Targeted optogenetic expression in the non-human primate central vestibular system

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Abstract (EN)

The vestibular system is essential to ensure that self-motion information is processed, giving rise to vital reflexes alongside perception. Here we investigated the feasibility of using viral vectors to express opsins within neurons in central vestibular pathways of non-human primates (NHPs). Importantly, we chose our vector constructs while considering the functional relationships and anatomical differences between our regions of interest, which span from the brainstem to the cerebral cortex. Using optogenetics would allow for fine manipulation of vestibular pathways *in vivo*, increasing the potential of our understanding of how vestibular neurons represent sensory input. Due to our shared bipedality and close evolutionary origins, NHPs are ideal model animals for studying the vestibular system. However, unlike applications in invertebrates and rodents, optogenetic techniques using NHPs have not been well established, requiring researchers to infer the best approach.

Our study induced optogenetic expression through injection of two opsin adenoviruses (serotype: AAV9) in different central vestibular brain regions of a cynomolgus macaque: the vestibular nuclei (VN) in the brainstem, and the parietoinsular vestibular cortex (PIVC). Specifically, to induce expression in VN, we injected a virus into the neighbouring abducens nucleus (ABD) with a promoter specific to neurons (hSyn) containing the gene from the red-shifted opsin Jaws. In PIVC, we injected a virus with a promoter specific to cortical GABAergic interneurons (mDlx) containing the gene for Channelrhodopsin-2. We evaluated and quantified optogenetic expression using immunohistochemical techniques, confocal fluorescence microscopy, and image processing. Our results demonstrate our chosen viral constructs' robust specificity and sensitivity to their respective target regions and neurons. With the histological confirmation of optogenetic expression in our brain areas of interest, our study will set in motion the application of optogenetic techniques to future NHP studies investigating coding and information feedback along central vestibular pathways.

Resume (FR)

Le système vestibulaire est essentiel pour s'assurer que les informations d'auto-mouvement sont préparés, donnant lieu à des réflexes vitaux en même temps qu'à la perception. Nous avons étudié ici la faisabilité de l'utilisation de vecteurs viraux pour exprimer des opsines dans les neurones des voies vestibulaires centrales des primates non humains (NHP). Surtout, nous avons choisi nos constructions vectorielles en tenant compte des relations fonctionnelles et des différences anatomiques entre nos régions d'intérêt, qui s'étendent du tronc cérébral au cortex cérébral. L'utilisation de l'optogénétique permettrait une manipulation fine des voies vestibulaires *en vivo*, améliorant ainsi notre compréhension de la manière dont les neurones vestibulaires représentent l'entrée sensorielle. En raison de notre bipédie commune et de nos origines évolutives proches, les NHP sont les animaux modèles idéaux pour l'étude du système vestibulaire. Cependant, contrairement aux applications chez les invertébrés et les rongeurs, les techniques optogénétiques utilisant les NHP n'ont pas été bien établies, ce qui oblige les chercheurs à déduire la meilleure approche.

Notre étude a induit l'expression optogénétique par l'injection de deux adénovirus d'opsine (sérotype : AAV9) dans différentes régions du cerveau vestibulaire central d'un macaque cynomolgus : les noyaux vestibulaires (VN) dans le tronc cérébral et le cortex vestibulaire pariéto-insulaire (PIVC). Spécifiquement, pour induire l'expression dans le VN, nous avons injecté un virus dans le noyau abducens (ABD) voisin avec un promoteur spécifique aux neurones (hSyn) contenant le gène de Jaws, une opsine décalée vers le rouge. Dans le PIVC, nous avons injecté un virus avec un promoteur spécifique aux interneurones GABAergiques corticales (mDlx) contenant le gène de la Channelrhodopsine-2. Nous avons évalué et quantifié l'expression optogénétique en utilisant des techniques immunohistochimiques, la microscopie confocale à fluorescence, et le traitement d'images. Nos résultats démontrent la spécificité et la sensibilité robustes des constructions virales que nous avons choisies pour leurs régions cibles et leurs neurones respectifs. Avec la confirmation histologique de l'expression optogénétique dans nos zones cérébrales d'intérêt, notre étude mettra en route l'application des techniques optogénétiques à des futures études de NHP portant sur le codage et la rétroaction de l'information le long des voies vestibulaires centrales.

