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Stochastic Noise Application for the Assessment of Medial Vestibular Nucleus Neuron Sensitivity In Vitro

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TITLE:

Stochastic Noise Application for the Assessment of Medial Vestibular Nucleus Neuron Sensitivity In Vitro

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KEYWORDS:

Stochastic resonance, stochastic noise, sinusoidal noise, vestibular system, medial vestibular nucleus, electrophysiology.

SUMMARY:

Galvanic vestibular stimulation in humans exhibits improvements in vestibular function. However, it is unknown how these effects occur. Here, we describe how to apply sinusoidal and stochastic electrical noise and evaluate appropriate stimulus amplitudes in individual medial vestibular nucleus neurons in the C57BL/6 mouse.

ABSTRACT:

Galvanic vestibular stimulation (GVS) has been shown to improve balance measures in individuals with balance or vestibular impairments. This is proposed to be due to the stochastic resonance (SR) phenomenon, which is defined as application of a low-level/subthreshold stimulus to a non-linear system to increase detection of weaker signals. However, it is still unknown how SR exhibits its positive effects on human balance. This is one of the first demonstrations of the effects of sinusoidal and stochastic noise on individual neurons. Using whole-cell patch clamp electrophysiology, sinusoidal and stochastic noise can be applied directly to individual neurons in the medial vestibular nucleus (MVN) of C57BL/6 mice. Here we demonstrate how to determine the threshold of MVN neurons in order to ensure the sinusoidal and stochastic stimuli are subthreshold and from this, determine the effects that each type of noise has on MVN neuronal gain. We show that subthreshold sinusoidal and stochastic noise can modulate the sensitivity of individual neurons in the MVN without affecting basal firing rates.

INTRODUCTION:

The vestibular (or balance) system controls our sense of balance by integrating auditory, proprioceptive, somatosensory and visual information. Degradation of the vestibular system has been shown to occur as a function of age and can result in balance deficits[1,2]. However, therapies targeting the functioning of the vestibular system are scarce.

Galvanic Vestibular Stimulation (GVS) has been shown to improve balance measures, autonomic functioning and other sensory modalities within humans[3-6]. These improvements are said to be due to the Stochastic Resonance (SR) phenomenon, which is the increase in the detection of weaker signals in non-linear systems via the application of subthreshold noise[7,8]. These studies have shown improvements in static[9,10] and dynamic[11,12] balance, and vestibular output tests such as Ocular Counter Roll (OCR)[13]. However, many of these studies have used different combinations of stimulus parameters such as white noise[9], colored noise[13], different stimulus frequency ranges and thresholding techniques. Therefore, optimal stimulus parameters remain unknown and this protocol can assist with determining the most effective parameters. Besides stimulus parameters, the type of stimulus is also important in therapeutic and experimental efficacy. The above work in humans was performed using electrical noise stimuli, whilst much of the in vivo animal work has used mechanical[14,15] or optogenetic[16] noise stimuli. This protocol will use electrical noise to examine the effects on vestibular nuclei.

Previously, application of GVS to stimulate primary vestibular afferents was been performed in vivo in squirrel monkeys[17], chinchillas[18], chicken embryos[15] and guinea pigs[14]. However, only two of these studies examined the effect GVS has on the gain of primary vestibular afferents[14,15]. These experiments were performed in vivo meaning that the precise patterns of stimulation imposed on vestibular nuclei cannot be determined. To our knowledge, only one other study has applied stochastic noise to individual enzymatically dissociated neurons in the central nervous system[19]. However, no experiments have been performed in the central vestibular nuclei to assess appropriate stimulus parameters and thresholding techniques, making this protocol more precise in determining stimulus effects on individual neurons within the vestibular nuclei.

Here, we describe how to apply sinusoidal and stochastic (electrical) noise directly to individual neurons in the medial vestibular nucleus (MVN), determine neuronal threshold and measure changes in gain/sensitivity.

PROTOCOL:

All experimental protocols described were approved by the University of Sydney Animal Ethics Committee (approved protocol number: 2018/1308).

1. Animals

NOTE: Mice were obtained from the Australian Rodent Centre (ARC; Perth, Australia) and held at the Medical Foundation Building Animal Facility at the University of Sydney.

1.1. Maintain the mice on a normal 12 h light/dark cycle with environmental enrichment.

1.2. Use male and female C57BL/6 mice (3–5 weeks old) for all experiments.

2. Preparation of solutions

2.1. Prepare 1 L of artificial cerebrospinal fluid (ACSF) composed of 29 mM $NaHCO_3$, 11 mM glucose, 120 mM NaCl, 3.3 mM KCl, 1.4 mM NaH_2PO_4, 2.2 mM $MgCl_2$, 2.77 mM $CaCl_2$.

2.2. Prepare 200 mL of sucrose-ACSF (sACSF) containing 29 mM $NaHCO_3$, 11 mM glucose, 241.5 mM sucrose, 3.3 mM KCl, 1.4 mM NaH_2PO_4, 2.2 mM $MgCl_2$, 2.77 mM $CaCl_2$. Prior to the inclusion of $CaCl_2$ to the ACSF and sACSF, gas the solutions with carbogen (95 % O_2 and 5 % CO_2) to establish a pH of 7.4 and avoid calcium precipitation (cloudiness).

2.3. Prepare K^+-based intracellular solution composed of 70 mM potassium gluconate, 70 mM KCl, 2 mM NaCl, 10 mM HEPES, 4 mM EGTA, 4 mM Mg_2-ATP, 0.3 mM Na_3-GTP; with a final pH of 7.3 (adjusted using KOH).

NOTE: It is recommended to filter intracellular solutions with 0.22 μm filters and store 0.5 mL aliquots of the solution at -20 °C.

3. Preparation of the brainstem

3.1. Prior to brainstem extraction, equilibrate the sACSF with carbogen and cool at -80 °C for 25 min so that an ice slurry is formed.

