The effect of the loss of the cholinergic alpha-9 receptor subunit and CGRP on vestibular processing: insights into the role of efferent feedback and novel approaches for characterizing the vestibular system

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Abstract

The role of the vestibular efferent system is presently unknown, but evidence based on physiological similarities with the auditory efferent system suggest that it may mediate vestibular end organ development – potentially altering transmission of vestibular information by afferents. To investigate the role of the efferent system, I characterized gaze stabilization and postural control pathways in transgenic mice lacking mediators of efferent signaling (α-9 and/or αCGRP). I found that loss of α-9 attenuated VOR gain, particularly at 4.0HZ, and loss of αCGRP caused motor deficits during both rotarod and balance beam tasks, consistent with the role of efferents in inner ear development. Finally, to directly assess how loss of cholinergic efferent signaling might influence information transmission I developed methods to record from vestibular afferents of 129SvEv mice during both sinusoidal and random noise stimulation. I found that afferent sensitivity and phase were comparable between both stimulus paradigms, with higher sensitivity and phase lead in afferents with more irregular spontaneous discharge. In contrast, computations of coherence and mutual information revealed that afferents with more regular spontaneous discharge encoded more information content over the natural frequency range of head motion in mice (≤4.0Hz; Beraneck et al., 2008). Taken together, the results suggest this approach will be useful in characterizing changes in sensory information coding in transgenic mice with an impaired efferent system. Overall, my research provides insight into vestibular efferent function and presents future directions for better understanding the underlying physiological changes that occur when this pathways is impaired.

Résume

Le rôle du système efférent vestibulaire est actuellement inconnu, cependant de récentes évidences fondées sur des similitudes physiologiques avec le système auditif efférent suggèrent que ce dernier peut moduler le développement des organes vestibulaires et potentiellement altérer la transmission de l'information vestibulaire afférentes. Afin d'étudier le rôle du système efférent, j'ai caractérisé la stabilisation des voies oculomotrices et des voies de contrôle postural chez des souris transgéniques dépourvues de médiateurs de la signalisation efférente (α-9 et/ou αCGRP). J'ai trouvé que la perte des mediateurs α-9 atténue le gain du réflexe vestibulo-oculomoteur (RVO), en particulier pour les stimuli de 4,0Hz. La perte de αCGRP cause des déficits moteurs lors de l'execution d'une tâche de rotarod et de poutre d'équilibre, ce qui est en accord avec le rôle des efferents dans le développement de l'oreille interne. Finalement, pour évaluer directement comment la perte de la signalisation efférente cholinergique pourrait influencer la transmission de l'information, j'ai développé des méthodes d'enregistrement pour les afférentes vestibulaires des souris 129SvEv, et ce, pendant une stimulation sinusoïdale et une stimulation bruit aléatoire. J'ai trouvé que la sensibilité et la phase étaient comparables entre les deux paradigmes. Toutefois, les afférentes avec une décharge spontanée irregulière avaient une plus grande sensibilité et un plu grande avance de phase comparativement aux afferents régulière. En revanche, les calculs de cohérence et d'information mutuelle ont révélé que les afférents avec décharge spontanée plus régulière encodaient d'information sur la gamme de fréquence naturelle des mouvements de tête chez les souris (≤4,0Hz; Beraneck et al., 2008). À cet égard, ces résultats suggèrent que cette approche sera utile pour caractériser les changements dans le codage de l'information sensorielle chez les souris transgéniques ayant un système efférente affaiblies. Dans l'ensemble, ma recherche donne un aperçu de la fonction vestibulaire efférente et présente des orientations futures afin de mieux comprendre les changements physiologiques sous-jacents qui se produisent lorsque ce réseau est altérée.

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CHAPTER 1: LITERATURE REVIEW

I. Vestibular System

The vestibular system plays an important part in our ability to maintain our posture and balance, to stabilize our gaze as we move our head through space, and to differentiate between our environment moving versus our own movements. This sensory system is able to detect the motion of our head through the use of two classes of sensory organs within the inner ear – the semicircular canals which detect rotational movements and the otoliths which detect linear acceleration, including gravity (reviewed in Cullen, 2012). Within these organs are hair cells, characteristically divided into type I and type II hair cells, which sit in a compartment filled with endolymph fluid. On the apical surface of each hair cell are stereocilia that bend and move with the endolymph in accordance to our own head movements (Fig 1B, C). Mechanoreceptors found on the stereocilia of hair cells open in response to this motion. This allows an influx of potassium which depolarizes the cell and causes an influx of calcium through voltage dependent calcium channels (VDCC). The calcium influx triggers the release of glutamate from hair cells onto excitatory amino acid receptors (EAA-R; Soto and Vega, 2010) on afferent ganglion cells, depolarizing the vestibular afferents as a result. Apart from their apparent morphological differences, type I and II hair cells generally innervate different types of afferents. Type II hair cells synapse onto bouton afferents with typically regular firing rates and type I hair cells synapse onto calyx afferents with typically irregular firing rates. Both types of afferents project to neurons within the vestibular nuclei (Fig1A, blue pathway) which in turn project to eye motoneurons, higher order centers and the spinal cord for gaze and postural stabilization (reviewed in Holt et al., 2011).

The vestibular system also has a feedback pathway – the vestibular efferent system – which innervates the peripheral sensory organs (Fig 1A, red pathway). Efferent feedback mechanisms for sensory processing is a common feature across sensory modalities, but one that projects to the level of the sensory cells is not as common (Ryugo, 2011). Although it was first discovery by transmission electron microscopy over 50 years ago (Engstrom, 1958), the functional role of this pathway has yet to be fully understood. The goal of this study was therefore to provide insight into the functional role of the vestibular efferent pathway. I begin by reviewing literature describing the anatomy of this pathway, looking at both known and proposed mechanisms of action of the vestibular efferent system. Followed by a review of the literature on the functional role of this pathway to date.

II. Vestibular Efferent Pathway

The vestibular efferent pathway, which co-evolved with the ear in vertebrates (Sienknecht et al., 2014), is comprised of neurons – known as group *e* – whose cell bodies are found in the brainstem just lateral to the abducens and genu of the facial nerve (Fig 1A; reviewed in Holt et al., 2011). They have a highly divergent pattern of innervation in which profusely branching axons synapse onto multiple hair cells and/or afferents (depending on the species; see red asterisks in Fig 1B and C) in both the ipsilateral and contralateral vestibular end organs (Sienknecht et al, 2014; Lysakowski, 2010). The primary neurotransmitter of the efferent system is acetylcholine, although other neuromodulators – such as calcitonin gene-related peptide (CGRP), ATP, GABA, substance P (subs P) and encephalin (enk) – are also present at the synapse (Fig 1B, C; reviewed in Holt et al., 2011; Soto and Vega, 2010; Yamashita et al., 1993).

Depending on the species, vestibular efferent activation will induce different afferent responses (reviewed in Holt et al., 2011; reviewed in Jordan et al., 2013). For example, semicircular canal afferents of frogs, turtles and birds can be both inhibited and excited by efferent stimulation, whereas canal afferents of fish and mammals almost exclusively experience excitation (reviewed in Jordan et al., 2013; Goldberg and Fernandez, 1980; Marlinski et al., 2004). Anatomical differences of efferent innervations in the vestibular end organ also exist between species. Since fish and amphibians do not have type I hair cells or calyx afferents, efferent synapses are restricted to type II hair cells and bouton afferents and in frogs, only type II hair cells have efferent innervation. In contrast, reptiles, birds and mammals exhibit efferent innervation on type II hair cells, bouton and calyx afferents. Reptiles, birds and mammals all have rare contact to type I hair as they are enveloped by calyces of irregular afferent endings (Species differences are summarized in table 1).

Importantly, these known differences in termination of efferent fibers on different targets do not explain the differences in afferent responses to efferent stimulation (inhibitory and/or excitatory) between species. For example, frogs exhibit both inhibitory and excitatory afferent responses despite only having efferent innervation on type II hair cells (Hillman, 1969; Lysakowski, 1996). Rather, these distinct afferent responses to efferent stimulation appear to be mediated by the expression of different post-synaptic acetylcholine receptors on both hair cell and afferent targets of the efferent pathway which differ between species (reviewed in Jordan et al., 2013).

III. Synaptic Mechanisms of Vestibular Efferents

As previously described, acetylcholine is the primary neurotransmitter of the efferent pathway in all species (reviewed in Holt et al., 2011; Soto and Vega, 2010; Yamashita et al., 1993). As such, it is believed that differences in afferent responses between species are mediated by the expression of diverse cholinergic receptors in efferent targets (reviewed in Jordan et al., 2013). Below, I will discuss the mechanisms underlying inhibition and excitation as well as the acetylcholine receptors responsible for each. In addition, I will review the literature concerning one of the best understood neuromodulators of acetylcholine found at the efferent synapse, CGRP, and how it affects efferent action.

The mechanism mediating inhibition at vestibular efferent synapses, identified at the afferent bouton endings of frogs and turtles, appears to be similar to the mechanism mediating the auditory efferent pathway (reviewed in Jordan et al., 2013; Kong et al., 2013). Inhibition at both vestibular and auditory efferent synapses is mediated by activation of α-9/10 nicotinic acetylcholine receptors found on hair cell targets (Fig 1B and C, pink). Activation of this receptor causes a brief influx of calcium. As the α-9/10 receptor is coupled to the SK channel (Fig 1B and C, dark green), it causes a consequent efflux of potassium that hyperpolarizes the hair cell. This reduces calcium influx through other calcium channels and inhibits hair cell transmitter release of glutamate onto afferents (Im, 2012; Kong et al, 2008; Turcan et al., 2010).

The mechanism mediating excitation seen at vestibular efferent targets was originally believed to be mediated by the uncoupling of α-9/10 receptors from SK channels. This theory was initially supported by studies showing that blocking SK channels switched efferent-mediated inhibition to excitation in efferent targets (Holt et al., 2006). However, excitation being mediated by uncoupled α-9/10 receptors was proven to be unlikely through studies in turtles looking at afferent responses. In turtles, studies showed that efferent stimulation causes a subset of afferents (BM afferents) to exhibit a short latency efferent-mediated inhibition, followed by a quick burst of increased afferent activity within about 40ms – termed post-inhibitory excitation (PIE; reviewed in Holt et al., 2011). In comparison, when α-9/10 receptors are blocked by nAchR antagonists, such as strychnine (STR) or tropisetron (ICS) within BM afferents in turtles, only excitation remained. Given the difference in responses between excitation due to efferent stimulation and excitation from uncoupling of α-9/10 receptors, it is more likely that excitation of efferent targets is mediated through a different nAchR from α-9/10 (Holt et al., 2006). A nAchR was identified using a selective pharmacological antagonist for α4/β2-containing nAchRs (dihydro-β-erythroidine), which consequently blocked excitation in BM afferents. This finding suggests that α4/ β2 nAchR mediates excitation in efferent targets (Fig 1B and C, red; reviewed in Jordan et al., 2013) rather than uncoupled α-9/10 receptors.

However, α4/β2 nAchR is not the only mediator of excitation in efferent targets. Efferent stimulation studies in squirrel monkeys, chinchillas and turtles have identified both a fast and slow component in efferent mediated excitation (Brichta and Goldberg, 2000; Holt et al., 2006; Goldberg and Fernandez, 1980; Marlinski et al., 2004). While the fast component (time constant of 10-100ms) can be characterized by α4/β2 nAchR action, the kinetics of the slow component (time constant of 20-40s) are distinctly different (Goldberg and Fernandez, 1980). The slow component takes approximately 40-50 seconds to peak and outlast the stimulus considerably, elevating the background rates of afferents for almost 200 seconds (reviewed in Jordan et al, 2013; Brichta and Goldberg, 2000). Currently, it is proposed that the slow component of excitation is mediated by muscarinic acetylcholine receptors (mAchR; Fig 1B and C, dark blue; Jordan et al., 2010; Li et al., 2007) since muscarinic agonists in frog crista has been shown to cause slow excitation (Bernard et al., 1985).

Thus, as reviewed above, prior experiments in frogs and turtles have shown that inhibition and excitation of efferent targets are mediated by different post-synaptic acetylcholine receptors. The expression of these post-synaptic receptors may also differ depending on the type of efferent targets (hair cell versus afferent) within the same species. This hypothesis is consistent with findings in a toadfish study by Boyle et al., 2009. By recording from afferents and hair cells simultaneously during efferent stimulation, they found that afferents experienced excitation while at the same time hair cells experienced inhibition (Boyle et al., 2009). If the same responses do occur in mammals as well, it would explain how efferent stimulation in squirrel monkeys, while increasing afferent discharge, decreases sensitivity to vestibular stimuli. Efferent-mediated excitation would be the cause for increased afferent discharge, while efferent-mediated inhibition of hair cell targets would explain decreased sensitivity to vestibular stimuli (Goldberg and Fernandez, 1980), much like what was seen in toadfish.

In addition to differential expression of acetylcholine receptors, efferent activity can also be affected by neuromodulators found at the efferent synapse. The most prominent and well understood neuromodulator is CGRP (Fig 1B and C, light blue). It is expressed within the central nervous system and profusely found in vestibular efferents projecting to the vestibular end organs (reviewed in Holt et al., 2011; Soto and Vega, 2010; Yamashita et al., 1993). Since it commonly co-localizes with choline acetyltransferase (CHAT) it was suggested to influence acetylcholine activity and there is increasing evidence for this. First, within cochlear hair cells, CGRP is able to increase calcium concentrations in response to acetylcholine release in Xenopus laevis (Adam et al., 1987). Second, in skeletal muscles, CGRP is able to modify expression of nAchR subunits. It does so through coupling with adenylate cyclase, which catalyzes the conversion of ATP to cAMP (Laufer and Changeux, 1987). cAMP dependent phosphosrylation of nAchRs plays a key role in regulation of these receptors (Wackym et al., 1993). These results provide evidence that CGRP has the capacity to upregulate the expression of nAchRs and modulate the sensitivity of these receptors to acetylcholine which makes its presence at the efferent synapse particularly important for regulation of efferent signals (Wackym et al., 1993; Xiaocheng et al., 2012).

IV. Vestibular Efferent Function

The presence of vestibular efferent feedback is best exemplified in efferent-mediated responses of afferents induced by vestibular stimulation in mammals. While placing the canal orthogonal to the plane of motion to null conventional afferent responses, it was found that the afferents in chinchillas and macaque monkeys demonstrated efferent-mediated responses that were excitatory for both directions of rotation (Plotnik et al., 2002; Sadeghi et al., 2009). Though this demonstrates the presence of an efferent feedback response to vestibular stimuli, the exact functional role of this feedback in day to day life is still unknown in mammals. To date, various hypotheses of possible functional roles have been proposed for the efferent vestibular system. It had originally been anticipated that the efferent pathway would serve a role in differentiating between active and passive movements at the periphery (Goldberg et al., 2000). This was however disproved given afferents in macaque monkeys similarly respond to active and passive self-motion (Cullen and Minor, 2002; Jamali et al., 2009). Another hypothesis suggested that the efferent vestibular system was perhaps involved in balancing of the left-right vestibular end organs. This was also disproved by using a unilateral labyrinthectomy monkey model which showed no significant changes in resting afferent discharge that would suggest the influence of efferent activity in this regard (Sadeghi et al., 2006).

Additional insight regarding the functional role of the efferent vestibular system potentially could be gained from our current understanding of the role of the auditory efferent pathway. As mentioned above, the inhibition induced by auditory efferents uses the same mechanisms (α-9 /10 mediated responses) as those seen in the vestibular efferent pathway. Notably, recent studies suggest that the auditory efferent system plays a role in providing local feedback that can potentially modulate synaptic strength during development. In a study looking at early stages of auditory efferent development, Kong et al (2013) found local feedback mechanisms at transient synapses between auditory efferents and hair cells. This feedback mechanism, known as retrograde facilitation, was mediated through release of nitric oxide from efferent targets in response to cholinergic efferent signals. This response served to increase efferent neurotransmitter release onto its targets and could potentially modify synaptic strength between efferents and hair cells. Taken together, it is plausible that this same mechanism of local feedback in modulating synaptic strength during development is present in the vestibular efferent pathway (Lysakowski and Singer, 2000).