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Contribution of Authors

All chapters of this thesis were written by Tabitha Bethany Jimenez and edited by Dr. Maurice Chacron and Tabitha Bethany Jimenez. Data collection, analysis, and figure generation were completed by Tabitha Bethany Jimenez.

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List of Abbreviations

AAV: adeno-associated virus

ABD: abducens nucleus

ChR2: Channelrhodopsin-2

FEF: frontal eye field

FTN: floccular target neuron

NHP: non-human primate

PIVC: parietoinsular vestibular cortex

PVP: position-vestibular-pause (neuron)

SC: superior colliculus

VN: vestibular nucleus

VO: vestibular-only (neuron)

VOR: vestibuloocular reflex

VSR: vestibulospinal reflex

VPL: ventral posterolateral (nucleus)

Introduction

Introduction to Optogenetics

In the study of systems neuroscience, optogenetic tools allow for fine, temporally precise manipulation of neural activity, advancing our understanding of the circuitry and coding of complex behaviours and sensory perception. Implementing optogenetics in mammals often begins with selecting a viral vector, which comprises a protein capsid containing a promoter and a gene for an opsin, a light-sensitive ion channel. The vector is injected into a targeted brain region, and if the vector can transduce target neurons successfully, opsin expression can be observed histologically or activated in vivo only several weeks later (Zhang et al., 2010).

Since the first application of optogenetics to a behaving animal in 2005, there has been an exponential increase in new optogenetic studies, primarily with invertebrates and small mammals such as mice and rats (Deisseroth, 2015). Optogenetics has allowed for unique insights into several categories of neuroscience research, such as cell and circuit connectivity, information storage in the brain, and neurological and psychiatric diseases (Deisseroth, 2015); for instance, the mechanisms behind odor discrimination in the olfactory bulb (Lepousez et al., 2013), the recall of fear memories through hippocampal engram stimulation (Liu et al., 2012), and the changes in cortical inhibition in Huntington's disease mouse models (Cepeda et al., 2013), respectively. The popularity and efficacy of optogenetics is possible due to the method's reliance on light to manipulate neural circuitry, allowing for reversible neuronal modulation with high temporal resolution. With the refinement of implementation techniques and the discovery of new gene promoters for specific cell types, optogenetics could be used to target

any neuron (Deubner et al., 2019). The future of optogenetics continues to lie in investigations of complex behavioural effects, perception, cognition, and treatment of human disease phenotypes.

Consequently, applying optogenetics to more complex mammals, such as non-human primates (NHPs), becomes imperative. This is because NHPs are much closer evolutionarily to humans (Harding, 2017; Gerits & Vanduffel, 2013). However, working with NHPs is a privilege, with few animals being used for any given study. It is thus imperative to ensure that experimental methods involving optogenetics are successful at the onset. To aid in the task of establishing methods for implementing optogenetic tools in NHPs, "toolbox" papers aim to provide clarity and provide discussion on implementation and critical considerations.

Optogenetics in the study of sensory systems

Many published NHP optogenetic studies often pertain to the visual system, as NHPs are vital model animals in the field due to anatomical and perceptual similarities of the primate visual system to that of humans. Visual system researchers have successfully used optogenetics to perturb primate visuomotor behaviour. For example, Jazayeri et al. (2012) found that optogenetic stimulation of primate V1 neurons induced saccades towards their respective neuron receptive fields (Jazayeri et al., 2012). In addition, Cavanaugh et al. (2012) detected deficits in saccade distance, latency, and velocity with optogenetic inhibition of superior colliculus (SC) neurons (Cavanaugh et al., 2012).

Other researchers have used optogenetics to tease apart relationships between brain areas or pathway-specific effects on saccadic behaviour. Inoue et al. (2015) found that an

injection of an optogenetic vector into the frontal eye field (FEF) resulted in optogenetic expression in connected SC neurons through FEF projections. Optogenetic stimulation of transduced SC neurons resulted in saccades towards the receptive field of a given SC neuron, elucidating the role of the FEF in generating saccades (Inoue et al., 2015). Furthermore, Amita et al. (2020) discovered through optogenetic stimulation of caudate tail neurons that the caudate tail enables saccades toward objects associated with a high-value award (Amita et al. 2020).