3.2. Anaesthetize the mouse with isoflurane (3–5 %) saturated in oxygen (3 mL/min). Once the hind paw reflexes are absent, decapitate the mouse with sharp stainless-steel scissors.

3.3. Expose the skull by making a sagittal incision in the skin using a razor blade (#22 rounded).

3.4. Using the pointed end of a pair of standard pattern scissors make a small incision at the lambda and cut along the longitudinal fissure.

3.5. Carefully reflect away the paired parietal bones and the occipital bones using a pair of shallow-bend Pearson rongeurs.

NOTE: During this whole procedure the brain is continuously bathed in situ using the previously prepared ice-cold sACSF slurry.

3.6. Isolate the brainstem from the forebrain and its bony encasing using a razor blade (#11 straight) to cut down the parieto-occipital sulcus and at the caudal medulla.

3.7. Mount the isolated brainstem ventral end down on a previously cut trapezoidal polystyrene block. Remove excess fluid around the dissected tissue with a wick of tissue paper to ensure good

tissue adhesion to the cutting stage.

NOTE: The polystyrene block is cut in a trapezoidal shape, to ensure the rostral end of the midbrain fits and tapers into the spinal cord.

3.8. Use cyanoacrylate glue to fix the polystyrene block with the attached brainstem rostral end down to the cutting stage.

3.9. Using an advance speed of 0.16 mm/s and vibration amplitude of 3.00 mm, prepare 200 µm transverse slices of the MVN.

NOTE: Location of the MVN is determined using the Paxinos and Franklin mouse brain atlas (Figures 79–89)[20]. The MVN (listed as MVe in atlas) lies immediately ventrolateral to the 4th ventricle and is largest right before the attachment of the cerebellum (between the inferior colliculi and the obex).

3.10. Use a plastic-trimmed pipette to transfer slices onto a filter paper disc sitting in carbogenated ACSF at 25 °C for at least 30 min prior to recording.

4. Instruments

4.1. Use a standard electrophysiological setup to perform whole-cell patch clamp techniques[21].

4.2. Prepare micropipettes using a two-step protocol (heat step 1: 70; heat step 2: 45) on a micropipette puller (see the **Table of Materials**). Micropipettes should have a final resistance ranging 3–5 MΩ with internal solution when placed in the bath.

NOTE: Settings used may vary depending on the temperature within room and can change quite frequently.

5. Whole-cell patch clamp electrophysiology

5.1. To obtain whole-cell patch clamp recordings from individual neurons in the MVN, a K^+-based internal solution is used within the recording pipette.

5.2. Transfer a single tissue slice from the incubation chamber to the recording chamber and secure the slice using a nylon thread on a U-shaped weight. Continuously perfuse the recording chamber with carbogenated-ACSF at 25 °C at a flow rate of 3 mL/min.

5.3. After filling a micropipette with internal solution, locate the MVN using a low power (10x) objective lens. Using a high-power (40x) objective, individual neurons within the MVN can be located.

NOTE: Cell quality is essential in ensuring quality recordings and durability of the cell when

attempting to achieve the whole-cell configuration. A good cell will demonstrate spherical shape, a reflective cell membrane and an invisible nucleus. A bad cell will have a large visible nucleus (egg-like) and a swollen/shrunken appearance.

5.4. Before breaching the tissue with the pipette, apply a small amount of positive pressure to push debris away from the pipette tip.

5.5. Move the pipette using the micromanipulator towards the chosen neuron and a small dimple should form on the neuronal membrane. Release positive pressure and apply a small amount of negative pressure.

5.6. Once a 1 GΩ seal is achieved, apply gentle short and sharp positive pressure to the pipette holder through the suction port to rupture the membrane and create a whole-cell configuration.

5.7. Make whole-cell current clamp recordings using standard techniques[21,22].

6. Applying sinusoidal and stochastic noise to individual medial vestibular nucleus neurons

6.1. Apply the stochastic and sinusoidal noise at a range of amplitudes from 3 to 24 pA to determine neuronal threshold and firing rate.

6.2. Determine the sensory threshold by grouping lower and higher stimulus intensities and perform an ANOVA to observe any differences (as shown in **Supplementary Figure 1**).

6.3. Calculate the average firing rate over the 10 s period where the depolarizing current step was/will be injected for each individual current level (i.e., 7 total episodes; **Figure 1**).

6.4. Use the average firing rate values to generate a firing rate versus current plot and perform a linear regression analysis to determine the gradient of the line of best fit. The gradient of the line of best fit is indicative of the neuronal gain[22].

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

Initial recordings can provide information about the effects that sinusoidal and stochastic noise have on basal firing rates of individual MVN neurons and how the stimuli effect the gain of neurons. **Figure 2** shows that neither sinusoidal nor stochastic noise change basal firing rates of MVN neurons when compared to control (no noise) recordings. This information is crucial for determining the threshold of the individual neurons. During the application of galvanic vestibular stimulation to humans, a sensory thresholding task is performed to ensure that the stimulus is subthreshold[13]. The subthreshold stimulus is an important component of the stochastic resonance (SR) phenomenon[7,8]. In vitro, this thresholding task needs to be performed differently and the activity or basal firing rate of neurons has been chosen for this. This ensures that the stimuli are as close to subthreshold as possible and therefore comparable to human studies.

Figure 2B highlights that the selected noise level (6 pA) is subthreshold, as it can be observed that average firing rate begins to increase from 12 pA (experimental threshold). This threshold was determined objectively by grouping stimulus levels above (18 and 24 pA) and below (3 and 6 pA) the 12 pA threshold and is shown in **Supplementary Figure 1**.

