acetate, then viewed and digitalized in a Hitashi H7100 microscope.

**Morphometry**

For quantification of hair and supporting cells within utricles, Metamorph software (Molecular Devices, Sunnyvale, CA) was used and cells were manually counted on
semi-thin sections. Collection of sections starting 100µm from the anterior edge of the sensory epithelium ensured a sampling of transverse section composed of both striolar and extrastriolar region (Desai et al. 2005b). The 25 µm spacing between each set prevents from counting twice the same hair cell in 2 distinct sets of serial sections (mean hair cell width = 10 µm, calculated from Desai et al., 2005b). Three serial sections were collected for each set of transverse section of epithelium to ensure acquisition of one full section per each set suitable for quantification. For each utricle, 3 sections collected from 3 different sets were quantified. Thus, a snap shot of around 400 hair cells, sampled within the first third part of each utricle and coming from both striolar and extrastriolar region were counted for each time point on 3 different animals. Quantification of the contralateral ears was pooled as control. Cells were classified based on morphological criteria, cell shape and position of the nucleus were the main ones. The following classification was settled: 1) type I hair cell when a pear shaped hair cell was observed with basal nucleus localization; 2) type II hair cell when an elongated hair cell with a highly positioned nucleus was observed; 3) Not-determined hair cell type when the swollen terminal around the cell prevented from determining the cell type based on its shape, or when an elongated cell has a nucleus in a more basal position; and 4) supporting cells forming the basal part of the epithelium. Note that calyx innervation is a classical criterion to identify type I hair cells, however our observation at this magnification prevents from a clear-cut identification of afferents terminals. Thus, we preferred avoiding the presence of calyx innervation as an identification criterion and only used it as a confirmatory criterion for hair cell type I classification when it was definitely observed. The number of counted cells was standardized to the length of the analyzed transverse section.
For electron microscopy quantification, the aim was the quantification of type I hair cells and their afferent terminals. Thus, only hair cells identified as type I (n = 117) by their pear shape and low-leveled nucleus were analyzed on ultra-thin sections from 3 different animals fixed 24 h after STTK. Quantification was done on 3 ultra-thin sections collected from 3 different sets to obtain a snapshot of type I hair cells and calyx innervation from striolar and extrastriolar region without overlapped counts. They were categorized and counted depending on the state of their calyx afferent innervation: 1) full if reaching their neck, 2) partial if present but not fully formed and, 3) absent if lacking.

Statistical Analysis
SigmaPlot Software (Systat Software, Chicago, IL) was used with appropriate statistical tests. For behavioral scoring, Friedman repeated measures analysis of variance (RM-ANOVA) was followed by Dunn’s method to analyze the vestibular dysfunction over time after the kainic acid injury. For comparison to saline control animals, Mann-Whitney Rank Sum Test was used. For histological analysis, one way analysis of variance (ANOVA) was followed by the Holm-Sidak method to determine significant difference in cell counts for the different time points. Comparison over time, with contralateral ear referenced as time 0 h, was performed from 0 h to 168 h for each type of hair cells.

Immunchemistry
Vestibular endorgans from animals perfused at 2, 7, 12 or 24 h after the STTK were embedded in 4% agarose, and 40 μm thick sections were cut with a vibratome (HM650V, Microm). The free-floating sections were first permeabilized with 4% Triton X-100, non-specific binding was prevented by a pre-incubation step in a blocking
solution of 0.5% fish gelatin, 0.5% Triton X-100 and 1% BSA. Samples were then incubated with primary antibodies diluted in the blocking solution. For control experiments, the investigated primary antibody was omitted, while the following procedures were unchanged. Specific labelings were revealed with fluorescent secondary antibodies (1:200) in the blocking solution combined to actin staining with Alexa 594- or Alexa 647-conjugated phalloidin (Fisher Scientific, Illkirch, France). Samples were observed with a laser scanning confocal microscope (LSM 5 LIVE DUO, Zeiss). Final image processing was done with Adobe Photoshop software (San Jose, CA). Control reactions were observed and processed with the parameters used for the stained sections.

Antibodies

Two different combinations of primary antibodies were used. Antibodies were used as cellular markers. In control ear, they stain the appropriate pattern of cellular morphology and distribution as demonstrated in previous publications. Table 2 gives details and the references linked. The first combination included the mouse monoclonal anti-synaptophysin, a protein expressed in small synaptic vesicles in efferent terminals and, at the top of calyx afferent terminals and, the goat serum anti-calretinin, cytoplasmic calcium-binding protein expressed in most type II hair cells and pure calyx. In vestibular epithelia of adult rodents, calretinin was shown to specifically label type II hair cells and some calyx nerve fibers and endings. This particular population of calretinin expressing calyces is restricted to striolar zone of maculae and central zone of cristae. This population is identified as the pure calyx one, it was shown to correspond to only calyx unit without bouton branching like dimorphic afferent fibers (Desai et al. 2005a; Desai et al. 2005b; Desmadryl and
Dechesne 1992). The other combination involved the rabbit serum anti-synapsin, phosphoprotein expressed in synaptic vesicles of efferent terminals and, the mouse monoclonal anti-neurofilament 200 KD, neuronal intermediate filaments of the cytoskeleton of afferent innervation. Fluorescent secondary antibodies were donkey sera conjugated to Alexa 488-, Alexa 594- and Alexa 647- anti-mouse, rabbit and goat IgG respectively (1:700, Fisher Scientific).
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COMPETING INTERESTS

A.S., A.B., A.B. and S.G-N. work for Sensorion, a biopharmaceutical company committed to finding treatments for inner ear disease. C.C. and S.G-N. have shares in the company.