Alternatively, researchers have used optogenetics in cortical areas to evaluate the effects of optogenetics on visual perception, as indicated through behavioural tasks used to gauge perception, instead of perturbing visuomotor behaviour such as in studies outlined prior. For instance, Fetsch et al. (2018) found that optogenetic inhibition of neurons in the extrastriate visual cortex results in visual stimuli direction discrimination that is less confident and is biased against the direction sensitivity of inhibited neurons. However, perceptual effects decreased with time over a given recording session, possibly due to cortical compensatory mechanisms acting against optogenetic inhibition (Fetsch et al., 2018). In contrast, De et al. (2020) found that visual cortex inhibition using an optogenetic vector with a promoter specific only to inhibitory GABAergic neurons resulted in sustainable impacts on visual perceptual tasks (De et al., 2020). Discoveries in the visual system using optogenetics range from answering functional and anatomical questions to answering questions pertaining to complex behaviour and perception.

Toolbox papers for applying optogenetics to NHPs

Many papers implementing optogenetics in NHPs emphasize questions related to methodology, exploring the efficacy of numerous vector constructs and injection modalities available commercially or easily built by researchers. Applying optogenetics to NHPs often begins with acquiring a vector construct with a chosen protein capsid, gene promoter, and opsin. Six weeks before an experiment start date, the vector construct is injected into areas of the brain targeted for optogenetic expression; usually, brain areas are key to specific sensory systems and modalities. Research often evaluates through a single or a combination of histological, electrophysiological, and behavioural results. Specifically, researchers can verify the location and prevalence of optogenetic expression in targeted brain areas using immunohistochemical techniques and confocal fluorescent microscopy. Additionally, researchers can validate optogenetic manipulation of neural circuitry and systems through electrophysiological recordings and observation of behavioural and perceptual effects (Tremblay et al., 2020).

For example, a toolbox paper by Dimidschstein et al. (2016) discovered that a promoter targeting genes for distal-less homeobox 5 and 6 (mDlx5/6) allowed for robust targeting of cortical GABAergic neurons for opsin expression. The promoter sufficiently targeted GABAergic neurons of four different species, including marmosets, an NHP (Dimidschstein et al., 2016). Furthermore, Yazdan-Shahmorad et al. (2018) produced a toolbox paper exploring the injection of a large volume of optogenetic viral vector through convection-enhanced delivery, previously seen in gene-therapy applications, in order to transduce larger areas of the brain. Traditionally, infusion time with smaller volumes is significantly longer, and damage to the brain is often

observed when targeting large areas of the brain, as it is necessary to inject using multiple cannula tracks (Yazdan-Shahmorad et al., 2018). Furthermore, a toolbox paper by Fortuna et al. (2020) compared resulting optogenetic expression patterns and trafficking via axons of injected vector constructs for opsins eNpHR3.0, ChR2, and C1V1 in rhesus macaques. After comparison, researchers observed varying expression patterns despite opsin expression being driven by the same promoter, CaMKIIα (Fortuna et al., 2020). Moreover, Mendoza et al. (2017) addressed a possible confounding factor to consider when using a given experimental animal for several optogenetic experiments, as is often the case when working with NHPs. They found that multiple injections of viral vectors with the same adeno-associated virus (AAV) serotype in a given animal can induce a humoral immune response in later injections, emphasizing the importance of using different serotypes in subsequent injections (Mendoza et al., 2017).

The presence of toolbox papers for optimal NHP optogenetics and knowledge of many unpublished attempts at implementing optogenetics to NHPs led Tremblay et al. (2020) to compile data taken from 45 laboratories. Data collected included NHP species, viral vectors, promoters, opsins, and other factors used in published and unpublished NHP optogenetics experiments. The study determined which factors and tools allowed for an ideal or strong effect on physiological, anatomical, or behavioural aims. For example, when used, the serotype AAV9 was found to result in a strong effect in a given aim 93% of the time despite only being used in 13% of the acquired experimental database, illustrating the importance of compiling a large data pool in order to determine the most effective methodological choice for a given NHP optogenetic experiment (Tremblay et al., 2020). One key finding is that 47% of collected

experiments either did not test for anatomical aims or observed only weak, mixed, or no effects on anatomical aims (Tremblay et al., 2020).