Next, neuronal gain was evaluated by subjecting neurons to a suite of depolarizing current steps (0–50 pA, increasing by 10 pA) with and without (control) noise (**Figure 1**). These results are crucial to determining the effect that stochastic noise may have on neurons in the central vestibular system and thus, potentially how GVS is eliciting its effects on human balance. **Figure 3** shows that sinusoidal (**Figure 3B**) and stochastic (**Figure 3A**) noise applied at subthreshold amplitudes of 6 pA can alter the gain of MVN neurons. These results were assessed by measuring the firing rate during each 10 s current step and performing a linear regression analysis to calculate the gain (gradient) from the line of best fit.

[Please **Figure 2** here]
[Place **Figure 3** here]

FIGURE LEGENDS:

Figure 1: Diagrammatic profiles of control, sinusoidal and stochastic noise protocols. (**A**) Control (no noise) protocols applied to MVN neurons. (**B**) Sinusoidal noise protocol with a frequency of 2 Hz. (**C**) Stochastic noise protocols where majority of the power spectrum is ≤2 Hz. Each protocol presented here has an amplitude of ±6 pA with a 10 s depolarizing current increasing by 10 pA up to 50 pA. The true stimulus does not have a depolarizing current step and is therefore the first episode of these protocols to determine neuronal gain changes.

Figure 2: The effect of sinusoidal and stochastic noise on MVN neuronal firing rate. (**A**) Stochastic (SN; middle trace) and sinusoidal noise (bottom trace) at a 6 pA amplitude show no significant effect on basal firing rate of an individual MVN neuron in comparison to control (no noise; top trace). (**B**) Firing rate of MVN neurons in response to control (n = 53), stochastic and sinusoidal noise protocols (with no current steps) of amplitudes 3 (SN, n = 30; sine, n = 6), 6 (SN, n = 46; sine, n = 17), 12 (SN, n = 13; sine, n = 4), 18 (SN, n = 5; sine, n = 0) and 24 (SN, n = 8; sine, n = 0) pA. Lines/whiskers indicate the maximum and minimum values, the box indicates the 25^{th}–75^{th} percentiles and the line within the box indicates the mean firing rate (spikes/s). The dashed line indicates experimental threshold, as chosen by pooling the mean firing rates within 3 and 6 pA (below 12 pA) and 18 and 24 pA (above 12 pA) shown in **Supplementary Figure 1**.

Figure 3: Sinusoidal and stochastic noise alter MVN neuronal gain. (**A**) MVN neuronal firing rate at each depolarizing current step and the corresponding gain calculation in response to stochastic noise. (**B**) The data presented were generated the same way as in **Figure 3A** but during the application of sinusoidal noise. (**C,D**) Graphs represent the gains calculated from the lines of best fit of **A** and **B**. Error bars indicate S.D. Statistical significance was determined by linear regression analysis comparing the gradients of the lines of best fit between control and experimental condition. **$p < 0.02$; ***$p < 0.01$.

Supplementary Figure 1: Objective determination of 12 pA threshold. Firing rates for less than 12 pA (3 and 6 pA) and more than 12 pA (18 and 24 pA) were pooled and averaged. These averages were then analyzed using an ANOVA and statistical significance between sham and >12 pA and between <12 pA and >12 pA. *$p < 0.05$.

DISCUSSION:

The effects of galvanic vestibular stimulation (GVS) on the vestibular system has been highlighted in vivo in humans[3,13,23], guinea pigs[14], rodents[18] and non-human primates[24]. However, none of these studies have assessed the direct impact of electrical noise on the sensitivity of individual neurons in the vestibular system. Here we demonstrate the first in vitro application of stochastic noise directly to individual medial vestibular nucleus (MVN) neurons.

The primary objective of applying stochastic noise directly to individual MVN neurons, is to determine whether the noise is exhibiting an effect on neuronal sensitivity directly. Thus, establishing how Stochastic Resonance (SR) impacts balance in humans. For SR to be evident, the stimulus needs to be subthreshold to ensure that individual neurons are not being overtly activated[7] (**Figure 2**). Therefore, the in vitro neuronal firing rate must remain comparable to control (no stimulus) conditions. This step is critical for the protocol in order to highlight the SR phenomenon, and may be different for other neuronal populations and therefore performed slightly differently.

Although this preparation provides clear advantages over previous in vivo work in animals[14,15,17,18], there are still some caveats. First, the stimuli are applied to individual neurons and therefore the thresholding of stochastic and sinusoidal noise may not represent what is occurring at a population level. However, using this protocol we are able to analyze changes at a single neuron level and use this information to subsequently model what may happen in behavioral studies. Second, these electrophysiological recordings are limited to neurons that display spontaneous activity or responses to direct current injection to simulate natural activity. This is one of the reasons for choosing the MVN as a target for testing the effects of these electrical stimuli, as it exhibits spontaneous neuronal activity[21].

An advantage of using whole-cell patch clamp recordings of individual MVN neurons is that the response can be more reliably linked to a specific output of the vestibular system. Behavioral studies are able to provide such information regarding the otolith-ocular pathway through the measurement of ocular vestibular-evoked myogenic potentials (oVEMPs) and ocular counter-rolls (OCRs) at a more macro level[13]. Through electrophysiological recordings, information regarding specific nuclei involvement and thus, the specific pathways involved can be elucidated. Further, previous work in stimulating primary vestibular afferents in vivo has provided important information into how GVS may be working but cannot directly assess how the central vestibular nuclei respond[14,15,17,18]. Therefore, highlighting the sensitivity and precision of whole-cell patch clamp recordings helps in elucidating how GVS may improve vestibular functioning.