AUTHORS CONTRIBUTIONS

C.T., A.S., A.B., A.B. and S.G-N. executed the experiments. S.G-N and C.C. analyzed the data, conceived and directed the project, and wrote the paper. All authors edited the manuscript prior to submission.

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Vestibular Dysfunction induced by a single trans-tympanic injection of kainic acid. As soon as 1 h after the injection, vestibular dysfunctions were induced. The peak crisis was maximal at 1.5 h and lasted until 7 h (* P < 0.05 vs 1 h, *** P ≤ 0.001 vs 7 h). Then, the cumulative score decreased and became no more statistically different from control animals at 96 h. Significance of the ANOVA followed by the Dunn’s method to analyze the whole time post-lesion, *** P ≤ 0.001, ** P ≤ 0.01. Mann Withney test was used to compare with control animals injected with the saline solution (n =8).
Figure 2

Excitotoxic injury in vestibular utricles observed 2 h after the lesion by electron microscopy. A, B: in control tissue, type I hair cells (I) surrounded by calyx nerve endings and cylindrical type II hair cells (II) were identified lying over the nuclear layer of supporting cells (sc). C-F: in injured inner ear, few type I hair cells were still identified by the calyx terminal. Most hair cell type became undetermined (asterisk) because of their distorted morphology induced by large swellings at their base (a). Localization and content in mitochondria characterized the swollen structures as afferent terminals (a). Scale bars = 10 µm in A-D, 2 µm in E-F.
Progression over time of the general organization of vestibular epithelia after induced excitotoxic injury. Gross morphology was observed on semi-thin sections of vestibular utricles. A: In control ear, the regular macro-organization of hair cells and supporting cells constitutes the sensory epithelium lying on a conjunctive tissue where innervating fibers arrived. B: 2 h after the STTK, large holes appeared between the layers of nuclei from hair and supporting cells. C-E: from 7 h to 12 h post-lesion, size of swellings progressively decreased. F: 24 h post-lesion, no more vacuole was observed in the sensory epithelium whose gross morphology appeared similar to the contralateral one. Notice the innervating fibers were always present underneath the injured sensory epithelium. Scale bar = 100 µm applies to all panels.
Figure 4

Density of hair and supporting cells in vestibular epithelia following the excitotoxic injury. Cells were counted on transverse semi–thin sections of utricles
from 3 different experiments for each time-point. Their density is expressed for 100 µm length of epithelium. **A**: Total numbers of supporting cells (Sup. cells), hair cells (total HC) and segregation between identified type I (type I HC), type II (type I HC) and undetermined hair cell type (Und. HC type) were compared between contralateral, 2 h (KA 2 h) and 24 h (KA 24 h) post-lesion. Statistical significance between contralateral, 2 h and 24 h following one way ANOVA with post-hoc analysis using Holm-Sidak method is expressed: *** P ≤ 0.001. **B**: Density of identifiable and undetermined hair cell type was analyzed from 1 h to 168 h post-lesion in utricles and compared to the contralateral ears, referenced as time 0 h for statistical analysis. Significance of the ANOVA followed by the Holm-Sidak method is presented: *: P<0.001 vs 0 h, 24 h and 168 h; #: P<0.001 vs 0 h and 168 h; †: P<0.001 vs 168 h.
Figure 5

Detailed morphology of vestibular epithelia 24 h after the excitotoxic injury. Utricles were observed by electron microscopy. A: at low magnification, some type I hair cells (I) were identified while other hair cells with undetermined type remains (asterisk). B-G: nerve terminals were detailed at higher magnification. B: typical ribbons (white arrow) facing postsynaptic densities on bouton afferent were normally observed. C, D: bouton–like terminals (a) or efferent terminals (e) unusually connected some type I hair cells. E, F: single or multiple ribbons face remaining pieces of calyceal membrane. G: typical feature of competition between efferent terminals and afferent fibers to contact hair cell facing ribbons were found. Scale bars = 10 µm in A, 1 µm in C, D, and 500 nm in B, E-G.
Figure 6