Due to the interest in the vestibular field in many small and isolated structures and regions spanning the brainstem, thalamus, and cortex, demonstrating robust optogenetic expression is critical for sustainable research and application of optogenetics to the non-human primate vestibular system. Current investigations of the NHP vestibular system involve the encoding of naturalistic vestibular stimuli, population coding, complex behavioural tasks, and perception, all of which can benefit from the future implementation of optogenetic tools.

Introduction to the Vestibular System

Vestibular system structure and function

Sight, sound, touch, smell, and taste are the classical five basic human senses, and they are known as such because of their distinguishable and easy-to-conceptualize nature. Any of these five senses can be observed in isolation from the others. On the other hand, vestibular sensations cannot be easily perceived in the absence of other senses and cannot be consciously manipulated but are, however, integral to many functions executed by our central nervous system (Angelaki & Cullen, 2008). In particular, the vestibular system is involved in activities requiring information from our visual system, such as gaze stabilization, and activities requiring information from our proprioceptive system, such as posture, balance, and perception of self-motion and spatial orientation (Cullen, 2012). Pathologically, our vestibular system can be the root of symptoms such as nausea, dizziness, and disorientation (Goldberg et al., 2012).

From a research perspective, the study of the vestibular system may lead to unique insights due to its multisensory nature, particularly in the field of neural coding. Questions in neural coding involve understanding the brain as a system in which stimuli inputs are processed to give rise to a measurable output, i.e., a sensation, perception, or behaviour. Neural coding can be investigated from the perspective of encoding, the modeling of brain activity with knowledge of a given input stimulus, or from the perspective of decoding, the determination of input stimulus qualities from observed brain activity (Holdgraf et al., 2017).

The vestibular sensing organs, located in the inner ear, are the beginnings of the vestibular system. These comprise five organs containing hair cells that sense motion along different planes of movement; three semicircular canals, which sense rotational motion orthogonally from each other, and two otolith organs known as the utricle and the saccule, which sense linear accelerations, including that of gravity, (Angelaki & Cullen, 2008; Goldberg et al., 2012). Hair cells give rise to afferents, nerves that directly receive information from sensory stimuli and transfer information to the central nervous system (Goldberg, 2000). From the vestibular sensors in the inner ear, afferent fibers synapse onto the vestibular nuclei (VN) in the brainstem. Downstream, VN synapses to several brain areas, such as the abducens nuclei (ABD), giving rise to gaze stabilization or the vestibuloocular reflex (VOR); the spinal cord, facilitating posture and balance or the vestibulospinal reflex (VSR) (Cullen, 2012); and the ventral posterolateral (VPL) nucleus in the thalamus which later synapses onto several cortical areas, but primarily to the parieto-insular vestibular cortex (PIVC), giving rise to the perception and estimation of self-motion (Cullen, 2019).

Vestibular brainstem nuclei

We are interested in two different brainstem nuclei, the first being the abducens nuclei (ABD), from which the sixth cranial nerve originates. It is situated in a small and isolated location, with a stereotyped firing rate, making it a simple landmark to map once found. Most importantly, it is directly responsible for an easily observable behaviour, the VOR, or horizontal eye movements via lateral and medial rectus muscles (Sylvestre & Cullen., 1999). The ability to target and perturb a relatively simple behavioural effect would be ideal for evaluating the efficacy of our optogenetic methods.