In addition, there is evidence that the auditory efferent system also plays a role in maturation of hair cell responses to calcium. Knock-out (KO) mouse models for the α-9 subunit of the α-9/10 nAchR revealed that cochlear hair cells did not mature from a linear to non-linear response to calcium as is typically seen in controls (Johnson et al., 2013). Gene profiling of α-9 null mutants also revealed altered expression of genes that would otherwise be normally present during early stages of development, including voltage-gated ion channels and increases in GABA receptor subunits (Turcan et al., 2010). These findings would suggest that a functional α-9/10 nAchR is essential for normal development of cochlear hair cells and could potentially have the same effect on vestibular hair cells.

Interestingly, these effects on hair cell development do not appear to be not limited to the early stages of development. Naturally, we experience gradual hearing loss as we age and our auditory hair cells deteriorate. Recent studies were able to show that when auditory efferent signaling was removed, synaptic loss that is attributed to cochlear aging and damage over time dramatically increased (Liberman et al., 2014). Given the anatomical similarities, it is possible that the vestibular efferent pathway could play a similar role in the development of efferent targets as well as preserving synaptic integrity within the vestibular end organ.

V. Mouse Models for Elucidating Efferent Function

As previously mentioned, a number of acetylcholine receptors are present at the efferent synapse. We can therefore gain general insight into overall efferent-mediated cholinergic signaling by targeting αCGRP expression in this pathway because of its role in modulating overall acetylcholine activity. Recent work from our group (Luebke et al., 2014) identified behavioural deficits in the vestibular ocular reflex (VOR) as a result of a loss of αCGRP. Null mutants had a significant attenuation in VOR gains by ~50% relative to controls without any effects on phase or saccadic eye movements which would suggest that loss of αCGRP impairs VOR efficacy.

To date, Luebke et al. (2014) has been the only published study investigating the functional role of the vestibular efferent pathway using an *in vivo* mammal model in which efferent pathways are impaired. The αCGRP transgenic model is ideal for understanding overall efferent function considering the role of αCGRP in modulating cholinergic activity, but may also have a slightly broader affect due to its presence in other central pathways (such as within neurons in the vestibular efferent nucleus; Chi et al., 2007). Given that cholinergic efferent activity in the inner ear is mediated by many different post-synaptic acetylcholine receptors, investigations of other transgenic mouse models with mutations for more specific cholinergic receptors in the inner ear could provide further insight into how the efferent system may affect vestibular end organ development.

In particular, it would seem α-9/10 nAchR has the most evidence for being involved in efferent-mediated vestibular end organ development and its mechanism is best understood. Both α-9 and α-10 expression is restrictedly largely to the hair cells of the inner ear (Elgoyhen et al., 1994, 2001; Hiel et al., 1996; Lustig et al., 2001), making it a more precise target. In addition, based on the studies mentioned above in which cholinergic signals in the auditory efferent pathway – mediated by α-9/10 nAchR – appear to be essential for modulating synaptic strength and protecting against ongoing deterioration of hair cells (Kong et al., 2013; Liberman et al., 2014). The role of vestibular efferents in vestibular end organ development is supported by evidence that transient efferent synapses similar to those previously described in the auditory efferent pathway can also be found between vestibular efferents and type I hair cells. During early stages of development in mammals, before calyces have yet to form, efferent and afferent synapses develop at the same time within the vestibular end organs. As such, both efferents and afferents have direct contact onto type I hair cells during this period (Favre and Sans, 1978). This suggests that vestibular efferent signaling through α-9/10 nAchRs could elicit similar modulations in synaptic strength during early stages of development like those of the transient auditory efferents (Kong et al., 2013). The α-9 subunit of the α-9 /10 nAchR in particular appears to play a role in auditory hair cell maturation and mediating a cascade of gene expression during development (Johnson et al., 2013; Turcan et al., 2010). Since α-9/10 nAchRs are also present in the vestibular end organ of mammals (Anderson et al., 1997; Hiel et al., 1996; Kong et al., 2006), it seems likely that α-9 mediates a similar efferent signal for vestibular end organ development. Altogether, these findings make α-9 null mutant mice an ideal model for investigating how loss of this receptor might impair development of the vestibular system.

VI. Characterizing Vestibular Dysfunction

Transgenic mouse models are useful for investigating the functional role of the vestibular efferent pathway. This has already been demonstrated in previous findings from our group which show αCGRP is necessary for optimization of VOR gain (Luebke et al., 2014). These results along with what is currently understood about the vestibular and auditory efferent system suggest that cholinergic efferent synapses are important for hair cell and afferent development and synaptic strength. As such, loss of efferent activity might alter the transmission of vestibular information by afferents, which would be consistent with the results from Luebke et al (2014). Considering its developmental role, α-9 transgenic mice should presumable exhibit similar attenuations in VOR responses as αCGRP transgenic mice if they are involved in vestibular end organ development. In addition, since the vestibular system performs other functions beyond gaze stabilization (recall Fig 2A), including postural regulation, loss of αCGRP should also affect other vestibular pathways. Considering vestibular efferents may affect afferent development, it would also be of interest to investigate afferent information transmission in mice to better understand how they might be affected by efferent impairment.

Thus, to evaluate vestibular dysfunction and characterize peripheral sensory processing in mice, I used three main approaches in my thesis: 1) recordings of eye movement responses to characterize the vestibulo-ocular reflex (VOR), 2) behavioural assays of postural regulation and head stabilization (i.e., balance beam and rotarod), and 3) single-unit recordings of afferent responses. The associated pathways and reasoning behind the methods for characterizing them will be described below.

A. Recordings of Eye Movement Responses to Characterize VOR

The purpose of the VOR (Fig 2A, B) is to stabilize our gaze in the event of head movements. For example, during rotational VOR which is mediated primarily by semi-circular canals, compensatory eye rotation that is equal and opposite to head rotation would occur. In the pathway mediating VOR, head velocity signals from peripheral sensors project through vestibular afferents to the vestibular nucleus (VN). These signals then project to the abducens nucleus which innervates oculomotor neurons that drive eye movements (Fig 2B, gray; Scudder and Fuchs, 1992). Altogether, this pathway is one of the fastest neuronal reflexes, taking less than 6ms in total as a result of the direct transmission through this three-neuron arc (Huterer and Cullen, 2002). It is because the dynamic response properties are easily quantified and the overall simplicity of this pathway that the VOR is often chosen to evaluate functional loss in the vestibular system in various species, including mice.

As the aim of this study is to identify the functional role of efferent signaling on inner ear development and information transmission in afferents, it is necessary to control for the possibility that these mutations may cause unforeseen defects downstream of the vestibular afferents (for example, in the VN or ABN; Fig 2B, blue and green asterisks). To control for this, two other pathways are also characterized: 1) the premotor pathways for vestibular quick phases (Fig 2B, blue), saccadic eye movements that re-center the eye during sustained head motion (Chun and Robinson, 1978; Scudder and Fuchs, 1992) and 2) the OKN (Fig 2B, green), a reflexive eye movement induced by “full-field” image movement across the retina which causes a retinal “slip” of the visual image (Wester et al., 2007). Both pathways converge with the pathway mediating the compensatory VOR downstream of vestibular afferents either at the vestibular nucleus (OKN; Fig 2B, green asterisk) or the abducens (quick phases; Fig2B, blue asterisk). As such, using these controls, we can confirm whether loss of efferent-mediated cholinergic innervations in these transgenic mice specifically effect the vestibular end organs (i.e., hair cell and afferent information transmission) or have unforeseen effects on the projections downstream of the vestibular afferents.

B. Behavioural Assays of Postural Regulation and Head Stabilization

Postural regulation and head stabilization are mediated by descending pathways from the vestibular nucleus to the spinal cord known as the vestibulospinal tract. This pathway is mediated by axons projecting both ipsilaterally and contralaterally to the medial and lateral vestibulospinal tract, each of which innervate motoneurons at distinct spinal segments. The medial vestibulospinal tract innervates the cervical segments to mediate spinal reflexes controlling primarily neck muscles (Kasumacic et al., 2010) and make up the vestibulocollic reflex which helps to stabilize the position of the head in space (Grande et al., 2010; Mitchell et al., 2013; reviewed in Goldberg and Cullen, 2011). The lateral vestibulospinal tract on the other hand innervates cervical and lumbar segments to mediate reflexes controlling the limbs for balance (Kasumacic et al., 2010).

Considering the impairments in gaze stabilization seen in αCGRP transgenic mice are hypothesized to be a result of deficits in the development of the inner ear impairing transmission of vestibular information through afferents, this raises the question of whether vestibular postural control pathways responsible for balance, regulation of posture, and head stabilization would show similar impairments. In order to address this question, I used two paradigms, the balance beam and accelerating rotarod paradigms which were chosen because of their standard use for characterizing balance and motor deficits in mouse models (Luong et al., 2011; Deacon, 2013). In addition to quantify dynamic behaviour while performing the balance beam task in mice, a novel method was introduced, wherein a micro-electromechanical systems module (refer to methods in Carriot et al., 2014) was attached to the head of the mouse. This module allows us to distinguish differences in the dynamics and frequency content of head motion in 6 axis while the mice perform different tasks and offer a novel method of characterizing posture and head stabilization.

C. Vestibular Afferent Responses

To build upon the behavioural components of this study and fully appreciate the cholinergic efferent impairments introduced in these transgenic mouse lines and their effects on development of hair cell to afferent synapses, it is necessary to have a thorough understanding of peripheral sensory processing in mice. In particular, *in vivo* characterization of vestibular afferents are invaluable for this purpose. Specifically, I wanted to test the hypothesis that transmission of information is impaired in afferents as a result of loss of cholinergic efferent signals, resulting in decreased sensitivity and loss of information content in vestibular afferents. As mentioned before, vestibular afferents have a bimodal distribution of firing regularity which separate them into regulars and irregulars. Regular afferents in mice characteristically have high firing rates (~55spks/s; Lasker et al., 2008) with low variation in inter-spike intervals while irregulars, in contrast, have lower firing rates (~37spks/s; Lasker et al., 2008) with drastically higher variation in inter-spike intervals. These afferents also differ in the hair cell inputs they receive (type I versus type II hair cells) and morphology, from their axon diameters to their synaptic endings (reviewed in Eatock and Songer, 2011 and Holt et al.,2011).

Previous works in characterizing vestibular afferents within mice have focused on responses to relatively simple stimulus paradigms: trapezoids or sinusoids of linear acceleration to drive otoliths or sinusoids of angular velocity to drive the canals (reviewed in Cullen, 2012; Lasker et al., 2008; Yang and Hullar, 2007). These paradigms allow for the characterization of dynamic firing properties of regulars and irregulars.

A limitation of these paradigms is that they average the response variability of the afferents. Therefore, the analysis of afferent properties through these paradigms provide limited information on neural variability and information content of the afferents. To address these limitations, Sadeghi et al (2007) stimulated vestibular afferents using a random noise stimulation paradigm in a primate model. Their study revealed that while irregulars best coded high frequency information, regulars encoded low frequency information and the detailed time course of stimuli due to their low variability.

Considering the added benefits of employing a noise stimulation paradigm, a random noise stimuli within the relevant frequency range of natural mouse head motion was used for the first time in mice in this thesis to evaluate if mice afferents exhibit similar characteristics to those seen in primates. This paradigm provides information on coherence and mutual information of vestibular afferents which will allow further understanding of sensory signal processing in mice and could be potentially applied in a transgenic mouse model to better understand the effects of vestibular efferent impairment on afferent information transmission.

VII. Summary of the Goals of this Thesis

Altogether, characterizing vestibular responses in transgenic mouse models presents important information for elucidating the complexities of neuromodulator and receptor interactions at the vestibular efferent synapse. The first aim of this thesis was to characterize oculomotor and postural control pathways in transgenic mouse lines with vestibular efferent impairments to understand how loss of cholinergic efferent innervations would affect hair cell and afferent transmission of vestibular information. In particular, I focused on the possible contribution of α-9 to the impairments in inner ear development and vestibular information transmission with a α-9 and α-9/αCGRP transgenic mouse line as well as how the deficits previously established in αCGRP transgenic mice is present in other vestibular pathways involving postural regulation and head stabilization. In meeting its first aim, this thesis also provides a novel means of quantitatively characterizing vestibulospinal reflexes relating to head and postural stabilization that has not been previously used during standard motor tasks with mice. The second aim of this thesis was to demonstrate the effectiveness of a noise stimulation paradigm when performing single-unit recordings in mouse models to obtain novel information on sensory processing in rodents that has not been done before. This information would be invaluable for characterizing changes in information transmission of vestibular afferents due to loss of cholinergic innervations in transgenic mice. Collectively, the findings of this thesis provides information for future directions using transgenic mouse models for determining vestibular efferent function and presents novel approaches for developing an understanding of the role of the vestibular efferent system.

CHAPTER 2: BEHAVIOURAL ASSAYS FOR VESTIBULAR FUNCTION IN MICE

I. Statement of Contributions

I collected and analyzed all data presented in this study with the exception of the optokinetic nystagmus gains for CGRP (+/+) mice which were obtained through the contributions of Lisa Sun to Dr. Cullen's lab.

II. Introduction

The functional role of vestibular efferent feedback to the peripheral sensory organs is poorly understood. It was previously thought that the vestibular efferent system might distinguish active/passive motion at the periphery (Goldberg et al., 2000) or perhaps balance the left-right labyrinths (Cullen and Minor, 2002). However, studies found that vestibular afferent responses were the same during active and passive motion (Cullen and Minor, 2002; Jamali et al., 2009). In addition, changes in afferent responses to balance left-right labyrinths did not occur even after unilateral labyrinthectomy (Sadeghi et al., 2006).

Recently, it has been hypothesized that the vestibular efferents may play an important role mediating the development of the vestibular system (Sadeghi et al., 2006). This hypothesis draws much of its insight from current literature on the auditory efferent system; a system which has similar synaptic mechanisms to the vestibular efferents. In particular, both systems are cholinergic and while the auditory efferents are mediated primarily by inhibition through α-9/10 nAchRs, these receptors can also be found at vestibular efferent synapses (Johnson et al., 2013; Turcan et al., 2010). Loss of cholinergic efferent innervation in the auditory system has been shown to result in changes in maturation of hair cell responses (Johnson et al., 2013) and alterations in gene expression in hair cells during early stages of development (Murthy et al., 2009; Turcan et al., 2010). These findings suggest that auditory efferent innervation is essential for normal development of efferent targets. Accordingly, based on similarities between these systems, vestibular efferents may play a similar role for development of hair cells, afferents and the synapses between them. Therefore, loss of cholinergic efferent activity may consequently alter transmission of vestibular information by afferents as a result of developmental changes. Consistent with this hypothesis, a recent study from our group targeting αCGRP – a neuromodulator of cholinergic activity at vestibular efferent synapses – in mice found that loss of αCGRP impaired vestibulo-ocular reflex (VOR) efficacy (Luebke et al., 2014).

Altogether, we hypothesize that loss of efferent activity might impair inner ear development and consequently alter the transmission of vestibular information by afferents. Considering the developmental role of α-9 in the auditory efferent system, we suggest that similar attenuations of VOR gain to our previous study would be seen in α-9 deficient mice. As such, in this study, I first characterized VOR responses (Fig 2B) in α-9 and α-9/αCGRP transgenic mice. Secondly, given our hypothesis, other vestibular pathways should be similarly impaired by the loss of αCGRP. To address this, I tested whether the vestibular dysfunction of αCGRP transgenic mice would also be seen in motor pathways related to postural regulation and head stabilization (Fig 3). This was done using a rotarod and balance beam paradigm. In addition, a novel method of measuring head motion was introduced during the balance beam task – using a micro-electromechanical systems module (iNEMO; refer to methods in Carriot et al., 2014) – allowing us to detect six axis of head motion dynamics.