Future studies could apply this protocol to other neuronal populations displaying spontaneous

activity. One study has applied stochastic noise to a non-spontaneously active neuronal population within the somatosensory and auditory cortices of rats[19]. However, this was performed in a cell suspension of enzymatically dissociated pyramidal neurons and was recording Na^+ currents specifically, which are taken from postsynaptic cells using voltage clamp experiments. In this protocol the spontaneous activity of MVN neurons was recorded from individual neurons within transverse slices of the brainstem using current clamp experiments.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

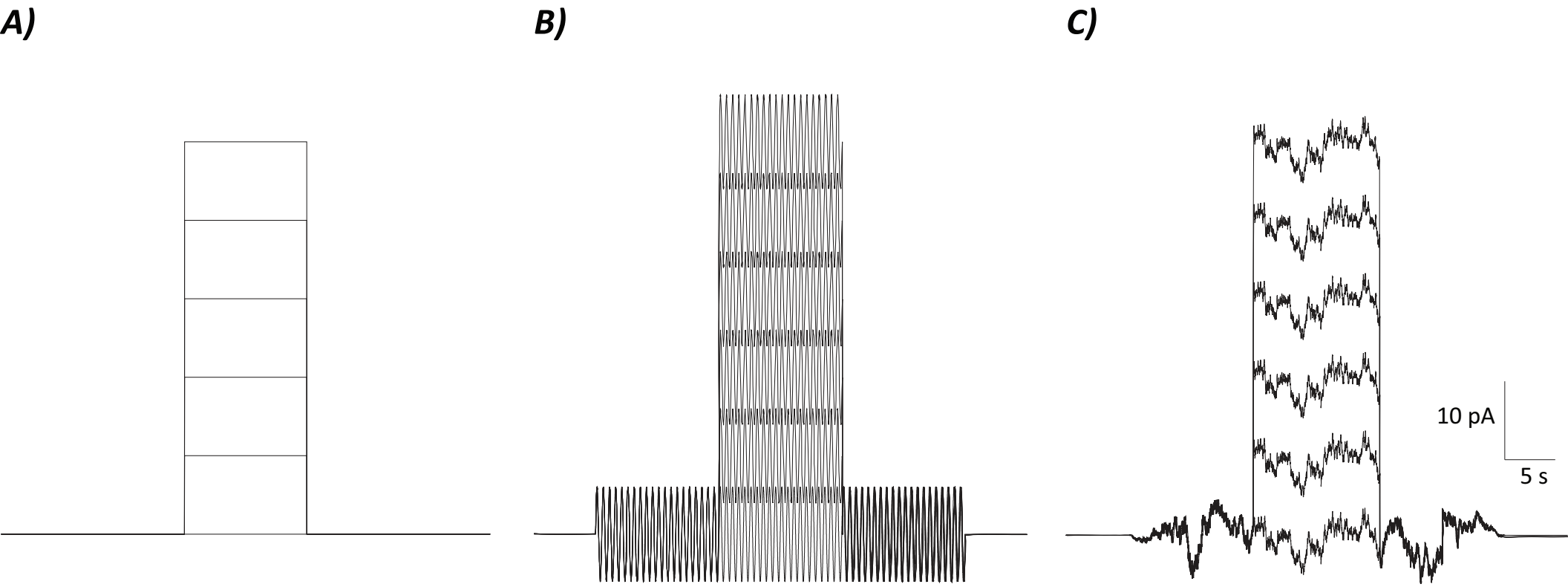
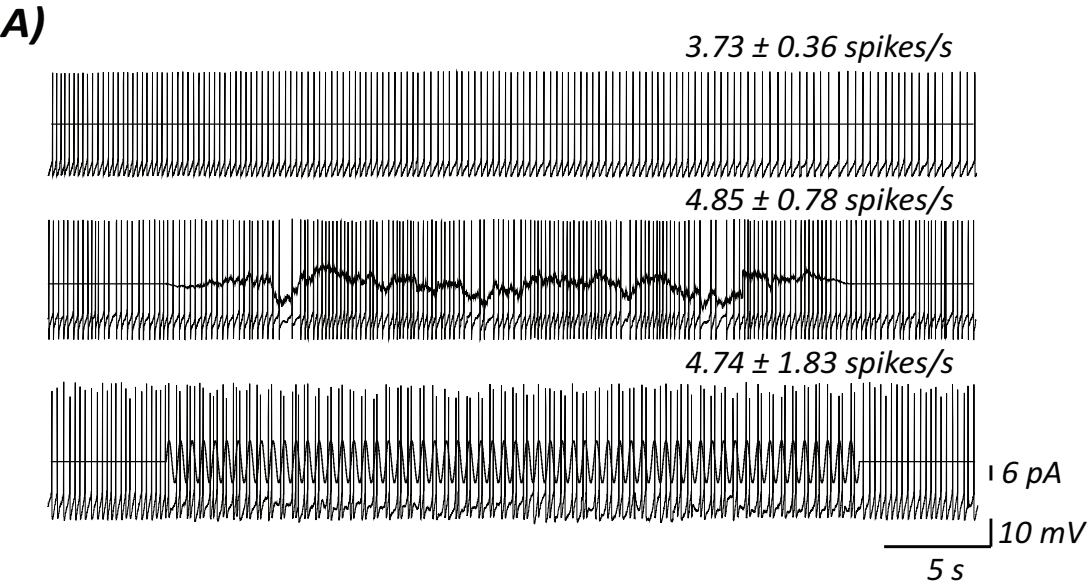