Secondly, in the brainstem, we are interested in the vestibular nucleus (VN), which, similar to ABD, is situated in a smaller and isolated location. The vestibular afferents from the brainstem synapse to three types of VN neurons; position-vestibular-pause neurons or PVP neurons, floccular target neurons or FTN, and vestibular-only neurons or VO neurons (Cullen, 2012). Both PVP neurons and FTN are active in response to head and eye movements and mediate reflexes such as the VOR, which is responsible for gaze stability, and later transfers information to the cerebellum and oculomotor plant. PVP neurons and FTN also directly synapse to ABD motoneurons, facilitating VOR (Cullen & Taube, 2017; Scudder & Fuchs, 1992). In contrast, VO neurons are only active in response to vestibular movements and are responsible for vestibular input that mediates balance and posture. VO neurons are also connected to the spinal cord and higher-order structures, such as the thalamus VPL and cortical PIVC, which mediate more complex movements and motion perception (Cullen & Taube, 2017; Cullen, 2019). In other words, VO neurons in VN encode for both translational and rotational motion, upstream perception. A target area upstream vestibular perception is ideal for

investigating feedback between the cortical and subcortical areas of the central vestibular system.

Targeting nuclei in the brainstem, situated in the pons, required specific consideration of components of our virus and viral construct. For our virus, we chose to use the serotype AAV9, which was found to be the most successful virus serotype in a database containing results of non-human primate optogenetics experiments from 45 laboratories (Tremblay et al., 2020), and a promoter for human synapsin 1, which is specific to neurons. For our opsin, we decided on a halorhodopsin, Jaws, a red-shifted chloride pump that inhibits neurons. As the brainstem is a critical area for everyday functions, we wanted to reduce the risk of optical damage. A red-shifted opsin would allow us to excite our nuclei of interest without high-intensity laser light. Adenoviruses carrying the gene for the red-shifted halorhodopsin Jaws with expression driven by hSyn were first shown to successfully transduce rodent motor cortex neurons in 2014 (Chuong et al., 2014). They were previously found to successfully inhibit the activities of primate eye-movement-sensitive neurons in the brainstem (Krauss, 2020). In summary, our goal in using this virus construct is direct optogenetic inhibition of brainstem neurons.

Vestibular cortex

Following, for our other brain area of interest, we move away from subcortical vestibular areas and into the parieto-insular vestibular cortex (PIVC), situated in the Sylvian fissure or the lateral sulcus, (Frank & Greenlee, 2018). As a critical area in the cortical vestibular network, PIVC has feedback with upstream subcortical vestibular structures such as VPL in the

thalamus and VN in the brainstem (Cullen, 2019). PIVC is essential for determining or perceiving spatial orientation during voluntary and involuntary self-motion. For example, when PIVC is stimulated at rest, participants perceive vestibular sensation (Cullen, 2019). Applying optogenetics to a higher-order and well-connected vestibular area opens the door for novel temporally-precise experiments involving perception, coding in other vestibular structures of interest, and information processing between connected areas of interest.

To target PIVC, we chose to use the same virus serotype used in the brainstem, AAV9, and a promoter, distal-less homeobox 5/6, or mDlx, which was found to only be specific to GABAergic interneurons in the cortex (Dimidschstein et al., 2016). For our opsin, we decided on Channelrhodopsin-2, a light-gated ion channel that excites neurons. Previous studies have shown that adenoviruses carrying the gene for Channelrhodopsin-2 with expression driven by mDlx can successfully transduce GABAergic neurons in non-human primate area V1 (De et al. 2020).

As potential experiments in PIVC involve perception, maximizing the likelihood of behavioural effect was vital. Using a promoter specific to one neuron type decreases the likelihood of cortical compensatory mechanisms against optogenetic stimulation, and using an ion channel (ChR2), as opposed to a chloride pump such as Jaws, increases excitation sensitivity. Previously, a study found that the effect of optogenetic inhibition on a perceptual task mediated by the opsin Jaws was transient and reduced with experiment length (Fetsch et al., 2018). In summary, using this virus construct, our goal is the indirect inhibition of cortical excitatory neurons through optogenetic excitation of inhibitory interneurons.

Hypothesis

We hypothesize that the injection of the viral construct AAV9-hSyn-Jaws-KGC-GFP in vestibular brainstem nuclei and AAV9-mDlx-ChR2-mCherry-Fishell-3 in PIVC will result in sufficient optogenetic expression in the targeted brain areas of interest, allowing for electrophysiological and behavioural experiments in the NHP vestibular system.