The findings from this study are consistent with the hypothesis that loss of cholinergic efferent innervation alters transmission of vestibular information by afferents, as seen in impaired responses in both VOR and spinal cord pathways. These effects appear to vary depending on the type of mutation, with greater affects in αCGRP null mutants compared to α-9 null mutants as seen in VOR responses. Even more surprisingly, loss of both neuropeptides appeared to rescue behavioural responses, suggesting far more complex interactions at the efferent synapse than what is currently understood.

III. Materials and Methods

Vestibular behavioural responses in wild-type and transgenic mice were tested using two main approaches: 1) eye movement recordings to quantify the efficacy of the vestibulo-ocular reflex and 2) motor tasks and head movement recordings to quantify postural control. The care and use of the animals in this study were approved by McGill University Animal Care Committee and in strict compliance with the guidelines of the Canadian Council on Animal Care.

A. Eye Movement Recordings in Mice

Transgenic mouse lines with altered efferent vestibular system signaling were tested in order to understand how α-9 in the vestibular efferent pathway affects optimization and efficacy of gaze stabilization in the same manner as αCGRP. Transgenic mouse lines with a target deletion of the α-9 subunit (VOR: 12-24 months; OKN: 5-20 months) or both α -9 and αCGRP (VOR and OKN: 16 to 20 months) were used.

1. Headpost surgery

In order to secure the head during testing, a custom-made aluminum headpost was fixed to the skull. Care was taken during the surgical procedure to avoid hypothermia or dehydration. The animal was anesthetized prior to surgery through an intraperitoneal injection of ketamine (10-1mg/g), atropine (5.10−4 mg/g), acepromazine maleate (2.5.10−2mg/g), xylazine (10−1mg/g), and sterile saline. An analgesic injection of carprofen (4mg/ml) was made subcutaneously 30 minutes prior to surgery. This surgery was done in accordance with the methods described by Luebke et al. (2014). Briefly, an incision was made to expose the surface of the skull. The pericranium was then removed and a custom-made aluminum headpost was secured to the bregma of the skull using adhesive cement (C&B Metabond). Analgesic injections of carprofen (4mg/ml) was administered subcutaneously every 24 hours for the next 48-72 hours while the animal recovered.

2. Eye Movement Data Acquisition

To record the VOR (α-9: n=20; α-9/αCGRP: n=16), the animal was restrained within a custom-made Plexiglass tube and their head secured with screws via the headpost while pitched at 35° to align the horizontal semicircular canals with the horizontal plane (Calabrese and Hullar, 2006; Vidal et al., 2004). The animal was then place onto a servo-motor turntable driven by a servo-motor on which an infrared camera system (iSCAN ETL-200) was secured to track and record their eye movements. VOR was evoked by sinusoidal rotations in the horizontal plane at 0.2, 0.5, 1.0, 2.0 and 4.0Hz in the dark at a peak velocity of 15°/s. To record the OKN (α-9: n=7; α-9/αCGRP: n=16), a drum with high contrast 5° width vertical stripes was set on the turntable enclosing the animal. The drum was sinusoidally rotated in light at 0.2, 0.5, 0.75, 1.0 and 2.0Hz at a peak velocity of 8°/s. Data acquisition and turntable movements were controlled using REX, a QNX-based real-time data acquisition system (Hayes et al, 1982). Pilocarpine (4% Alcon Canada) was applied to the target eye to maintain a consistent pupil size while testing (Iwashita et al., 2001). All eye movement recordings were taken from the left eye of each animal for consistency.

3. Eye Movement Analysis

Analysis was done using methods similar to those described in Luebke et al. (2014). Briefly, VOR and OKN gain and phase were determined by the least-squared optimization and plotted as mean ± SEM for all animals. All data was analyzed using MATLAB and significance was determined using a two-way ANOVA with Bonferroni corrections for VOR/OKN gain and phase. The quick phases of the vestibular and optokinetic nystagmus was removed when analyzing VOR and OKN. To compute the dynamics of quick phases in control and null mutant animals, I measured the peak velocity and amplitude of each quick phase. Peak velocity was then plotted against the peak amplitude (i.e., main sequence) and the best linear fit was computed. An unpaired T-test was used for the main sequence analysis to determine significance.

B. Motor Tasks and Head Movement Recordings in Mice

In order to investigate whether vestibular deficits from efferent impairment can be seen in pathways involving postural control, 129SvEv mice (Jackson laboratories; 1-4 months of age, n=3) and αCGRP null mutants (2 years of age, n=3) were used. Postural control during locomotion and balance were compared during balance beam and rotarod paradigms and head motion dynamics were compared during the balance beam task.

1. Headpost Surgery

Methods are the same as those described previously for eye movement recordings (Section A1).

2. Accelerating Rotarod Task

The mouse is placed on the rotarod for 5 minutes to acclimate to the environment. For each trial, the rotarod accelerates from 3 to 33rpm with a ramp of 180 seconds and maximum duration of 5 minutes. 6 trials are performed and between each trial the mouse is returned to its caged and allowed free access to food and water for 5 minutes. This is repeated for 4 days to evaluate both motor coordination and motor learning. All trials were recorded by video. The time and speed at which the animal fell off the rotarod were plotted as mean ± SEM and compared in EXCEL. T-tests were used to determine significant differences.

3. Balance Beam Task

For this task, a balance beam 1m in length with a diameter of 14mm made of wood was used. A card board box was used as the goal and covered to provide more incentive for the mouse. The start was set at 52.5cm above the ground while the goal box was placed at 60cm above the ground at a distance of 80.3cm to create an incline. A start and finish line was marked 60cm apart on the balance beam. To measure head motion while traversing the balance beam, the iNEMO – a micro-electromechanical systems module (refer to methods in Carriot et al., 2014) containing three linear accelerometers and three gyroscopes – was secured onto the headpost of the mouse. Once the iNEMO device was secured, the mouse was placed in the goal box for 2 minutes to acclimate to the environment. To train the mouse to perform the task, the mouse was placed just outside the goal box and encouraged to traverse the balance beam and return to the goal box. A 1 minute rest period was then granted for the mouse in the goal box. This process was repeated, while placing the mouse progressively further from the goal box, until it was able to traverse from the start to finish line without any help. 5 trials were conducted with 1 minute rest periods in between during which the mouse was in the goal box. All trials were recorded by video and the data from the six sensors of the iNEMO was sampled at 100Hz and recorded on a mircroSD card for analysis. Time of traversal were compared in EXCEL and head motion dynamics were analyzed through MATLAB. Both were plotted as mean ± SEM. Power spectrums were de-trended before comparison and T-tests were used to determine significant differences.

IV. Results

Recent findings from our group demonstrated the role of αCGRP – a neuromodulator of cholinergic efferent transmission – in mediating VOR efficacy (Luebke et al., 2014). In order to build on these findings, the first goal of my study was to characterize eye movement responses in α-9 and α-9/αCGRP transgenic mice. Since α-9 co-localizes with αCGRP at efferent synapses and is expressed specifically on irregular afferents, type I and type II hair cells (Kong et al., 2006), I hypothesize that the loss of α-9 would also impair the efficacy of the VOR as a consequence of developmental deficits in the inner ear. The second goal was to address whether αCGRP transgenic mice would have impairments in other vestibular pathways by characterizing postural regulation and head stabilization during the accelerating rotarod and balance beam tasks.

A. Eye Movement Dynamics in Mice

The VOR was measured and quantified in α-9 and α-9/αCGRP transgenic mice using a horizontal sinusoidal rotation paradigm. OKN and quick phase saccadic eye movements were characterized as controls for possible impairments downstream of vestibular efferent innervation either at the vestibular nuclei (OKN) or abducens (quick phase).

1. Alpha-9 (-/-) show VOR attenuation

Vestibular function in α-9 null mutants was first tested by quantifying VOR dynamics. Example traces of eye velocity and head movement velocity during VOR stimulation in the dark at 0.2Hz are shown in figure 3B. Quick phase eye movements were removed prior to computing gain (Fig 3B). While α-9 controls had gains ranging from 0.21±0.04 at 0.2Hz to 0.92±0.09 at 4.0Hz, α-9 null mutants had gains ranging from 0.15±0.03 at 0.2Hz to 0.68±0.06 at 4.0Hz (Fig 3C). Significant attenuation in VOR gain was only seen at 4.0Hz ($P<0.05$) within α-9 null mutants (%22.65) contrary to previous findings by our group with the αCGRP transgenic mouse line (Luebke et al., 2014). Interestingly, the phase of the VOR was observed to be unaffected by this mutation (Fig. 3D), displaying a standard trend of decreasing phase lead from 20.9±3.70° at 0.2Hz to a phase lag of -6.19±3.92° at 4.0Hz in null mutants, similar to αCGRP null mutants. Thus, looking at the attenuation in gain, these results suggest that the α-9 subunit mainly influences high frequency (>4.0Hz) optimization of VOR responses.

2. Alpha-9 strain exhibit visual-motor impairments

To ensure that these mutations did not affect neuronal projections downstream of vestibular afferents in the VOR pathway, two control paradigms were used. First, quick phase eye movements were analyzed. This was done by comparing the peak velocities and amplitudes of saccades that occurred during the VOR in a main sequence analysis (Fig 5E; Hubner et al., 2013). The results showed no significant differences ($P=0.66$) between the mutant mice and controls in this regard, where mutants had a slope of 47 while controls had a slope of 52. As a second control, the OKN response was also characterized. As reviewed above, the OKN pathway serves to stabilize gaze in response to visual motion cues and converges with the VOR pathway at the vestibular nuclei (recall Fig 2B, green). Recordings of OKN were made in a subset of alpha-9 mice (Fig 6). Notably, the OKN gains of both null mutants and controls of the α-9 mouse strain were much lower as compared with αCGRP controls. These results reveal that visual-motor impairments are present even in the controls for this mouse strain. This then indicates that this mouse model is not useful for our research, since the control mice do not appear to be “normal”.

3. Alpha-9/CGRP (-/-) show no attenuation

Vestibular function in a second transgenic mouse line with a loss of both αCGRP and α-9 subunits was also characterized by VOR and OKN responses (Fig 7). Although these mice did not exhibit any visual-motor impairments (Fig 7C,D), as was seen in the α-9 strain, the null mutants lacking both peptides did not display any attenuation in VOR gains nor any changes to phase (Fig 6A,B; P>0.05). These results are surprising in light of our recent study showing that αCGRP loss resulted in significant attenuation of VOR gains (Luebke et al., 2014). Thus while independent loss of αCGRP and α-9 causes attenuation in VOR gain, our results demonstrates that loss of both αCGRP and α-9 together appears to “rescue” the VOR impairments seen previously. Altogether, these findings suggest a far more complex interaction between neuromodulators and receptors at the efferent synapse than previously known.

B. Motor Tasks and Head Movement Dynamics in Mice

The second goal of this study was to determine whether other vestibular pathways involved in postural regulation and head stabilization in αCGRP mice would be impaired as well, given the hypothesis that loss of efferents result in impaired inner ear development and deficits in transmission of vestibular information by afferents. Note, I did not fully test the α-9 mice given the issues with the littermate controls discussed above. Motor coordination was characterized both by observed behaviour, as is the standard, and through the iNEMO device which recorded 6 dimensions of head motion (recall Fig 4).

1. CGRP (-/-) show impairment in motor tasks

Both the time and speed when the mouse fell of the rotarod was recorded and compared. αCGRP null mutants performed significantly poorly compared to their controls ($P<0.001$), with an average time of fall of 70.20±3.35 seconds and an average speed when falling of 5.92±040 rpm. In comparison, 129SvEv controls had an average time of fall of 171.00±4.32 seconds and their average speed when falling was 24.84±1.74 rpm. Overall, 129SvEv controls could out last the αCGRP null mutants on the accelerating rotarod task on average by 100.76±6.82 seconds and could handle speeds 18.92±2.03 rpm faster. 129SvEv controls also showed improvements on the 2nd day of testing both in time and speed ($P<0.05$), whereas αCGRP (-/-) mice showed no improvements across all 4 days of testing.

Similarly, αCGRP null mutants performed far worse on the balance beam as compared to their controls. Mice were timed while traversing a distance of 60cm on the balance beam to reach the goal box. In comparing the time to traverse the balance beam (Fig 8A) between 129SvEv control strains and αCGRP null mutants, it was evident that null mutants were significantly slower (9.27±0.53 seconds) relative to controls (4.20±0.40 seconds; $P < 0.0001$) by an average of 5.07±0.55 seconds. αCGRP null mutants were noticeably less stable while performing the balance beam task and much slower in comparison to the 129SvEv controls.

2. CGRP (-/-) have different head motion dynamics during balance beam task

Through the micro-electromechanical systems module (iNEMO; Carriot et al., 2014) containing three linear acceleration and three angular velocity sensors, six axis of motion (fore aft, lateral, vertical, roll, pitch and yaw) were recorded. The head motion dynamics were characterized by computing power spectrum densities. For comparison, the average power at frequencies of ≤2.0Hz in the axes of translation (fore aft, lateral, and vertical) and frequencies of ≤10.0Hz in the axes of rotation (roll, pitch, and yaw) were computed (Fig 8B). These ranges were chosen because they had the most power for translation and rotation, respectively(Fig 8A, grey area). During the balance beam task, 129SvEv control strains showed little power (Fig 8A and B, blue), especially in the fore aft, lateral and vertical dimension, and tended to have relatively flat power spectrums. On average, αCGRP null mutants had approximately 5 times more power during translation and 2 times more power during rotation (Fig 8 B). These findings provide additional information regarding the poor performance of the αCGRP mice and their higher power spectrum densities illustrate that these mice are much more unstable while performing the balance beam task.

IV Discussion

The purpose of this study was to characterize oculomotor and postural control pathways in transgenic mouse models with impaired vestibular efferent systems. Based on recent literature, we hypothesized that loss of cholinergic efferent innervation would result in deficits in the development of the inner ear and consequently impair transmission of vestibular information by afferents. To test this, I characterized vestibular function by testing VOR responses and performance on the rotarod and balance beam. The principal findings of this study were that (1) loss of the α-9 subunit impairs VOR efficacy, though not to the same degree as αCGRP null mutant mice, (2) null mutations for both α-9 and αCGRP appear to "rescue" gain attenuation in VOR, and (3) vestibular dysfunction in αCGRP transgenic mice is also observed in vestibulospinal pathways involving postural control.

A. Alpha-9 and CGRP in the VOR pathway

The VOR pathway stabilizes gaze in the event of head motion and is a very well-studied pathway mediated by a fast neuronal reflex through a simple three-neuron arc (Huterer and Cullen, 2002). Due to the ease of testing this pathway, it is often used to evaluate vestibular dysfunction, which was the case for a previous study from our group (Luebke et al., 2014). This study was one of the first published works to characterize this pathway in the efferent impaired αCGRP transgenic mouse model in order to understand the functional role of αCGRP in the vestibular system. Characterizations of VOR dynamics in αCGRP transgenic mice showed significant attenuation in VOR gain over the frequency range tested (0.2 to 2.0Hz) in null mutants. Based on these findings, our group concluded that loss of αCGRP impaired VOR efficacy (Luebke et al., 2014) which is consistent with our hypothesis regarding the role of efferent innervation in inner ear development.