Figure 2



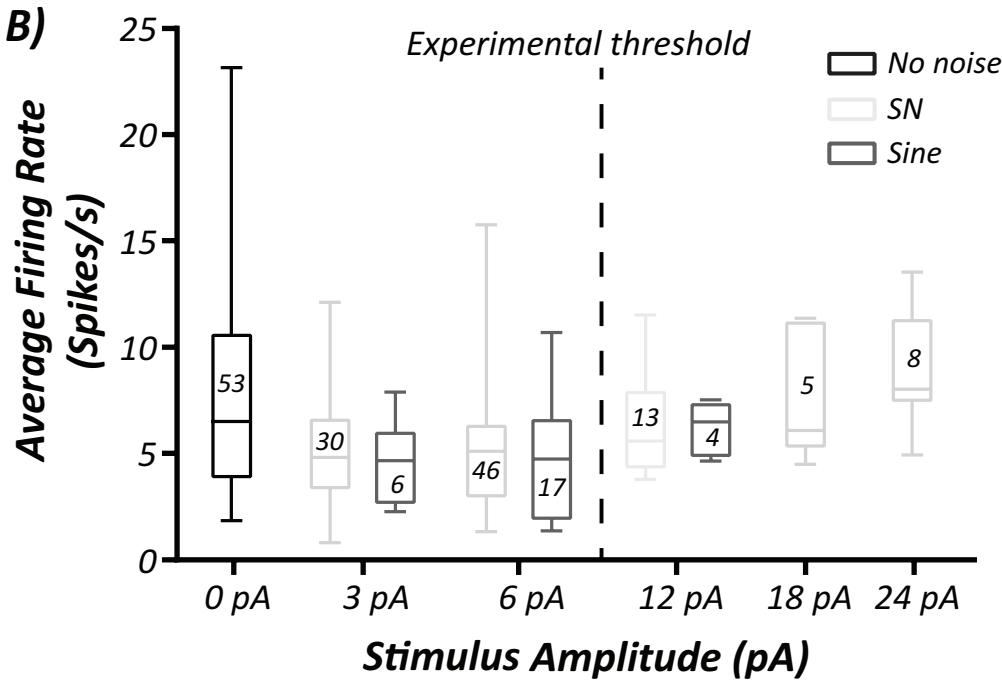


Figure 3

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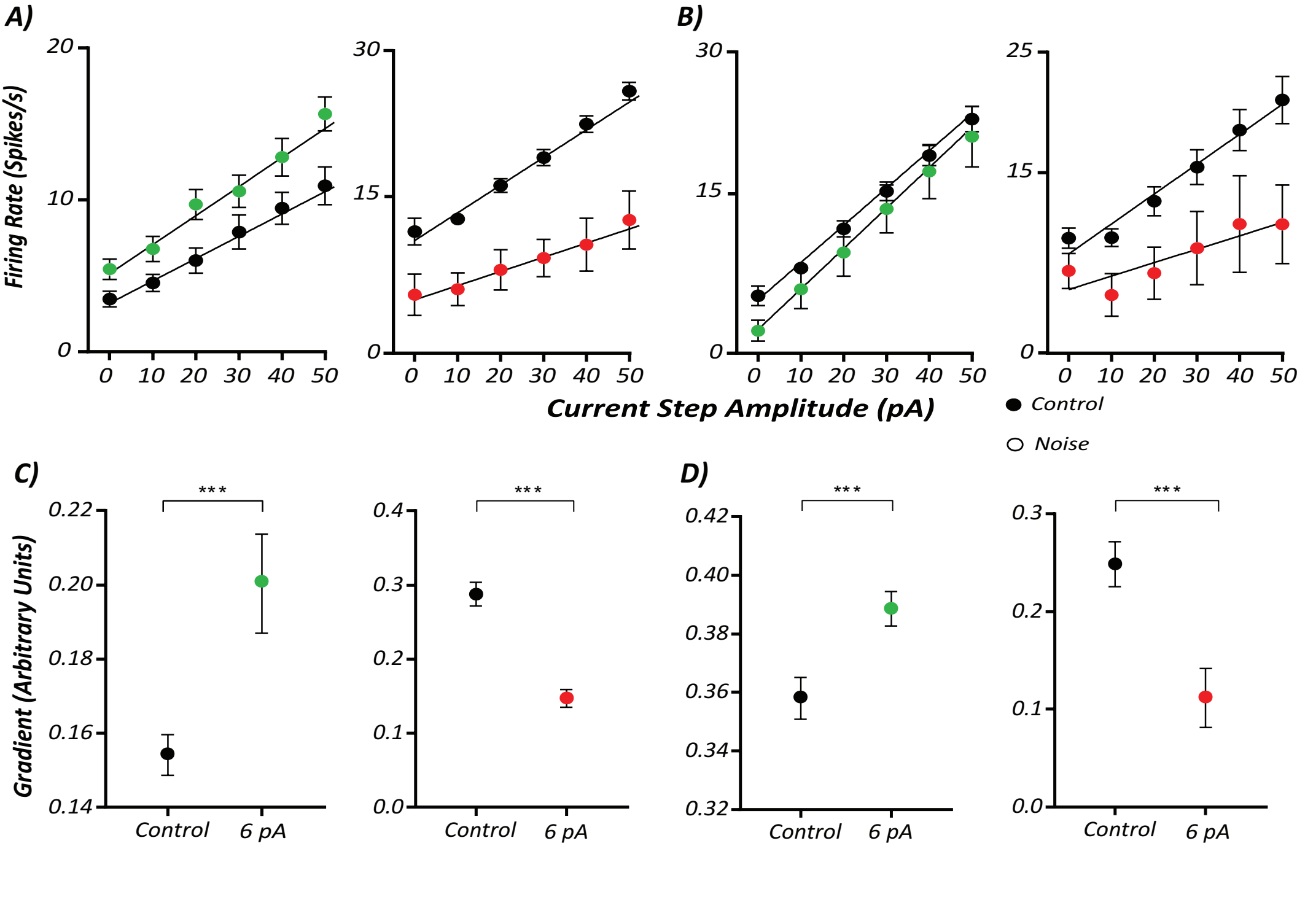