Histology of sensory epithelium 1 week after the excitotoxic injury. A: transverse semi-thin sections of the injured utricle, 168 h post-lesion shows a normal gross morphology and organization of hair and supporting cells, beneath the conjonctive tissue where innervating fibers are observed. B, C: electron microscopy
shows that morphology of type I (I) and type II (II) hair cells is normal, terminal afferents are present. D, E: at higher magnification, regular afferent terminals are observed: calyx connect type I hair cells (c), efferent terminals contact calyx afferents (e) afferent fibers are passing (a) and bouton afferent terminals (b) contact facing ribbons (white arrow) of type II hair cells. Scale bars = 100 µm in A, 10 µm in B, C, and 1 µm in D, E.
Figure 7

Progression of synaptophysin expression over time after the excitotoxic injury. Immunolabelling in transverse section of crista observed by confocal microscopy. A: in the control ear, counter-labeling of transverse sections with phalloidin (Phal, red) and antibodies anti-calretinin (Cal, blue) highlights hair bundles of sensory cells and, most type II hair cells and pure calyx of the central zone. Immunolabeling with antibodies anti-synaptophysin (Synapto, green/white) locates small synaptic vesicles in normal vestibular sensory epithelium. High magnification of the inset (black and white panel) shows precisely that synaptophysin is normally expressed in efferent terminals (arrows) and at the top of calyces (arrowheads) in this adult tissue. B-E: from 2 h to 24 h after the STKK, gross morphology of the sensory epithelium was distorted as evidenced by the actin labeling and, synaptophysin expression changed. B, C: At 2 h and 7 h synaptophysin got mostly disorganized with a disappearance at the top of calyces. D, E: from 12 h to 24 h, expression increased in innervating fibers and hair cells. Scale bars = 10 µm applied to all panels.
Figure 8

Progression of synapsin expression over time after the excitotoxic injury.

Immunolabelling in transverse section of crista observed by confocal microscopy. **A, B**: in the contralateral ear, counter-labeling of transverse sections with phalloidin (*Phal*, blue) and antibodies anti-neurofilaments 200 KD (*N52*, green) highlight hair bundles of sensory cells, and afferent fibers. Immunolabeling with antibodies anti-synapsin (*Synapsin*, red) locates small synaptic vesicles in normal vestibular sensory function.
epithelium. B. High magnification shows precisely that synapsin is normally expressed in efferent terminals (arrows) contacting afferent fibers. C, D: 12 h after the STTK, synapsin expression got disorganized and increased. E, F: 24 h after the STTK, synapsin also appeared in afferent terminals (arrowheads). Scale bars = 10 µm in A applies to C, E, in B applies to D, F.
Figure 9

Illustration of the correlation between vestibular dysfunction and changes at the vestibular afferent terminals. Diagram parallels the time course of behavioral vestibular dysfunctions induced by trans-tympanic injection of kainic acid (curve) and the change occurring at calyx terminals after excitotoxic injury (draw). The acute crisis peaking at 1.5 h and the progressive recovery period overlaid the lesion (red), terminal resorption (orange) and repair (yellow). Replenishment of proteins associated with small synaptic vesicles is pointed-out.
## TABLE 1. Vestibular Tests and Scoring criteria

| Behavioral Observation | Score criteria | Score = 0 | Score = 2 | Score = 4 |
|------------------------|----------------|-----------|-----------|-----------|
|                        | Score criteria |           |           |           |
| Head bobbing           | No bobbing     | Marked up and down uncontrolled move of the head, low frequency and small amplitude | Frequent and large amplitude of the bobbing move |
| Circling               | No circling    | Few but marked and uncontrolled circling around hips | Uncontrolled, repetitive and frequent circling around hips |
| Retro-pulsion          | No backward walk | Few backward steps | Long backward walk |
| Tail-hanging           | Instant forelimb extension | Forelimb extension with torsion of the body | Sustained body twist and turn |
| Contact-inhibition     | Instant release of grip | Slow and progressive release of grip | No release of grip with confinement between grid and ground |
| Air-righting           | Landing on all four feet | Disturbed body repositioning resulting in flank landing | Body torsion without repositioning resulting in random landing |
### TABLE 2. Antibodies Used in These Studies

| Antigen     | Host  | Description of immunogen                                                                 | Source                      | Dilution | Reference                  |
|-------------|-------|------------------------------------------------------------------------------------------|----------------------------|----------|----------------------------|
| Calretinin  | Goat  | Rat calretinin                                                                           | Millipore (AB1550)         | 1:350    | Hurley et al, 2006         |
| Neurofilament | Mouse | C-terminal segment of enzymatically dephosphorylated pig neurofilament 200.            | Sigma-Aldrich (N0142, clone N52) | 1:350    | Gaboyard et al, 2002       |
| Synaptophysin | Mouse | Vesicular fraction of bovine brain                                                      | Millipore (MAB5258, clone SY38) | 1:200    | Gaboyard et al, 2002       |
| Synapsin    | Rabbit| Residues surrounding Gln483 of human synapsin-1 protein                                 | Cell Signaling Technology (5297) | 1:200    | Scarfone et al, 1991       |