αCGRP lacks specificity however given its role in modulating cholinergic activation of the diverse acetylcholine receptors expressed at the vestibular efferent synapse. Of these receptors, α-9/10 nAchR has the most evidence for being involved in inner ear development based on studies in the auditory efferent system. Notably, α-9 appears to be involved in maturation of auditory efferent targets as well as gene expression during early stages of development (reviewed in Johnson et al., 2013; Turcan et al., 2010). Since the α-9/10 nAchR is expressed in efferent targets of the vestibular system as well, it was an ideal candidate for investigating the developmental role of vestibular efferents (reviewed in Jordan et al., 2013; Kong et al., 2013). In working with a line of α-9 transgenic mice, this study found that these mice similarly showed significant attenuation in VOR gain specifically at 4.0Hz. Though the attenuation in α-9 null mutants was not as drastic (~22.65%) as those seen in αCGRP null mutants (~50%), these findings demonstrate that loss of α-9 similarly reduces VOR gain. It is apparent, however, that the attenuation seen in α-9 null mutants constitutes only a portion of the effects seen in αCGRP transgenic mice and infers that other cholinergic signals modulated by αCGRP may be involved in vestibular end organ development, contrary to what is seen in the auditory efferent system.

Interestingly, when working with a transgenic mouse line deficient in both α-9 and αCGRP, this study found that loss of both peptides did not affect VOR dynamics. This contradicts our results described above involving α-9 as well as previous work from our group involving αCGRP mice (Luebke et al., 2014). Previous findings would suggest that loss of both neuropeptides would cause more – if not the same level – of attenuation in VOR gain. As such, it is particularly surprising that loss of both peptides appears to “rescue” the impairments seen when only α-9 or αCGRP is lost. Altogether, this emphasizes a lack of understanding in current literature of the interactions that take place at the vestibular efferent synapse between the different cholinergic efferent receptors. It would be of particular interest in the future to investigate other combinations of null mutations in efferent signaling to better understand how they influence each other and contribute overall to the development of the inner ear.

B. Concerns with the alpha-9 mouse model

Through my study, I identified a major concern regarding the use of one of the mouse models for the purposes of vestibular research. In particular, while characterizing the oculomotor pathways, this study found that this particular α-9 transgenic mouse line – commonly used to investigate auditory efferent responses – shows visual-motor impairments in their control littermates. Characterization of OKN responses in α-9 control mice across a range of ages (5 – 20 months) demonstrated a significant attenuation in OKN gain when compared with normal mice in the literature (Faulstich et al., 2004; Andreescu et al., 2005; Katoh et al., 2005; Alphen et al., 2010) as well as the αCGRP control mice – clearly illustrating that the α-9 controls are not “normal”.

These results indicate that this mouse model cannot be used to investigate vestibular function. Firstly, visual-motor impairments suggest mutations in the OKN pathway, which converges with the VOR pathway at the level of the vestibular nuclei. As such, unforeseen mutations in the vestibular nuclei in this transgenic mouse line could influence the VOR responses observed here. Secondly, vision has been demonstrated to play a role in affecting gaze stabilization dynamics. For example, studies have shown specifically that visual impairments during development can contribute to overall instabilities in gaze in adulthood (Kompf and Piper, 1987; Schneider et al., 2013). Notably, vision can also mediate adaptation in VOR responses, as demonstrated in a study by Kimpo et al (2005). By either rotating the visual surround with the head motion or opposite to the head motion, visual stimuli will down or up regulate (respectively) VOR gains through training. VOR dynamics therefore change in response to visual stimuli. As such, the poor vision of the α-9 transgenic mouse model could affect how VOR responses are naturally shaped from birth to adulthood. Though the attenuation seen in this model proves promising for determining its role in efferent signaling, this study illustrates a necessity to re-derive a cleaner α-9 transgenic mouse line to better understand the role of α-9 in vestibular efferent function in the future.

C. CGRP (-/-) perform poorly during motor tasks

By observation alone of foraging behaviours of the αCGRP transgenic mice, it was not apparent that these mice exhibited any deficits in locomotion or postural control compared to other transgenic mouse models of vestibular impairment such as mice deficient in Raldh3, which have tendencies to display tilting/circling behaviour (Romand et al., 2013). However, as previously mentioned, αCGRP null mutants have shown significant attenuation in VOR gain from 0.2 to 4.0Hz (Luebke et al., 2014). Given the apparent influence of efferent-mediated responses in the VOR efficacy consistent with how loss of efferents might alter information transmission by afferents, we predict that these animals would also show impairments in behaviours controlled by other vestibular pathways (e.g. vestibulospinal pathways).

To address this possibility, standard motor tasks – the accelerating rotarod and balance beam – were used to compare performance of 129SvEv controls to the αCGRP transgenic mouse line. The findings of this present study show that the αCGRP transgenic mouse line performed significantly worse compared to the 129SvEv controls during both the accelerating rotarod and balance beam task. During the rotarod task, αCGRP transgenic mice were less responsive to the motion stimuli of the rotarod and unable to keep up with the rotarod acceleration. Similarly, these mice were considerably less stable (exhibiting more paw slipping) while traversing the beam. These results suggest that loss of αCGRP, an important neuromodulator of cholinergic signals in the efferent pathway, not only impairs VOR efficacy (Luebke et al., 2014) but also impairs postural controls related to balance in the vestibular system, consistent with our hypothesis.

D. CGRP (-/-) exhibit different head motion dynamics

Through the use of the iNEMO module (Carriot et al., 2014), in combination with the standard balance beam task, I was able to characterize head motion dynamics in six axis in αCGRP transgenic mice while their postural reflexes were challenged. The use of the iNEMO provides a novel approach to quantitative measure of the mouse's motor performance during a vestibular dependent behaviour. More specifically, it can quantify head stability which is mediated by the vestibulocollic reflex (Grande et al., 2010; Mitchell et al., 2013; reviewed in

Goldberg and Cullen, 2011). During foraging, wild-type mice head movements typically have the most power at frequencies of ≤4.0Hz in all axes (Beraneck et al., 2008). During the balance beam task however, both 129SvEV controls and αCGRP null mutants appeared to have the most power at lower frequencies during translation (<2.0Hz) and higher frequencies during rotation (<10Hz) relative to the foraging head dynamics.

As previously mentioned, when looking at the traversal time of both controls and null mutants across the balance beam, it is clearly evident that αCGRP null mutants perform significantly worse in comparison. Similarly, these differences can be seen in the head motion dynamics of αCGRP null mutants while on the balance beam. With significantly higher power overall at low frequencies (<2.0Hz for linear motion, <10.0Hz for angular motion), the instability across all dimensions of head motion in αCGRP null mice when traversing the balance beam is apparent in the power spectrum densities. This finding provides additional information on motor performance through the iNEMO and illustrates that αCGRP null mutants perform poorly overall during balance tasks, which suggest that loss of αCGRP not only affects eye movement pathways of the vestibular system but also balance. This is consistent with hypothesis that information transmission by afferents is impaired in these mice.

While apparent age-related controls were not available to better compare the results of the αCGRP null mutants, these findings do illustrate that when the vestibular system is challenged head motion dynamics will change noticeably in αCGRP null mice. A difference that is apparent when observing the balance beam task, but not during natural foraging behaviours. With the additional information gained from measuring head movements, the potentially different effects on motor performance in transgenic mice can be more accurately quantified. For example, given the differences in the frequency range of attenuation of VOR gain for αCGRP and α-9 transgenic mouse lines, it would be of interest to look at how these mutations might have different effects on head motion dynamics.

V. Conclusion

The use of transgenic mouse models for the purpose of investigating the effects of vestibular efferent impairment has not been widely used. As such, this study hoped to gain more insight into the functional role of this pathway by performing behavioural assays of vestibular functions in αCGRP and α-9 transgenic mouse models. Given the apparent role of α-9 in the inner ear development of the auditory system (Johnson et al., 2013; Turcan et al., 2010), it was presumed that α-9 would likely cause similar attenuation in VOR dynamics to what was seen previously in αCGRP null mice (Luebke et al., 2014). This study concludes that loss of the α-9 subunit appears to impair VOR efficacy particularly at 4.0Hz (22.65%), which can only explain a portion of the attenuation seen in αCGRP transgenic mice (~50%) contrary to what we expected given the literature on the role of α-9 in the auditory efferent system. However, due to the visual-motor impairments in this transgenic mouse line (as seen in the both controls and null mutants alike), it is necessary to re-derive a cleaner model for the purposes of our research to better understand the role of α-9 in the development of vestibular hair cells and afferents.

Furthermore, while attenuation in VOR dynamics was seen in both α-9 and αCGRP transgenic mouse models, a deficiency of both neuropeptides appeared to “rescue” the VOR dynamics. This contradiction infers a more complex network of signaling occurring at efferent synapses that is not presently understood. Behavioural assays of postural control pathways in the αCGRP transgenic mouse line also indicate that loss of αCGRP not only impairs VOR efficacy, but also impairs regulation of posture and head stabilization. These are consistent with the hypothesis that cholinergic efferent transmission is necessary for development of hair cell to afferent synapses wherein loss of efferent innervations would alter information transmission by afferents. By characterizing these vestibular functions, not only can we better understand the influence efferent-mediated cholinergic responses has in the vestibular system, but combinations of transgenic mouse models of essential efferent synaptic mechanisms can be tested to further refine the present insights on vestibular efferent function and its role in hair cell and afferent development and synaptic strength.

CHAPTER 3: VESTIBULAR AFFERENT RESPONSE DYNAMICS IN MICE

I. Statement of Contributions

All data presented in this study was collected with the help of Vladimir Grouza and Yiran Chen. In addition, the analysis of vestibular afferent responses to Gaussian noise stimulation was completed with the help of Yiran Chen.

II. Introduction

Peripheral sensory organs detect head motion and orientation relative to gravity through hair cells. These hair cells in turn transfer the information to primary afferent neurons through chemical synaptic transmission. In most vertebrate species, afferent neurons have a bimodal distribution of resting rate firing regularity such that they can be separated into regulars and irregulars. These two types of afferents are not only characterized by their distinct spontaneous discharge properties but also by morphological features at peripheral terminations (reviewed in Holt et al., 2011; Eatock and Songer, 2011).

Single-unit recordings from primary afferent neurons *in vivo* in mice have provided valuable insight into how sensory information is encoded in the vestibular system. In addition, with the ability to perform genetic manipulations in mouse models, the effects of vestibular impairments and their underlying physiology can be easily studied through afferent recordings in mice (Lasker et al., 2008). As such, this provides an approach for investigating how a lack of efferent innervations might cause developmental changes that alter transmission of vestibular information by afferents in transgenic mice. Conventionally, relatively simple stimulus paradigms have been used to characterize vestibular afferent properties. In particular, studies have applied sinusoids of linear acceleration to drive otoliths or sinusoids of angular velocity to drive the canals (reviewed in Cullen, 2012; Lasker et al., 2008; Yang and Hullar, 2007).

The use of these stimulation paradigms can provide information on sensitivity and phase of vestibular afferents. Overall these studies have found that regular afferents in mice tend to have lower sensitivities and higher phases lead as compared to irregular afferents. However, a limitation of the use of sinusoidal stimulus to characterize afferents is that their response variability is averaged out so little information is extracted about neural variability or the information content of the afferents. Taking these limitations into consideration, Sadeghi et al. (2007) applied a random head motion stimuli with a Gaussian noise distribution instead to stimulate afferent responses in macaque monkeys. In doing so, they were able to not only determine sensitivity and phase of afferent response dynamics, but also information transmission of afferent neurons.

Here I used a similar random noise stimuli over the relevant frequency range of a mouse's natural head motion (≤4.0Hz; Beraneck et al., 2008) to stimulate afferent neurons during single-unit recordings. I found that sinusoidal stimulation at discrete frequencies within this same range was also used as a comparison. Sensitivities and phase were comparable to those seen during sinusoidal stimulation and consistent with those described in normal mice in previous studies (Lasker et al., 2008; Yang and Hullar, 2007). In addition, using information theoretical analysis, I further show that regular afferents consistently transmitted more information than irregular afferents in mice at ≤4.0Hz, similar to those in macaque monkeys. This study shows for the first time that, like primates, differences in neural variability contributes to the neural code for vestibular sensory processing in rodents. Altogether these findings are useful for characterizing how loss of efferent signals might alter transmission of vestibular information by afferents.

III. Methods

129SvEv mice (Jackson laboratories; 3-4 months) were used to characterize vestibular afferent signaling through single-unit recordings in response to random yaw head motion and sinusoidal yaw rotations. A total of 22 afferents (regulars: n=18; irregulars: n=4) that innervated the left semicircular canals were recorded during noise stimulation and a total of 4 afferents (regulars: n=2; irregulars: n=2) that also innervated the left semicircular canals were recorded during sinusoidal stimulation in six 129SvEv mice. The care and use of the animals in this study were approved by McGill University Animal Care Committee and in strict compliance with the guidelines of the Canadian Council on Animal Care.

A. Single-Unit Afferent Recording Surgery

In order to access the nerve, the mouse was anesthetized with an intraperitoneal injection of ketamine (10-1mg/g), atropine (5.10−4 mg/g), acepromazine maleate (2.5.10−2mg/g), xylazine (10−1mg/g), and sterile saline. An analgesic injection of carprofen (4mg/ml) was made subcutaneously 30 minutes prior to surgery. Care was taken during the surgical procedure to ensure the animal did no experience hypothermia or dehydration. The surgeries were done in accordance to the methods described by Lasker et al (2008). Briefly, the interparietal and occipital area on the left side of the skull were exposed. A craniectomy was performed to reveal the cerebellum. The inferior semilunar lobule, flocculus and paraflocculus were aspirated to expose the temporal bone. The brainstem was then retracted to reveal the vestibular nerve as it exits the internal auditory meatus.

B. Single-Unit Recording Data Acquisition

Once exposed, glass electrodes (with impedences of 10-20 MOhms) filled with 3M NaCl were inserted into the nerve in order to obtain single-unit recordings. The electrode was positioned in place using a manipulator and advanced into the vestibular nerve as it exited the internal auditory meatus using a microdrive mounted onto the stereotaxic frame. The stereotaxic frame was attached to a servo-motor turntable, that was computer controlled (REX real time operating system). Signals were amplified and band-pass filtered (400Hz-5000Hz). An external auditory speaker and oscilloscope were used to identify neuronal activity during recording sessions. Single-unit activity was recorded through a Plexon system at a sampling rate of 20kHz for offline analysis. Angular head velocity was recorded using a gyroscope mounted onto the turntable. In order to drive afferent activity, Gaussian noise stimuli from at ≤4.0Hz with an SD of 10°/s was used. This noise was chosen because it spanned the natural frequency range of motion produced by mice (≤4.0Hz; Beraneck et al., 2008). For comparison, sinusoidal stimuli of 0.2, 0.5, 1.0, 2.0, and 4.0HZ with a peak velocity of 10°/s were also applied. Raw spike signals were then sorted offline and analyzed through MATLAB. Coefficient of variance (CV) and CV* calculations (refer to methods in Lasker et al., 2008) are used to differentiate between regular and irregular afferents.

C. Single-Unit Recording Data Analysis

All data was analyzed using MATLAB and sensitivity, phase lead, coherence and mutual information were all plotted as mean ± SEM. Significance between the sinusoidal stimulation paradigm and the random noise stimulation paradigm was determined by comparing nonlinear fits of sensitivity and phase in regular and irregular afferent populations (Lasker et al., 2008). Responses to noise stimuli were also characterized using two alternate measures as described by Sadeghi et al., 2007. The first was coherence, *C(f)*, which compares the head velocity and spike train to measure the strength of the response to the stimulus at a given frequency. The second was the mutual information (MI) rate density, which was normalized by the mean firing rate.

IV. Results

The goal of this study was to characterize vestibular afferent responses through single-unit recordings in normal 129SvEv wildtype mice (Fig 10A) using noise stimuli to provide novel information regarding vestibular sensory processing in mice. Specifically, we applied a random noise and sinusoidal stimuli within the frequency range of natural mouse head motion (≤4.0Hz; Beraneck et al., 2008). We then computed the sensitivity and phase of the afferent responses to both stimulus paradigms and compared them to ensure that afferent response dynamics are comparable between these paradigms. Lastly, we computed coherence and mutual information in response to vestibular stimulation for the first time in mice to determine the information content of regular and irregular afferents. By quantifying coherence and mutual information of afferents, we can better understand how loss of efferent innervation might affect transmission of vestibular information by afferents.