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
CaCl	Scharlau	CA01951000	Used for ACSF and sACSF
D-(+)-Glucose	Sigma	G8270	Used for ACSF and sACSF
EGTA	Sigma	E0396-25G	Used for K-based intracellular solution
HEPES	Sigma	H3375-25G	Used for K-based intracellular solution
KCl	Chem-supply	PA054-500G	Used for ACSF, sACSF and intracellular solution
K-gluconate	Sigma	P1847-100G	Used for K-based intracellular solution
Mg-ATP	Sigma	A9187-500MG	Used for K-based intracellular solution
MgCl	Chem-supply	MA00360500	Used for ACSF and sACSF
Na3-GTP	Sigma	G8877-100MG	Used for K-based intracellular solution
NaCl	Chem-supply	SO02270500	Use for ACSF and intracellular solution
NaH2PO4.2H2O	Ajax	AJA471-500G	Used for ACSF and sACSF
NaHCO3	Sigma	S5761-1KG	Used for ACSF and sACSF
Sucrose	Chem-supply	SA030-500G	Used for sACSF
Isoflurane	Henry Schein	1169567762	Used for anaesthetising mice
EQUIPMENT			
Borosilicate glass capillaries	Warner instruments	GC150T-7.5	1.5mm OD, 1.16mm ID, 7.5cm length
Data acquisition software	Axograph		Used for electrophysiology and analysis
Friedmen-Pearson Rongeurs	World precision instruments	14089	Used for dissection
Micropipette puller	Narishige	PP-830	Used for micropipette
Multiclamp amplifier	Axon instruments	700B	Used for electrophysiology
pH meter	Sper scientific	860033	Used for internal solution
Standard pattern scissors	FST	14028-10	Used for dissection
Sutter micromanipulator	Sutter	MP-225/M	Used for electrophysiology
Upright microscope	Olympus	BX51WI	Used for electrophysiology
Vibratome	Leica	VT1200	Used for slicing brain tissue

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Dear editor,

We thank you and the reviewers for the comments and insights provided, we believe that the manuscript is much improved for them. For the most part we have amended the manuscript as suggested by the reviewers, and we detail these amendments below. Where we have chosen not to modify the manuscript, justification has been provided.

Editorial comments:

- ***Textual overlap*:**

  Information amended on *lines 150-151* and now reads: *"Use a standard electrophysiological setup to perform whole-cell patch clamp techniques can be used"*.

  Micropipette formation procedure has been amended on *lines 153-159* and now reads: *"Prepare micropipettes using a two-step protocol (heat step 1: 70; heat step 2: 45) on a micropipette puller. Note: Settings used may vary depending on temperature within room and can change quite frequently. Micropipettes should have a final resistance ranging from 3 – 5 MΩ with internal solution when placed in the bath.".*

- ***Introduction*:**

  Introduction has been amended to include advantages over alternative techniques and broader literature in *lines 51-73* and now reads:

  "Previously, application of GVS to stimulate primary vestibular afferents has been performed in vivo in squirrel monkey, chinchillas, chicken embryos and guinea pigs. However, only two of these studies examined the effect that in vivo GVS has on the gain of primary vestibular afferents. These experiments were performed in vivo meaning that the precise impact of stimulation on vestibular nuclei neurons cannot be determined. To our knowledge, only one other study has applied stochastic noise to individual enzymatically dissociated neurons in the central nervous system. However, no experiments have been performed in the central vestibular nuclei to assess stimulus parameters and thresholding techniques, making this protocol more precise in determining stimulus effects on individual neurons within the vestibular nuclei.".

- ***Protocol language:***

  All protocol language amended to be imperative.

- *Protocol details:*

  1) *Line 111* now reads: *"anaesthetize the mouse with Isoflurane (3-5%) saturated in oxygen (3 mL/min)"*.

  2) *Line 135* amended to include glue used and now reads: *"use cyanoacrylate glue to fix the polystyrene block with the attached brainstem rostral end down to the cutting stage".*

3) *Lines 153-159* amended to describe micropipette formation and puller settings (see second comment under textual overlap).

- ***Discussion:***

  Discussion has been amended to highlight the points mentioned. Limitations amended in *lines 281-289*, advantages amended in *lines 291-300*, future applications indicated on *lines 302-308* and critical steps *lines 277-279*.

- ***Figures/table legends*:**

  Statistical tests and asterisk allocations amended on *lines 261-263* and now read: *"Error bars represented as S.D. Statistical significance determined by linear regression analysis comparing the gradients of the lines of best fit between control and experimental condition. ** = p < 0.02. *** = p < 0.01"*.

- ***Commercial language*:**

  Commercial language (MATLAB, Narishige etc) has been amended throughout the manuscript.

Reviewer 1:

- ***"Although the discussion section is adequate and the references are enough to understand the importance of the main findings it would be relevant to contrast this manuscript with previous reports about stochastic resonance in the peripheral vestibular system and the central nervous system":***

  We thank the reviewer for pointing this out and we have included previous work performed *in vivo* in the peripheral vestibular system and the advantages of this technique in more accurately determining the effects of GVS on the central vestibular system. *Lines 296-300* now read:

  "further, previous work in stimulating primary vestibular afferents in vivo has provided important information regarding the mechanism of action of GVS but cannot directly assess how the central vestibular nuclei respond to this stimuli. This highlights the sensitivity and precision of whole-cell patch clamp recordings in elucidating how GVS improves vestibular function".

- ***"The authors claim that their protocol is the first in vitro application of stochastic noise directly to individual MVN neurons"*:**

  We thank the reviewer for bringing this work to our attention. The paper highlighted (Remedios et al 2019) was published after submission of this manuscript and therefore we were unaware of any other publications applying noise to individual neurons at the time. However, this paper recorded from enzymatically dissociated neurons of the cerebral cortex, as opposed to recording directly from individual neurons within the tissue slice. *Lines 69-71*

read: ("*To our knowledge, only one other study has applied stochastic noise to individual enzymatically dissociated neurons in the central nervous system*") and *lines 303-308* read:

"One study has applied stochastic noise to a non-spontaneously active neuronal population within the somatosensory and auditory cortices of rats. However, this was performed in a cell suspension of enzymatically dissociated pyramidal neurons and was recording Na+ currents specifically, which are taken from postsynaptic cells using voltage clamp experiments. In this protocol the spontaneous activity of MVN neurons is being recorded from individual neurons within transverse slices of the brainstem using current clamp experiments".