Methods

Materials

Reagent type	Product	Source	Concentration	Cat #	Lot #
Virus	AAV9-mDlx-ChR2-mCherry-Fishell-3	Addgene	2.3E13 GC/mL		V95369
Virus	AAV9-hsyn-Jaws-CAG-ERP	Laval Vector Core	3.2E12 GC/mL		AAV1867
Primary antibody	mCherry polyclonal antibody, rabbit	Invitrogen	1:1000, 1:500	PA5-34974	WD3241274E
Primary antibody	Anti-green fluorescent protein, rabbit IgG fraction	Invitrogen	1:1000	A11122	2273763
Primary antibody	Monoclonal anti-parvalbumin antibody produced in mouse	Sigma	1:10,000, 1:5000	P3088	097M4834V
Secondary antibody	Alexa Fluor™ 488 donkey anti-rabbit IgG	Invitrogen	1:800	A21206	2256732
Secondary antibody	Alexa Fluor™ 568 donkey anti-rabbit IgG	Invitrogen	1:800	A10042	2207536
Secondary antibody	Alexa Fluor™ 647 donkey anti-mouse	Invitrogen	1:800	A31571	1984047
Blocking serum	Normal donkey serum	Jackson Immuno-Research	10%, 5%	017-000-121	153804
Neuron marker	NeuroTrace™ 435/455 blue fluorescent Nissl stain	Invitrogen	1:200	N21479	2174901
Auto-fluorescence quencher	TrueBlack® Plus Lipofuscin Autofluorescence quencher	Biotium	1:40	23014	20T1202

Injection

One female cynomolgus macaque, aged 5 years old, participated in this study. All experimental protocols were approved by the McGill University Animal Care Committee

(#2001– 4096) and were complied following the guidelines of the Canadian Council on Animal Care.

Methods for the preparation of the animal and the injection was previously described by Wetzel et al. (2019). The injection was accomplished using a frameless stereotactic targeting robot, (Wetzel et al., 2019). Targeted areas of interest ABD and PIVC were identified during a pre-operative MRI. A syringe (Hamilton) was mounted on an injection pump (Model UltraMicroPumps III, World Precision Instruments), attached to the arm of the targeting robot, and virus was injected at a rate of 0.5 μL per minute. Virus volume totaled 10 μL in the ABD (1 track, 3 sites) and 20 μL in the PIVC (2 tracks with 10 μL each, 5 sites per track). Injection cannulas were allowed to rest for 5 minutes prior to removal.

Histopathology

Six weeks after injection, the monkey was euthanized and perfused with 4% paraformaldehyde in phosphate buffer solution. The brain was removed and post-fixed in excess solution overnight, then cryoprotected in 30% sucrose in phosphate buffer until sunken at 4C, taking about 1 week. Afterwards, brain tissue was sectioned using a sliding microtome or cryostat into slices 50 μm thick. Slices were collected free-floating in phosphate buffer solution (PBS) in 6-well plates.

Immunohistochemistry

Unless stated otherwise, all subsequent washing and incubating steps were done on a shaker at room temperature for 10 minutes per wash.

After collection, slices were washed 3x in PBS, then washed 1x in PBS with 0.2% Triton-X (PBS-T). Slices were blocked with 10% normal donkey serum (NDS) (Jackson Immuno-Research) for 1 hour. To target our proteins of interest, slices were incubated in a solution of required primary antibodies and 5% NDS at 4C overnight. Specifically, brainstem slices were incubated with an rabbit anti-GFP primary antibody (1:1000) and cortex slices were incubated with rabbit anti-mCherry (1:500) and mouse anti-Parvalbumin (1:5000) primary antibodies.

The following day, to remove excess primary antibody, slices were washed 3x in PBS-T. To amplify detection of proteins of interest, slices were incubated in a solution of required fluorescent secondary antibodies against the species of the chosen primary antibodies (AlexaFluor, Invitrogen) and 435/455 blue fluorescent Nissl stain (1:200) (NeuroTrace, Invitrogen) for 2 hours. Following secondary antibody incubation, slices were washed 1x in PBS-T and 3x in regular PBS.