A. Static Firing Properties of Afferent Population

Results from single-unit afferent nerve recordings were obtained from 129SvEv control mice. Example static firing properties of a regular and irregular afferent are shown in figure 10, panel B to F. The resting rate for regular afferents was 51.88±16.24 spikes per second (mean±SD, n=18) and the resting rate for irregular afferents was 42.06±22.93 spikes per second (mean±SD, n=4). Regular afferents typically showed less variability in their inter-spike interval (Fig 10B) which is also reflected in their spike train power spectrum (Fig 10C) by the peaks at their resting firing rate (~50Hz) as well as at integer multiples of that frequency. In contrast, irregular afferents showed greater variability (Fig 10E) as reflected the lack of peaks in their spike train power spectrum (Fig 10F). Overall, static firing properties of the afferent neurons were robust and consistent with those described in Lasker et al (2008), whose surgical procedure was replicated for this study. The average CV* for regular and irregular afferents was 0.06±0.01 and 0.24±0.16 respectively (Fig 11A).

B. Afferent Response Dynamics to Sinusoidal and Noise Stimuli

Response dynamics of the afferent responses to sinusoidal stimulation (Fig 10B, C) recorded in this study were comparable to those described in Lasker et al (2008) as well. Irregular afferent responses were increasingly more sensitive to higher frequencies whereas regular afferents had lower sensitivities that were relatively consistent across all tested frequencies (Fig 11B). In addition, irregular afferents consistently had higher phase lead compared to regular afferents (Fig 11C). Similarly, afferent responses to noise stimulation showed the same trends. Irregular afferents had sensitivities ranging from 0.18 to 0.34 spikes per second/degree per second (Fig 11D, black) while regular afferent sensitivities remained between 0.15 to 0.19 spikes per second/degree per second across all tested frequencies (Fig 11D, grey). Irregulars also consistently had higher phase leads compared to regular afferents (Fig 11E). In comparing the nonlinear fits of afferent responses to sinusoidal and noise stimulation, regular and irregular afferent responses showed no significant differences ($P>0.05$) in both sensitivity and phase. Overall, these findings demonstrate that the use of noise stimuli did not affect the response dynamics of the afferent population when compared with conventional sinusoidal stimuli in mice.

C. Mutual Information and Coherence

By using a random noise stimulus, information content of regular and irregular afferents can be characterized in a way that is not feasible through sinusoidal stimulation paradigms alone. More specifically, we can calculate coherence and mutual information for regular and irregular afferent populations to determine the information content across all frequencies. Looking at coherence and mutual information obtained from the noise stimulation paradigm, it is apparent that regular and irregular afferents not only have different response dynamics, but also have different information content (Fig 12A, B; coherence and mutual information respectively). Over the frequency range (≤4.0Hz) that was tested, regular afferents consistently transmitted more information as compared to the irregular afferents. These findings are consistent with data collected from the macaque monkey, in which regulars consistently contained more information in their rate coding compared to irregulars, particularly at lower frequencies (<5Hz; Sadeghi et al., 2007). Altogether, these results show for the first time that differences in neural variability contribute to the neural code for vestibular sensory processing in mice.

V. Discussion

Although behavioural tasks (e.g., balance beam) are able to characterize functional changes in the vestibular system in transgenic mice easily and non-invasively, they provide relatively limited information regarding the underlying physiology causing these changes. For the purposes of my thesis, it is not possible to identify precisely how cholinergic efferent innervations are changing the peripheral sensory system and altering transmission of vestibular information by afferents through these methods alone. To gain insight into the effects of cholinergic efferent innervations, single-unit afferent recordings were used since they offer more insight into the effects of vestibular efferent impairment at the peripheral synapses between hair cells and afferents. Specifically, the goal of this study was to characterize the transmission of vestibular information by afferents in normal mice to gain insight on how afferent responses might be affected by cholinergic efferent innervation.

Most conventional stimulation paradigms of mouse vestibular afferents have used relatively simple motion stimuli, such as sinusoids of linear acceleration or angular velocity (reviewed in Cullen, 2012; Lasker et al., 2008; Yang and Hullar, 2007). Although sensitivity and phase can be computed from these paradigms, they provide limited information on information transmission by afferents since response variability of the afferents is averaged. To address this, Sadgehi et al (2007) used a random noise stimulation paradigm in macaque monkeys to compute both coherence and mutual information. These computations allowed them to demonstrate that response variability plays a role in neural coding in primates. Accordingly, in order to obtain novel information on the information content of afferents in mice, a similar Gaussian noise stimuli was used in this study.

This study found resting rate properties for regular and irregular populations similar to those described in Lasker et al (2008). Sensitivity and phase of afferent responses in 129SvEv control mice were also comparable to Lasker et al (2008) and no significant differences were found when comparing afferent response dynamics between noise and sinusoidal stimuli. More importantly, looking at coherence and mutual information at frequencies ≤4.0Hz, it was evident that regular afferents had higher information content compared to irregular afferents – similar to those in macaque monkeys (Sadeghi et al., 2007). Comparisions with the mutual information of afferents in the primate model did also reveal that primates consistently encoded more information, especially in regular afferents at higher frequencies (~60% more at 4.0Hz; Sadeghi et al., 2007), demonstrating differences in the information content of vestibular afferents between species.

Overall, this study illustrates for the first time that differences in neural variability contribute to the neural code for vestibular sensory processing in a rodent model. Interestingly, previous work in primates have shown that efferent-mediated responses in regular afferents are smaller (2 spks/s) than those seen in irregular afferents (10 spks/s; Sadeghi et al., 2009). However, since information content within the natural frequency range of mouse head motions (≤4.0Hz) was mostly encoded in regular afferents, our findings suggest that loss of cholinergic efferent innervations would have a greater impact on the transmission of vestibular information by regular afferents compared to irregular afferents. Although these findings may seem inconsistent, work by Sadeghi et al (2007) have shown that addition of spikes effect information content of regulars much more than irregulars. As such, it is possible that smaller efferent-mediated inputs to regular afferents are enough to influence their information transmission. Based on these findings, I hypothesize that loss of cholinergic efferent innervation would result in a decrease in information content, particularly in regular afferents, and attenuation in sensitivity, particularly in irregular afferents. Random noise stimulation during single-unit afferent recordings in transgenic mouse lines with impaired vestibular efferent innervations would provide more insight into how development of the inner ear might be altered by the vestibular efferent system.

VI. Conclusion

Altogether, more information can be gained from using a random noise stimulation paradigm compared to sinusoidal stimulation. This study found that noise stimulation paradigms allows us to compute information content of regulars and irregulars while also robustly characterizing sensitivity and phase responses. It was shown for the first time that regular afferents encoded more information at ≤4.0Hz as compared to irregular afferents in mice, similar to afferent responses in macaque monkeys (Sadeghi et al., 2007). This characterization of afferents can help identify even more specific changes in transmission of vestibular information by afferents and possibly elucidate how α-9 and αCGRP affect the development of regular and irregular afferent responses differently given the differences in their impact on vestibulo-ocular reflex efficacy.

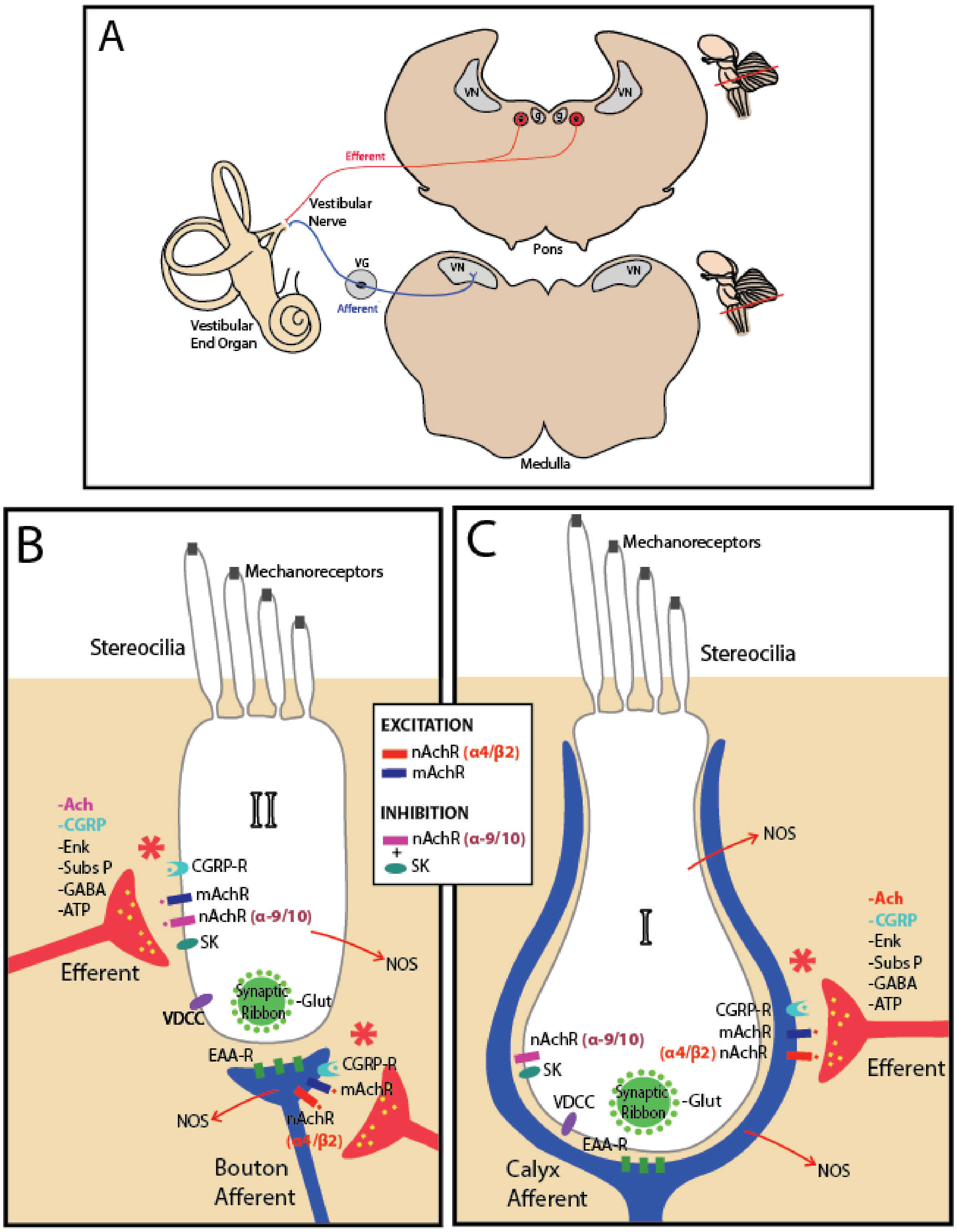

Fig 1. Schematic of vestibular efferent and afferent projections in the central nervous system (A) and in vestibular sensory epithelium (B: Type II hair cell; C: Type I hair cell). Efferent innervation is found on type II hair cells, bouton and calyx afferents (Red asterisks). Efferent synapses (Red) are mainly cholinergic (Ach) and produce a variety of neuromodulators including CGRP, enkephalin (Enk), substance P (Subs P), GABA and ATP. Ach synapses onto muscarinic and nicotinic acetylcholine receptors (mAchR; nAchR) found on efferent targets to elicit either inhibitory (ex. Hyperpolarize hair cells which decrease voltage-dependent calcium channel [VDCC] activity and glutamate [Glut] release on excitatory amino acid receptors [EAA-R]) or excitatory post-synaptic potentials (ex. Increase afferent discharge).

Species	Efferent-mediated Afferent Response	Mature Efferent Innervations			
		Type I HC	Type II HC	Calyx Aff. (Type I)	Bouton Aff. (Type II)
Frogs	**Inhibition/Excitation**	-	√	-	-
Turtles	**Inhibition/Excitation**	-	√	√	√
Birds	**Inhibition/Excitation**	-	√	√	√
Fish	**Excitation**	-	√	-	√
Mammals	**Excitation**	-	√	√	√

Table 1. Summary of differences between species in afferent responses to efferent stimulation and efferent innervations in the vestibular end organ (“ - ” means no efferent innervation; “ √ ” means there is efferent innervation).

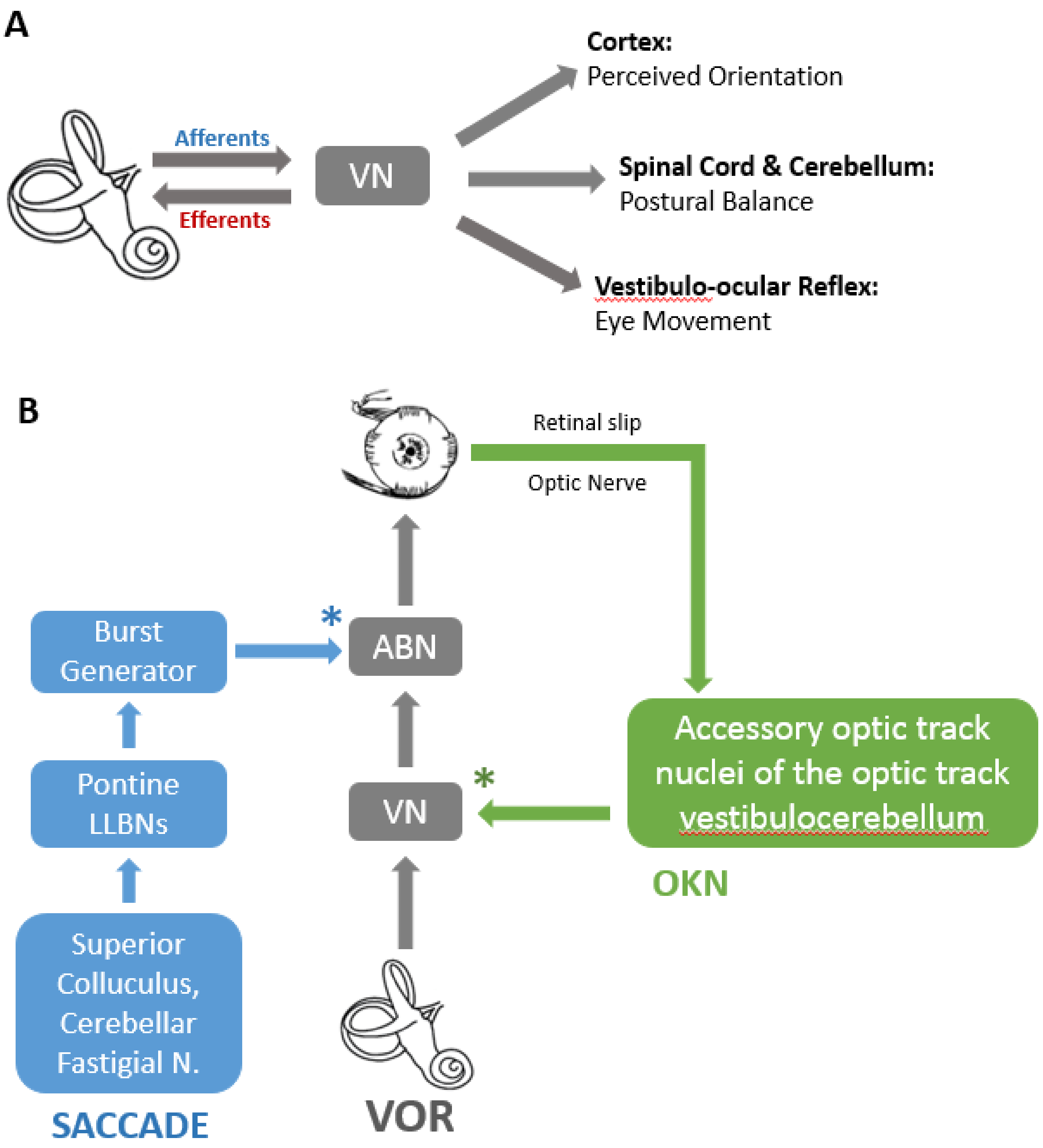

Fig 2. (A) Overview general vestibular pathways and their functions. (B) Oculomotor system pathways for gaze stabilization. VOR (Gray) and OKN (Green) pathways induce eye movements to stabilize gaze. The slow phase of VOR (Gray) consist of a three-neuron arc projecting from the vestibular end organs to the vestibular nucleus (VN), abducens (ABN) and oculomotor neurons. The quick phase of VOR (Saccade, Blue) is mediated by burst neurons which receive vestibular and visual inputs regarding eye position. The OKN pathway (Green) is induced by retinal slip and projects from the retina, to the optic track before synapsing onto the vestibular nucleus and projecting through the same pathway as the slow phase of VOR. Sites of convergence between pathways are noted in asterisks (saccades: blue; OKN: green)