- ***"Because in the literature of stochastic resonance different types of noise can be applied; e.g., mechanical, electrical, optical, optogenetic-noise-photostimulation, etc, the authors must specify through the text the type of noise employed in their study."*:**

  We thank the reviewer for this comment and have amended the manuscript to make clear that electrical noise was used within this protocol. The point of using different stimulation techniques is an important one methodologically, and such we have made reference to this fact in *lines 60-63* which now read: "*The above work in humans was performed using electrical noise stimuli, whilst much of the in vivo animal work has used mechanical or optogenetic noise stimuli. This protocol will use electrical noise to examine the effects on vestibular nuclei*". For our work however we focus on electrical stim due to the non-invasive therapeutic options and the established *in vivo* human and animal work using galvanic vestibular stimulation.

- ***"The manuscript could be improved if the authors include a brief introduction and discussion about the findings of stochastic resonance in the peripheral vestibular system.":***

  We thank the reviewer for their comment regarding previous *in vivo* studies examining the effects of GVS on the peripheral vestibular system. We have included a brief introduction and discussion to some of these works (Kim and Curthoys 2004, Flores et al 2016) on *lines 65-73*. These lines read:

  "*Previously, application of GVS to stimulate primary vestibular afferents has been performed in vivo in squirrel monkeys, chinchillas, chicken embryos and guinea pigs. However, only two of these studies examined what effect GVS has on the gain of primary vestibular afferents. These experiments were performed in vivo meaning that the precise patterns of stimulation imposed on vestibular nuclei cannot be determined. To our knowledge, only one other study has applied stochastic noise to individual enzymatically dissociated neurons in the central nervous system. However, no experiments have been performed in the central vestibular nuclei to assess appropriate stimulus parameters and thresholding techniques, making this protocol more precise in determining stimulus effects on individual neurons within the vestibular nuclei*".

Reviewer 2:

- ***"One set of studies in animals that might be worth mentioning has shown that different regularities of primary neurons stimulated by GVS similar to humans might contribute to oculomotor and spinal reflexes differentially (e.g., Kim and Curthoys, 2004, Kim, 2013).":***

We thank the reviewer for the comments and have included additional studies (including those suggested) examining the effects of GVS on primary vestibular afferents *in vivo* on *lines 65-73* in the introduction and *lines 296-300* in the discussion. (see comment above and *lines 296-300* in the discussion extracted below):

"Further, previous work in stimulating primary vestibular afferents in vivo has provided important information into how GVS may be working but cannot directly assess how the central vestibular nuclei respond. Therefore highlighting the sensitivity and precision of whole-cell patch clamp recordings in elucidating how GVS may improving vestibular functioning".

- ***"I personally do not see the limitations as real limitations per se.":***

Limitations have been amended and added to on *lines 281-289*. However, the point of noting the limitation of only stimulating a single neuron was to highlight that there is a population of neurons within the medial vestibular nucleus and therefore act in concert to produce the behavioural effects observed *in vivo*. *Lines 281-289* now read:

"Although this preparation provides clear advantages over previous in vivo work in animals, there are still some caveats. First, the stimuli are applied to individual neurons and therefore the thresholding of stochastic and sinusoidal noise may not represent what is occurring at a population level. However, using this protocol we are able to analyze changes at a single neuron level and use this information to subsequently model what may happen in behavioral studies. Second, these electrophysiological recordings are limited to neurons that display spontaneous activity or responses to direct current injection to simulate natural activity. This is one of the reasons for choosing the MVN as a target for testing the effects of these electrical stimuli, as it exhibits spontaneous neuronal activity".

Reviewer 3:

- Abstract:

- ***"low amplitude (6 pA) sinusoidal and stochastic noise can modulate the sensitivity of individual neurons in the MVN without affecting basal firing rates.":***

We appreciate the reviewers comment, and have amended *line 43* to read: *"We show that subthreshold sinusoidal and stochastic noise can modulate the sensitivity of individual neurons in the MVN without affecting basal firing rates"*. In addition, elsewhere in the paper (*lines 236-240*) we also state that the subthreshold noise (below an objective threshold of 12 pA) modulated neuronal sensitivity and not firing rate of individual neurons.

- Introduction:

- ***"This does not discuss previous work done using galvanic vestibular stimulation":***

We thank the reviewer for the comments and in line with both reviewer 1 and 2 we have amended *lines 65-73* to include literature regarding *in vivo* application of GVS to primary vestibular afferents. It now reads:

"Previously, application of GVS to stimulate primary vestibular afferents has been performed in vivo in squirrel monkey, chinchillas, chicken embryos and guinea pigs. However, only two of these studies examined the effect that in vivo GVS has on the gain of primary vestibular afferents.".

In addition, *lines 51-62* highlight galvanic vestibular stimulation experiments that have been performed in humans, which is the most relevant literature regarding this topic. However, no studies regarding GVS have been performed *in vitro* and the purpose of the no noise/stimulus control in this protocol is to highlight basal functioning of the MVN, which has been demonstrated (Camp et al 2006, Camp et al 2010).

- ***"Authors need to clearly state what are the differences in the stimuli parameters used in the references 8-11 claiming the application of GVS to improve balance control to show rationale of development of protocol to compare their effectiveness in their invitro preparation."*:**

We thank the reviewer for the comments and have amended *lines 56-58* to read:

"However, many of these studies have used different combinations of stimulus parameters such as white noise, colored noise, different stimulus frequency ranges and thresholding techniques.". Few human studies assessing the benefits of GVS have used 'random' stimuli, such as those exhibiting Brownian motion or a 1/f-type noise (colored or pink noise). We have elected not to describe the stimulus development in full since it is under patent application.