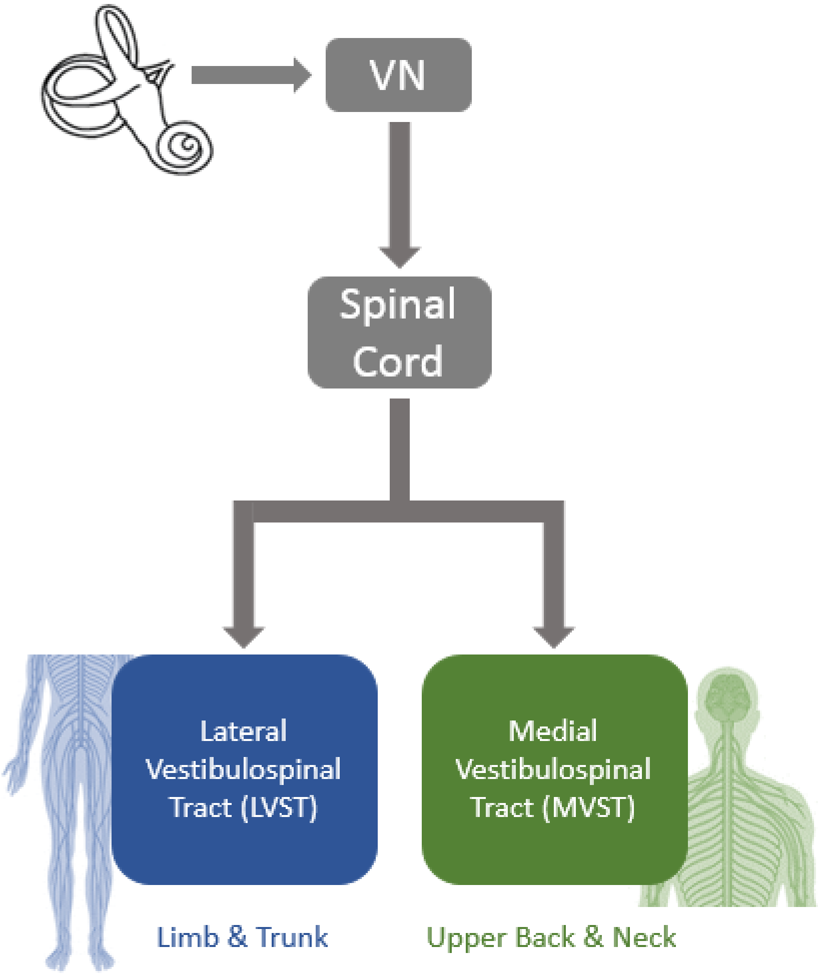

Fig 3. Vestibulospinal pathway and their functions. Vestibular sensory information project to the medial and lateral spinal cord pathways to regulate posture (Limb and trunk; Blue) and head stabilization (Upper back and neck; Green) respectively.

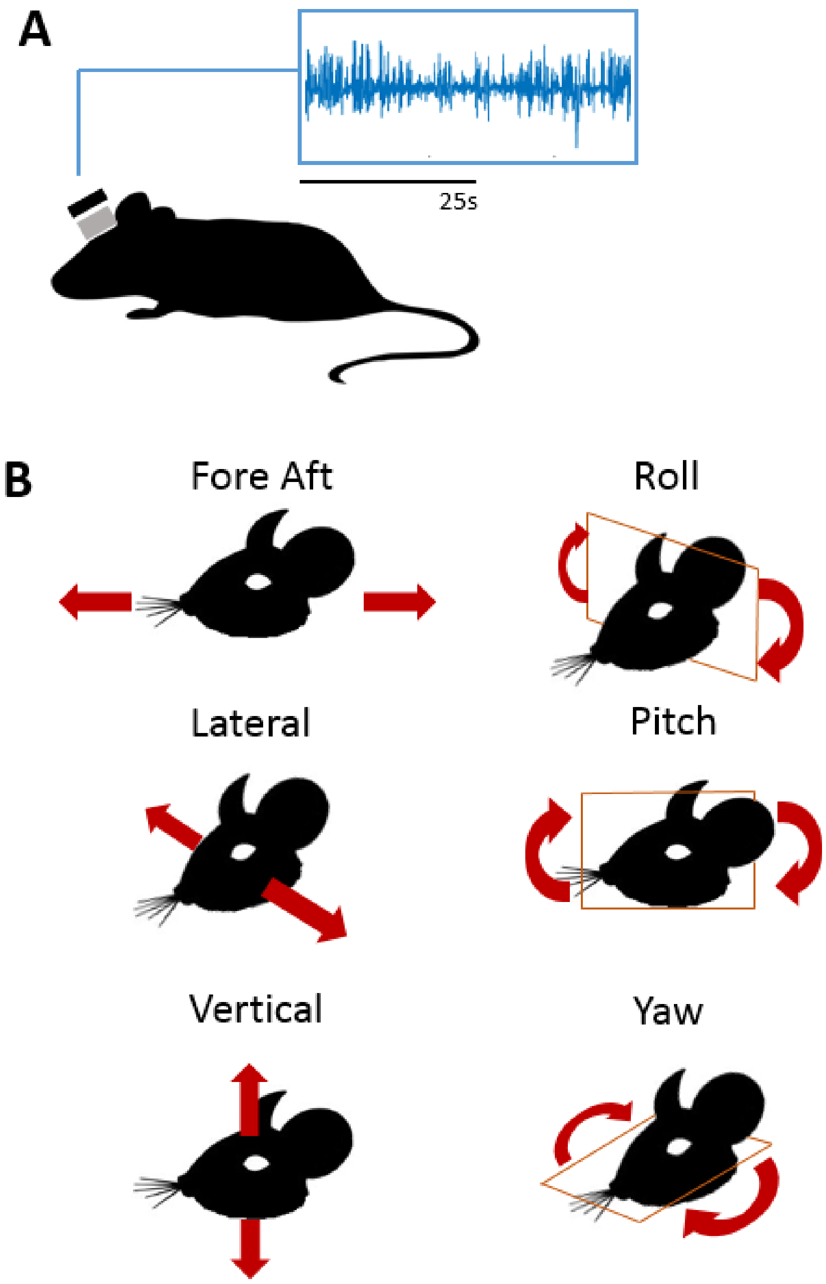

Fig 4. iNEMO head motion dynamics recording device. (A) Schematic of iNEMO secured onto headpost of mouse which records acceleration (for fore aft, lateral and vertical dimensions) or velocity (for roll, pitch and yaw). An example velocity trace is shown. (B) Schematic representation of each direction of motion that is recorded through the iNEMO device.

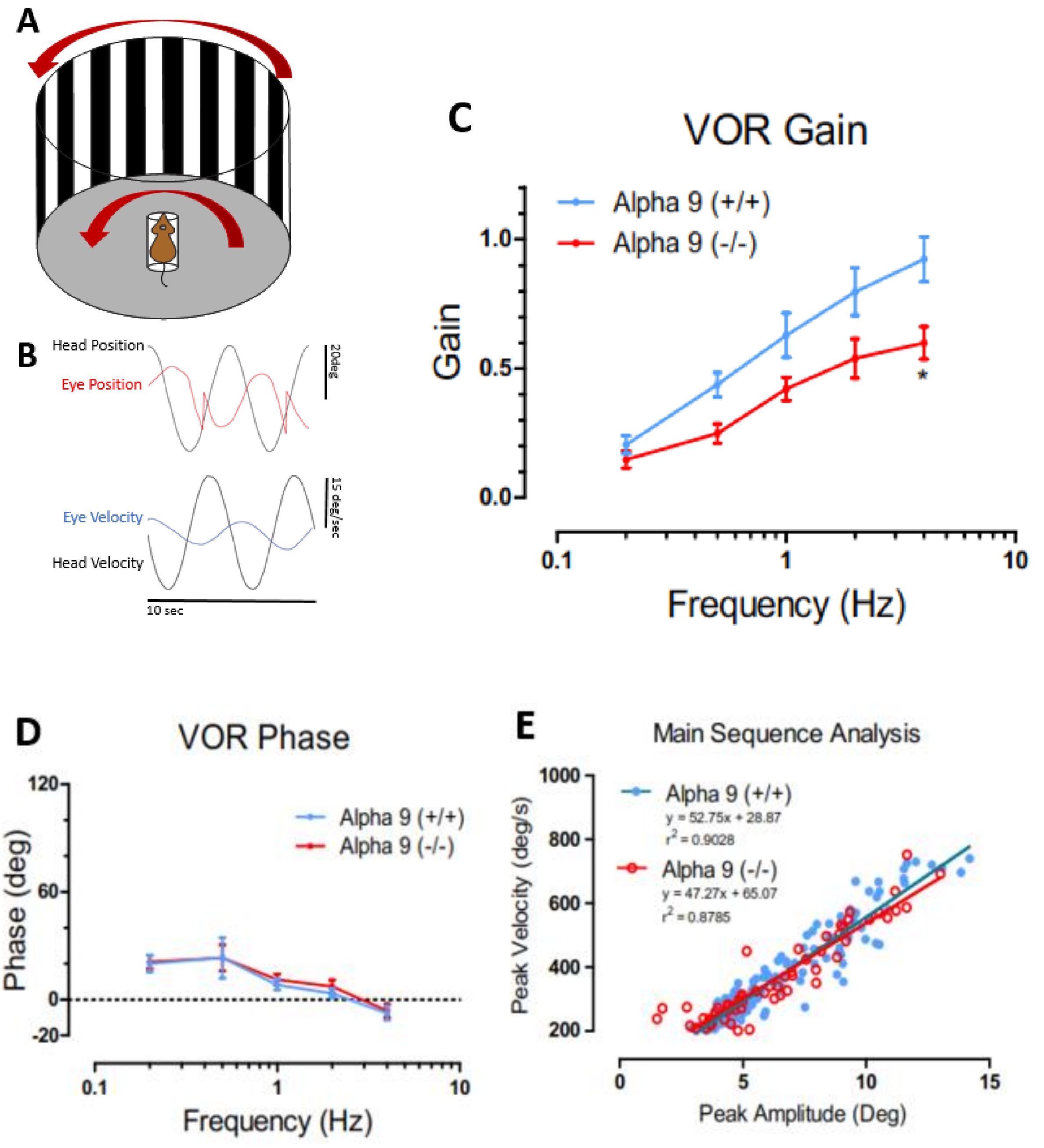

Fig 5. Characterization of VOR for alpha-9 mouse strain. (A) Schematic of recording set-up for VOR stimulation in dark. (B) Example position (upper) and velocity (lower) traces of alpha-9 control eye movements during VOR stimulation at 0.2Hz. Saccadic eye movements have not been removed from position trace. (C) Mean VOR Gain at 0.2, 0.5, 1.0, 2.0 and 4.0Hz for alpha-9 (+/+) (Blue; n=10) and alpha-9 (-/-) (Red; n=10). Significant decrease in VOR gain only at 4.0Hz ($P<0.5$) with an attenuation of %22.65. (D) Mean VOR Phase at each frequency. (E) Main sequence analysis comparing peak velocity and amplitudes of saccadic eye movements for each strain.

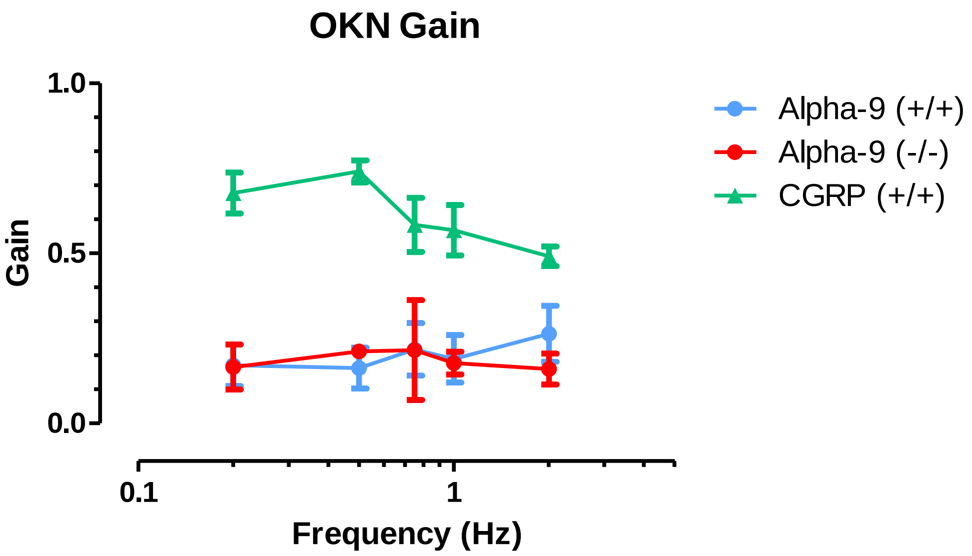

Fig 6. Mean OKN gain of alpha-9 (+/+) (Blue; n=5) and alpha-9 (-/-) (Red; n=2) in comparison with the CGRP (+/+) (Green; n=5) strain. Alpha-9 strain shows visual-motor impairments in both null mutants and their littermate controls.

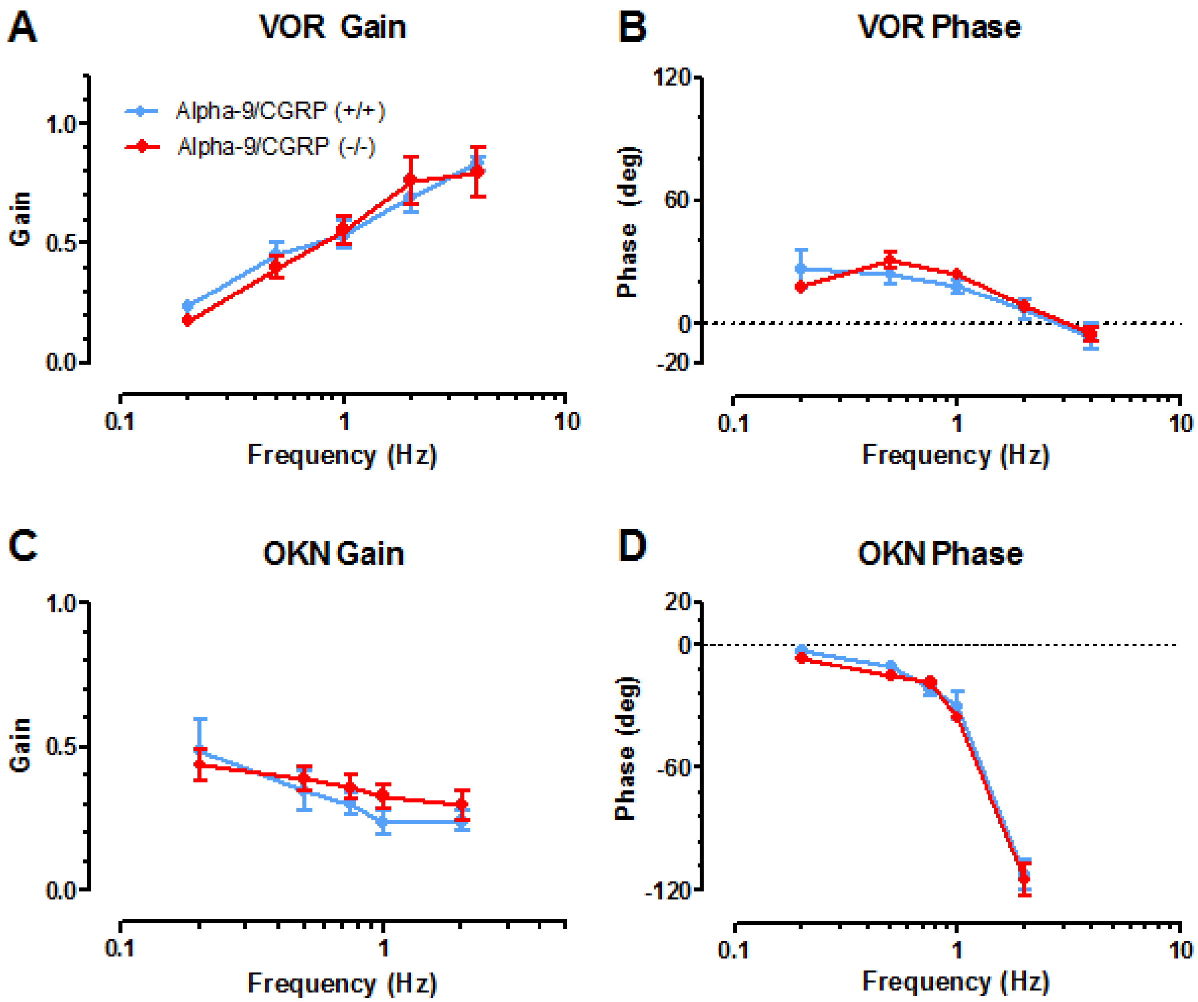

Fig 7. Characterization of VOR in mice with loss of both the alpha-9 subunit and CGRP. (A) Mean VOR gains of alpha-9/CGRP (+/+) (Blue; n=8) and alpha-9/CGRP (-/-) (Red; n=8) and (B) mean VOR phase of alpha-9/CGRP (-/-) strains show no significant difference from controls. (C) Mean OKN Gains and (D) mean OKN Phase of alpha-9/CGRP mutant strains also do not show any differences from controls. OKN Phase of alpha-9/CGRP (-/-) mutants have been shifted by -5 deg to better distinguish the data sets.

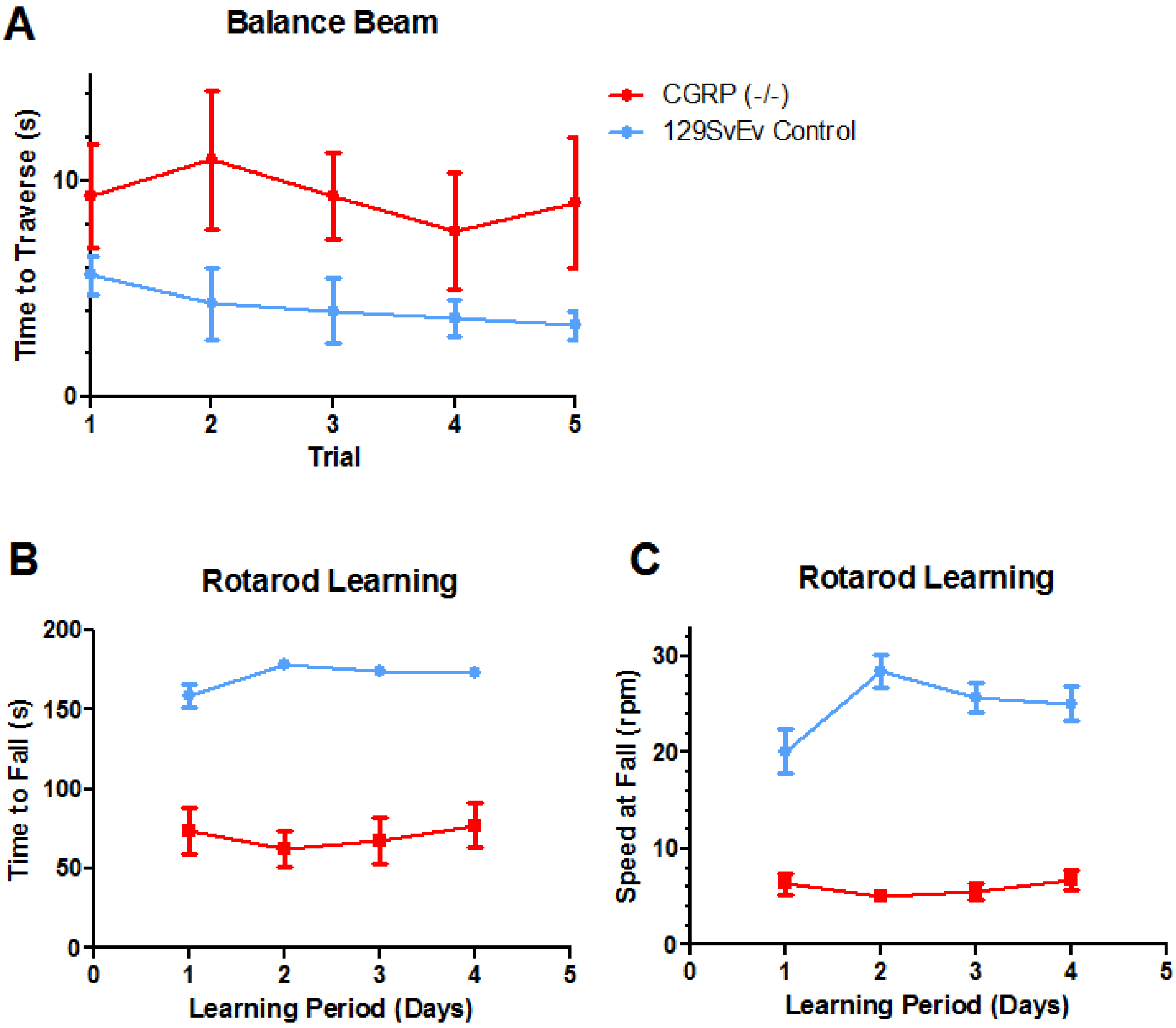

Fig 8. Behavioural response during (A) balance beam task and (B) accelerating rotarod task. (A) Mice repeated the task over 5 trials and mean traversal time was plotted per trial. 129SvEv controls (Blue; n=3) consistently perform better than CGRP (-/-) (Red; n=3) mice. For the accelerating rotarod task, mice repeated the task for 6 trials per day over the course of 4 days. Time to fall (B) and maximum speed at time of fall (C) were recorded. Overall, 129SvEv controls could traverse the balance beam faster and last longer, up to higher speeds, on the accelerating rotarod as compared to CGRP (-/-) mice.

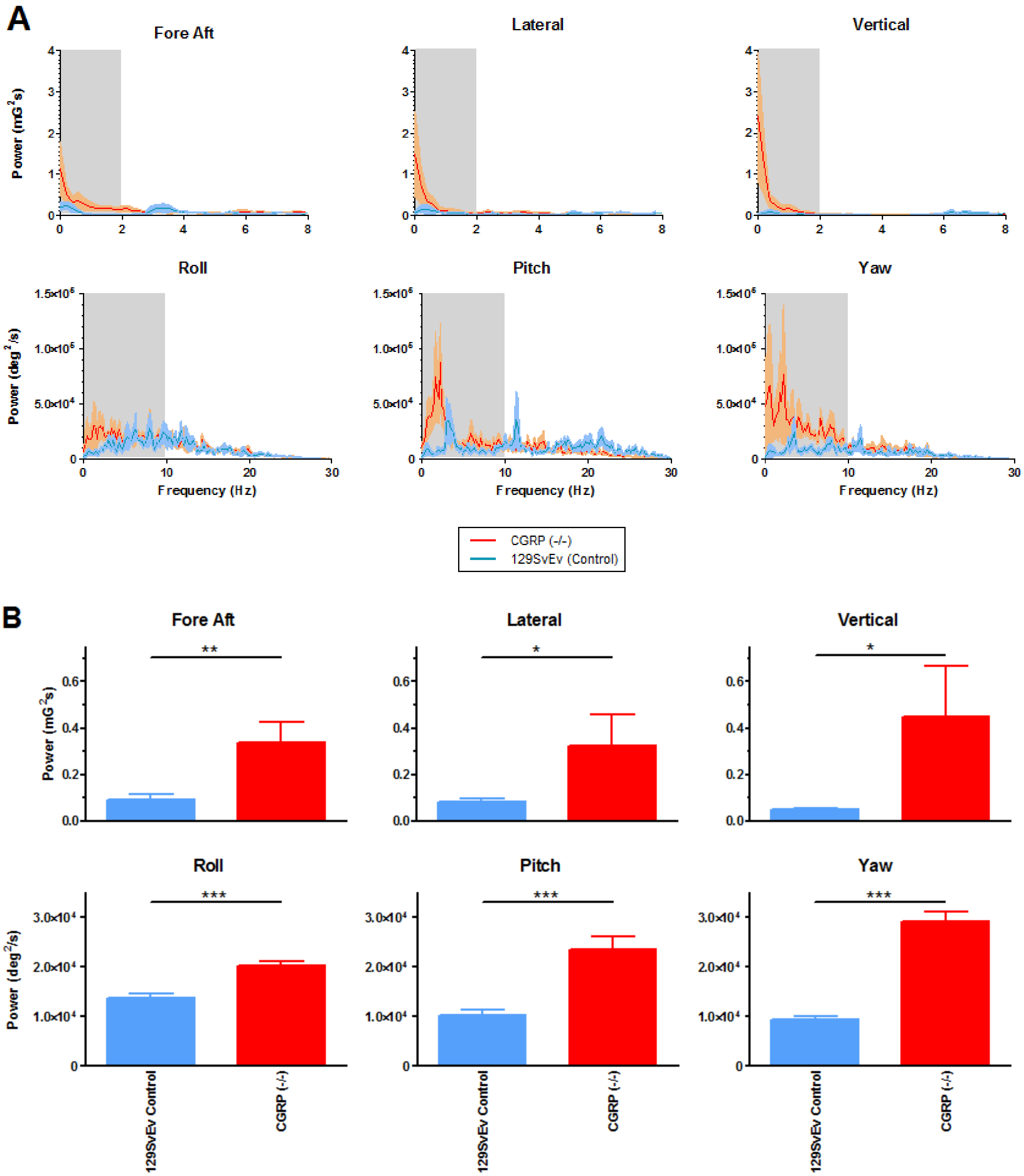

Fig 9. Head motion dynamics during balance beam task in 129SvEv controls (Blue, n=3) and CGRP (-/-) (Red, n=3). (A) Mean power spectrum across all 5 trials of head motion dynamics during balance beam task in fore aft, lateral, vertical, roll, pitch and yaw. Grey area used to calculate average power in panel B. (B) Average power at ≤2.0Hz for fore aft, lateral and vertical directions and ≤10.0Hz for roll, pitch and yaw. Overall, CGRP (-/-) had significantly higher power at lower frequencies compared to 129SvEv controls.

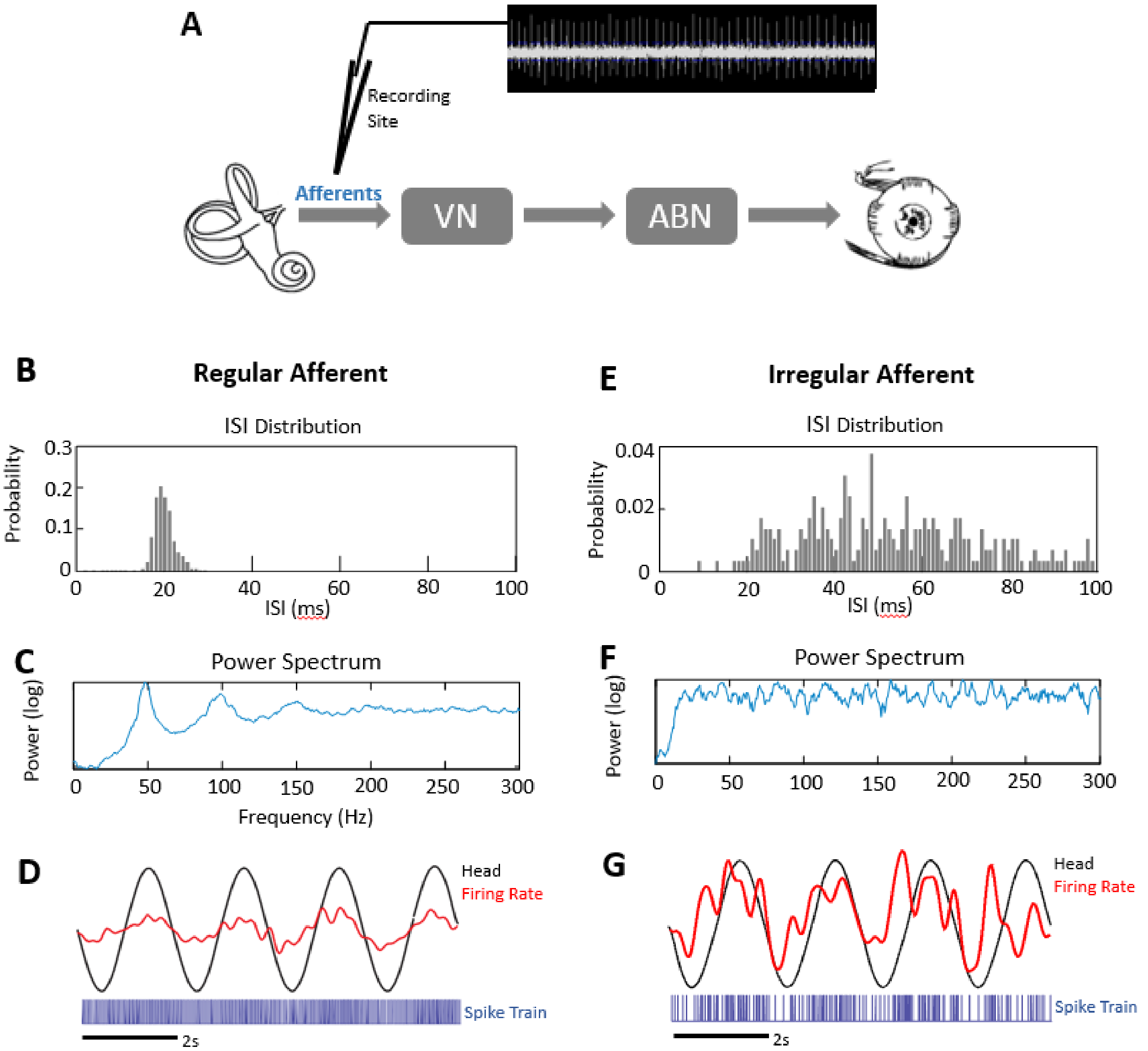

Fig 10. (A) Schematic of vestibular afferent recording, relative to VOR pathway, and example single-unit afferent raw spike trace. Example resting rate properties of regular (B, C, D) and irregular (E, F, G) afferents. (B) and (E) histograms illustrate inter-spike interval distributions for regular and irregular afferents respectively. (C) and (F) show differences in power spectrum for each afferent type. (D) and (G) are example traces of regular (C) and irregular (F) responses to sinusoidal stimuli at 0.5Hz showing head velocity (Black trace), afferent firing rate (Red trace) and the spike train (Blue).