- Protocol:

- ***"Please state How many M and females assuming 6 total":***

We thank the reviewer for the comments, however, we believe the reviewer has misunderstood the figures and subsequently the numbers of animals used and thus cells recorded from. For the data in question the more important consideration is the number of cells recorded, since multiple can come from a single animal. The data presented here comes from 13 Male and 7 female mice (not 6 mice) as interpreted from figure 2B. In **Figure 2B** the n-values for each experimental group have now been included within their respective boxes, as well as in the legend (*lines 247-250*). In some instances data points correspond to the same control value since recordings were made from the same cell (i.e. 3 and 6 pA stochastic and sinusoidal stimuli applied to the same neuron are compared to the same control value).

- ***"In item 7, Generation of stochastic noise signal needs reference for the generation of the Brownian motion like paradigm":***

We thank the reviewer for their comment and have now removed the reference to Brownian motion from the manuscript.

- ***"Sinusoidal noise protocol usage has been linked to positive controls?":***

We thank the reviewer for their comment and agree that terming 'sinusoidal noise' a positive control may be misleading. *Line 194* has been amended to reflect this and terming of sinusoidal noise a positive control removed from the manuscript throughout.

- ***"Authors state citation of Moss et al. 2004 - adding sensory noise to weak signals results in enhanced signal detection, a phenomenon termed "stochastic resonance" (Moss, Ward & Sannita, 2004)…"***

  We thank the reviewer for the comment, however, we believe that **Figure 1** has been misread. The baseline mean of each stimuli are not changed within **Figure 1**, as each depolarising current step is a separate trace and noise profile. This was necessary for calculating the gain of individual MVN neurons either in the presence or absence of noise. The true stimuli, are the lowest lines of these figures where there are no current steps and therefore no change in the average baseline of the signal. Figure 1 was added to demonstrate how gain was calculated by applying a 10s current step that started at 0 pA (no step, normal stimulus) and increased by 10 pA up to 50 pA.

- ***"Figure 1 indicates control protocols with no noise were applied but in the Figure 2 the effect of the control is not shown at the same amplitude of stimulation - controls are all bunched to one end":***

  We thank the reviewer for their comment and we believe the above comment will sufficiently answer this query.

- Results:

- ***"Have you checked if the variability of firing rate is affected by application of stimulus...":***

  We thank the reviewer for highlighting this error. The wrong figure legend was attached to the figure displayed and may have generated confusion. We have amended *lines 247-253* of the **Figure 2** legend. It now reads:

  "Firing rate of MVN neurons in response to control (n = 53), stochastic and sinusoidal noise protocols (with no current steps) of amplitudes 3 (SN, n = 30; sine, n = 6), 6 (SN, n = 46; sine, n = 17), 12 (SN, n = 13; sine, n = 4), 18 (SN, n = 5; sine, n = 0) and 24 (SN, n = 8; sine, n = 0) pA. Lines/whiskers indicate the maximum and minimum values, the box indicates the 25th - 75th percentiles and the line within the box indicates the mean firing rate (spikes/s). The dashed line indicates experimental threshold, as chosen by pooling the mean firing rates within 3 and 6 pA (below 12 pA) and 18 and 24 pA (above 12 pA) shown in ***supplementary file 1"***.

  To answer the reviewers query of the variability of firing rate, we found no changes in variability to the firing rate in the absence and presence of stimuli (**supplementary file 1**).

  We have added a supplementary file to show the analysis of the groups above, and below, the 12 pA threshold showing a clear delineation at this amplitude, highlighting the choice of

this amplitude as our threshold. Also, we believe that the use of a box plot best represents this increasing trend and is best supplemented by supplementary file 1.

In addition, for clarity **Figure 2B** has been amended ('control' to '0 pA 'on x-axis and 'control' to 'no noise' in the key).

- ***"Line 238: Figure 2B shows the average firing rate and not the variability.":***

Please see the above comment.

- ***"Average firing rate and Neuronal sensitivity are shown but not presented in protocol as to how they were determined?":***

We thank the reviewer for the comment and we have amended *lines 199-204* to show how neuronal firing rate and sensitivity was calculated. They now read:

"Calculate the average firing rate over the 10 s period where the depolarizing current step was/will be injected for each individual current level (i.e. 7 total episodes; Figure 1).

Use the average firing rate values to generate a Firing rate vs. current plot and perform a linear regression analysis to determine the gradient of the line of best fit. The gradient of the line of best fit is indicative of the neuronal gain".

- Discussion:

- ***"Central to this paper is the statement on line 274: impact of noise on the sensitivity of individual neurons in the vestibular system. Is this the goal of the paper?":***

The author is correct and this is highlighted on *lines 75-77* which read:

"Here, we describe how to apply sinusoidal and stochastic (electrical) noise directly to individual neurons in the medial vestibular nucleus (MVN), determine neuronal threshold and measure changes in gain/sensitivity."

- ***"What is noise - Here defining noise as used in this paper is important.":***

We thank the reviewer for their comment and believe that previous amendments and explanations to comments 4 and 5 within the protocol section, should provide sufficient explanation to this query.

- ***"Figure 2 B results discussion state there is no significant difference between 6 pA and up to 24 pA with only an increasing trend.":***

We thank the reviewer for their comment and have included a supplementary file (**supplementary file 1**) to highlight how we objectively determined the threshold. We grouped the values that are below (3 and 6 pA) and above (18 and 24 pA) the 12 pA threshold and performed an ANOVA on these averages. We found that the average of those

values above the threshold are significantly different to both the control and below threshold values as shown in **supplementary file 1**.