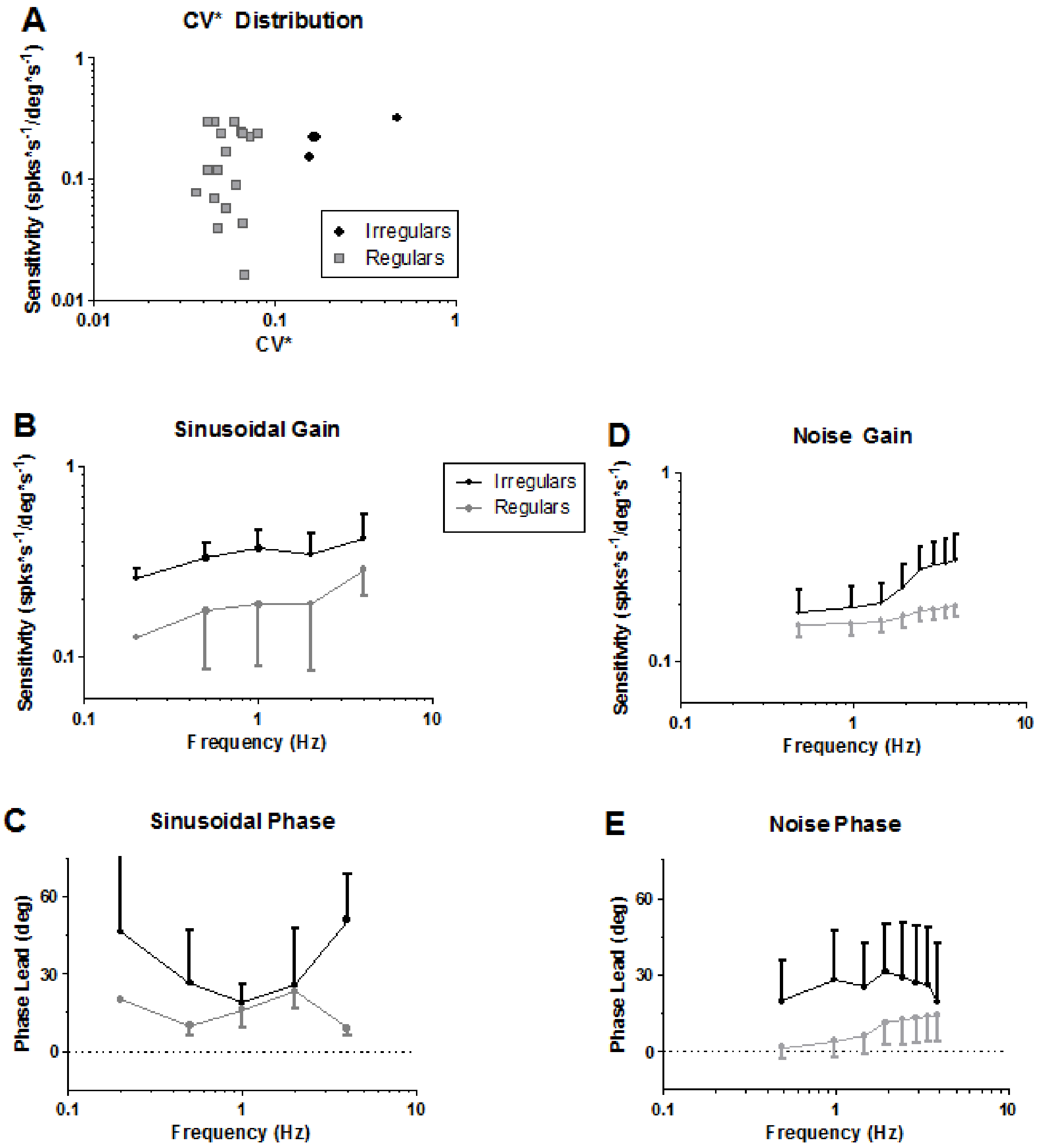

Fig 11. CV* distribution (A) and vestibular afferent response dynamics of 129SvEV control mice. (B, C) Response dynamics of vestibular afferents to sinusoidal stimulation (Irregulars, n=2; Regulars, n=2). (D, E) Response dynamics of vestibular afferents to Gaussian noise stimulation (Irregulars, n=4; Regulars, n=18).

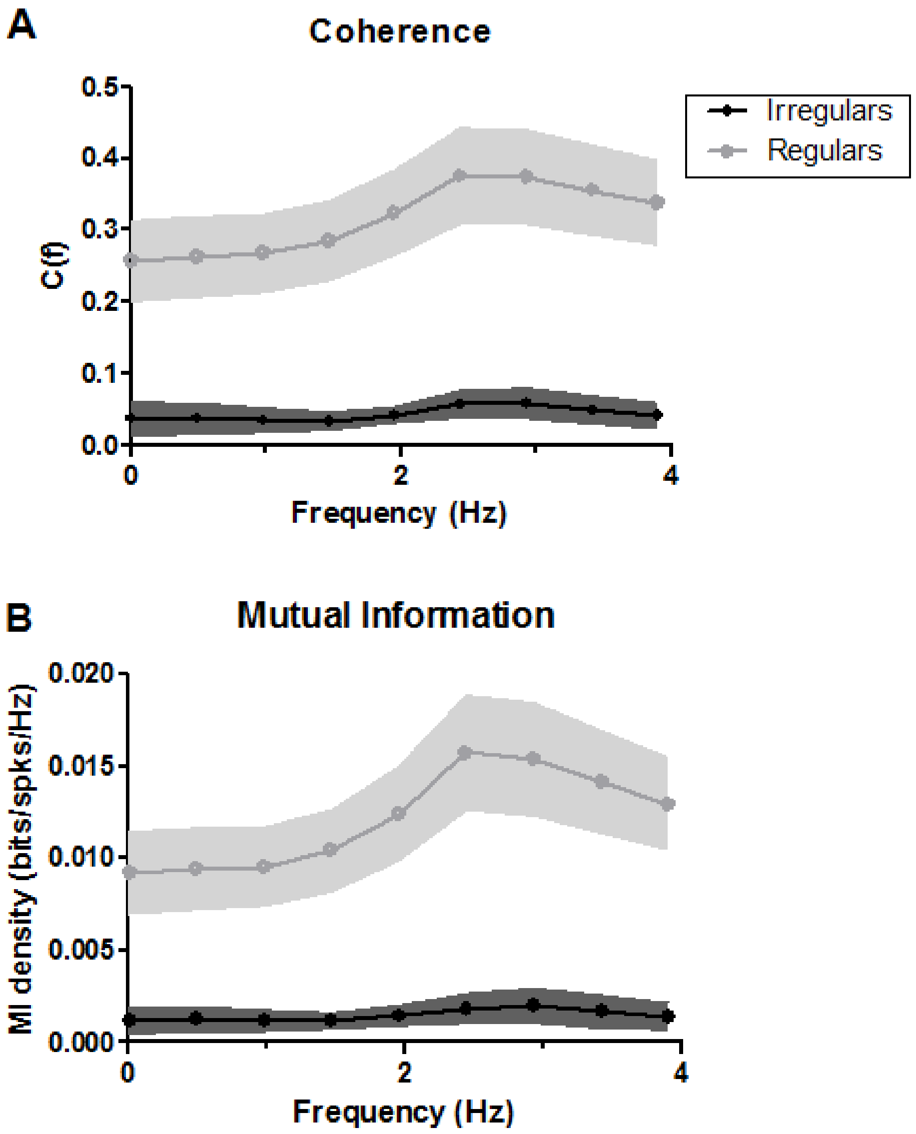

Fig 12. Coherence (A) and mutual information (B) of regular (Gray, n=18) and irregular (Black, n=4) afferents as determined from response dynamics during the random noise stimulation paradigm. Regular afferents consistently encode more information than irregular afferents.

CHAPTER 4: GENERAL DISCUSSIONS AND SUMMARY

The purpose of this thesis was to elucidate the role of the vestibular efferent system in development and address the hypothesis that loss of cholinergic efferent innervations might alter the transmission of vestibular information by afferents. The two main goals of this thesis were: 1) to characterize gaze stabilization and postural control pathways in transgenic mice lacking mediators of efferent signaling to understand their effects on vestibular dependent behaviours and 2) to characterize vestibular afferent populations in normal mice using random noise stimuli to better understand peripheral sensory processing and potentially gain insight on how they might be influenced by cholinergic efferent innervations.

My results show that loss of α-9 in the efferent pathway causes attenuation in VOR while, surprisingly, loss of both α-9 and αCGRP "rescued" this attenuation. This raised the question of how efferent signaling might affect other vestibular pathways, such as postural control, if proper hair cell and afferent development does indeed require efferent innervation. This study characterized vestibulospinal reflexes in αCGRP mice performing rotarod and balance beams tasks and found that αCGRP null mutants performed significantly worse than controls, consistent with my hypothesis. Lastly, to better understand the underlying mechanisms, single-unit afferent recordings were used to characterize afferent response dynamics in normal mice. A random noise stimulation paradigm was used for the first time to characterize the information content of regular and irregular afferents in normal mice revealing the contribution of neural variability to the neural code of vestibular sensory processing in rodents. These findings and their implications in elucidating vestibular efferent function are discussed below.

I. VOR Responses in Transgenic Mouse Models

To understand how loss of cholinergic efferent transmission might affect vestibular dependent behaviours, I first characterized vestibulo-ocular reflex (VOR) response dynamics in α-9 and α-9/αCGRP transgenic mice by computing gain and phase. Eye movements were recorded by an infrared camera system and gyroscope respectively. VOR responses were elicited by horizontal sinusoidal rotations at frequencies between 0.2 to 4.0Hz and peak head velocity was kept at 15^{o}/s. As controls, quick phase and optokinetic nystagmus (OKN) responses were also characterized. VOR responses in α-9 null mutants showed significant attenuation, particularly at 4.0Hz, without affecting phase or saccadic eye movements. The attenuation of VOR gains in α-9 mice is consistent with my hypothesis as well as previous findings from our group with αCGRP transgenic mice (Luebke et al., 2014). However, attenuation was noticeably lower in α-9 null mutants (~23%) as compared to αCGRP null mutants (~50%), suggesting that other cholinergic receptors that are modulated by αCGRP are involved in vestibular end organ development, contrary to what is seen in the auditory system.

Interestingly, characterization VOR responses in a transgenic mouse line lacking both α-9 and αCGRP did not reveal any changes in VOR. Characterization of VOR responses in α-9 mice in this study and αCGRP mice by our group (Luebke et al., 2014) would suggest that loss of both peptides would cause VOR gain attenuation. Thus, it comes as a surprise that loss of both neuropeptides would “rescue” the impairments seen when only α-9 or αCGRP is lost. These results suggest a more complex interaction between these neuropeptides that is not well understood in current literature. It would be of interest in the future to investigate how other combinations of null mutations in vestibular efferent transmission would contribute to the overall role of efferents in the development of the inner ear.

This study also found that both α-9 null mutants and their littermate controls showed visual-motor impairment which is a major limitation for using this particular mouse model in vestibular research. Since the OKN pathway converges with the VOR pathway at the vestibular nuclei (VN), it becomes difficult to discern if mutations in the VN might be influencing VOR responses. In addition, visual stimuli can cause adaptation in VOR responses, which may result in more variable VORs within the mouse strain (Kompf and Piper, 1987; Schneider et al., 2013). As such, although the gain attenuation suggests that α-9 plays a role in VOR efficacy, a cleaner α-9 transgenic mouse model should be re-derived to better understand the role of α-9 in vestibular efferent function.

II. Postural Regulation in Transgenic Mouse Models

As mentioned before, αCGRP null mutant mice show approximately 50% attenuation in VOR gain (Luebke et al., 2014). This prompted the question of whether these changes can be seen in other vestibular pathways that mediate postural regulation and head stabilization, if indeed efferent innervations play a role in the development of hair cell and afferents in the inner ear. To assess this, the performance of αCGRP transgenic mice was evaluated during the accelerating rotarod (3 to 33 rpm in 180 seconds) and balance beam task (traversing across 60cm at an incline). While these mice did not display obvious motor deficits during natural foraging behaviours, αCGRP transgenic mice did perform significantly worse (e.g., slower and less stable) than 129SvEv controls during both tasks. In addition, a micro-electromechanical module (iNEMO; refer to methods in Carriot et al., 2014) – which can measure both linear acceleration and rotational velocities in six axes – was used to obtain additional information on head motion dynamics during the balance beam task. Using this approach I found that αCGRP null mice showed greater power at low frequencies in all axes, suggesting more instability while performing balance tasks when mice lack αCGRP. These findings are consistent with the VOR deficits seen across all tested frequencies in αCGRP transgenic mice (Luebke et al., 2014).

It is important to note that while these findings are interesting, the age gap between our null mutants and controls may bias some of our results as an age-dependent decrease in performance on the balance beam and rotarod have been shown in mice (Tung et al., 2014). Nevertheless, these results do suggest that when the vestibular system is challenged, head motion dynamics will noticeably change in αCGRP null mutants. A difference that is apparent when observing their performance on the rotarod and balance beam task, but not during foraging behaviours. Characterization of head motion dynamics measured by devices such as the iNEMO could be used to more accurately quantify differences in performance between different transgenic mouse lines. For example, given the differences in the frequency range of attenuation for α-9 and αCGRP transgenic mice, these mice strains may exhibit different head motion dynamics.

III. Vestibular Afferent Response Dynamics in Normal Mice

Behavioural tasks, though useful for characterizing functional changes in the vestibular system, are limited in the information they can provide regarding the underlying physiology causing the changes described above. As such, to gain insight into the effects of cholinergic efferent innervations in the inner ear, single-unit afferent recordings were used in this study. Specifically, we characterized the transmission of vestibular information in normal mice to gain insight on how afferent responses might be affected by cholinergic efferent innervation.

A random noise stimulus was used, similar to what is described in Sadeghi et al (2007), over the natural frequency range of head motion in mice (≤4.0Hz; Beraneck et al., 2008) to obtain novel information on the information content of afferents in a mouse model. This study found that sensitivity, phase and resting rate properties in 129SvEv control mice were all comparable to those seen in C57BL/6 mice in Lasker et al (2008), with no significant differences when comparing afferent response dynamics between noise and sinusoidal stimuli. More importantly, computations of coherence and mutual information revealed that regular afferents had higher information content compared to irregular afferents – similar to those in macaque monkeys (Sadeghi et al., 2007). Although mutual information of afferents in mice appear to be consistently less than what is seen in primate models (~60% less at 4.0Hz; Sadeghi et al., 2007), these findings illustrate that differences in neural variability contribute to the neural code for vestibular processing in rodents as well.

Overall, based on the literature reviewed above and our preliminary findings, it suggests that vestibular efferent signals can shape behaviour in mice and may potentially mediate vestibular end organ development. Interestingly, considering how VOR attenuation is seen at all frequencies ≤4.0Hz in αCGRP mice, this might suggest that loss of efferent innervations has a greater effect on regular afferents since they encode more information within this frequency range. Sadeghi et al. (2009) did report however that irregulars showed greater efferent-mediated responses (10 spks/s) than regulars (2 spks/s). Altogether, I propose that efferent innervations have different effects on regular and irregular afferents and hypothesize that the loss of cholinergic efferent innervation would result in a decrease in information content, particularly in regular afferents, and an attenuation in sensitivity, particularly in irregular afferents. For future directions, single-unit recordings in transgenic mouse lines during random noise stimulation would provide greater insight into how development of the inner ear might be altered by the vestibular efferent system.

IV. Concluding Remarks

Given the feedback signal the efferent pathway provides for the vestibular system, it is evident that understanding of the efferent pathway will provide greater insight into vestibular afferent signal processing with clinically relevant applications (i.e., motion sickness). To date, studies in mammals have focused on identifying potential mechanisms of efferent signaling using electrical stimulation paradigms, pharmacology and immunohistochemistry. This study builds on these previous works and the work by Luebke et al (2014) by using transgenic knock-out mouse models to manipulate prominent neuropeptides found at efferent synapses (α-9 and αCGRP) to better understand, not just the molecular mechanisms, but the functional role these cholinergic signals play in modifying behaviour and afferent signaling within an *in vivo* model that could potentially be applied to other mammals.

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