To remove auto-fluorescence by lipofuscin proteins, slices were quenched, off the shaker, with TrueBlack Plus (1:100) (Biotium) with the necessary volume to cover slices evenly for 10 minutes. After a final washing 2x with PBS, slices were ready for mounting on gelatin subbed slides with use of a small paintbrush. After mounting, slices were allowed to dry until excess PBS evaporated, then coverslipped with Aqua-Poly/Mount (Polysciences Inc.) as a mounting medium. Slides were allowed to dry on a flat surface, at 4C for 2 days or until coverslips were secure prior to subsequent handling.

Imaging and pre-processing

To gather images and evaluate the fluorescent results of immunohistochemical processing, slides were imaged using a Zeiss AxioImager M2 Imaging microscope and a Zeiss Axiocam 506 Color camera with Zen Blue software. Images were captured using 2.5x, 10x, and 20x objectives and Tiling and Z-Stack modes. Z-Stacks were merged with Orthogonal Projection to result in images with clear neuronal outlines.

Post-processing and analysis

Czi files from the preprocessed images were further processed using Fiji/ImageJ software, (Schindelin, 2012). Steps included Image > Adjust > Brightness/Contrast, Process > Subtract Background, Image > Type > converting to 16 and 8 bit images, Image > Adjust > Threshold, Process > Noise > Despeckle and finally using the multipoint tool to manually count neurons with distinct outlines. Counting was done for each relevant fluorescent colour channel, and signals were considered to be colocalized if signal was found at identical locations in two compared channels. Data points were exported using Analyze > Measure into Excel files, and modified for import into MATLAB. X-Y data points could then be visualized through a chart using MATLAB for ease of viewing.

Expression values were quantified by comparing ratios of fluorescence positive neurons in one channel to another channel, allowing for measures of virus sensitivity and specificity to virus targets.

For Figure 4, transduction area in mm^2 was measured in in Fiji, with the approximate coronal axis (x-axis) determined per slice after comparison of slice morphology to MRI images of the given animal's brain.

Results

We targeted two central vestibular brain areas with different gene constructs contained in the adeno-associated virus, AAV9. In the brainstem, abducens nucleus neurons (ABD) were targeted with a gene for the halorhodopsin *Jaws* conjugated with GFP, with expression driven by the neuron-specific promoter human synapsin I (hSyn). Conversely, parietoinsular vestibular cortex (PIVC) neurons were targeted with a gene for channelrhodopsin-2 conjugated with mCherry, with expression driven by the promoter distal-less homeobox 5/6 (mDlx), specific to cortical GABAergic neurons.

Expression in brainstem abducens nucleus (ABD) and vestibular nucleus (VN)

To evaluate if we can adequately drive optogenetic expression in brainstem vestibular brain areas, we injected the viral construct AAV9-hSyn-Jaws-KGC-GFP into the abducens nucleus of a female cynomolgus macaque using an automated injection system (see Wetzel et al. 2019 for details) and examined the resulting optogenetic expression histologically, (**Figure 1**).

We specifically aimed to induce expression in the abducens nucleus (ABD), as targeted and injected with our stereotactic injection system programmed with MRI data. However, we only saw robust expression of Jaws-GFP in neurons within the neighbouring vestibular nuclei (VN), seen in green and outlined in orange in **Figure 1A**. In brainstem slices more rostral and caudal to slices featuring robust Jaws-GFP expression, diffuse expression of Jaws-GFP can be seen in a more medial area of the brainstem, much like the area of the targeted abducens

nucleus (**Figure 1B**). The localization of diffuse expression of Jaws-GFP seen in slices more rostral and caudal to the injection site suggests that the viral vector was accurately injected in the target area ABD. ABD and VN are key interconnected regions of the central vestibular system. At a higher magnification, diffuse Jaws-GFP expression in a slice caudal to the injection site can be seen sparingly and does not clearly outline neuronal bodies or axons. (**Figure 1C**)

As the most robust Jaws-GFP expression is not found in our targeted site ABD, we instead quantified expression found in the VN, (**Figure 2**). At a higher magnification, Jaws-GFP expression is found to be expressed in both neuronal bodies and axons connecting said neuronal bodies (**Figure 2A**). When evaluating the sensitivity of our virus in a representative region, 39 of 114 (34%) NeuroTrace-positive neurons were positive for Jaws-GFP, (**Figure 2B**).

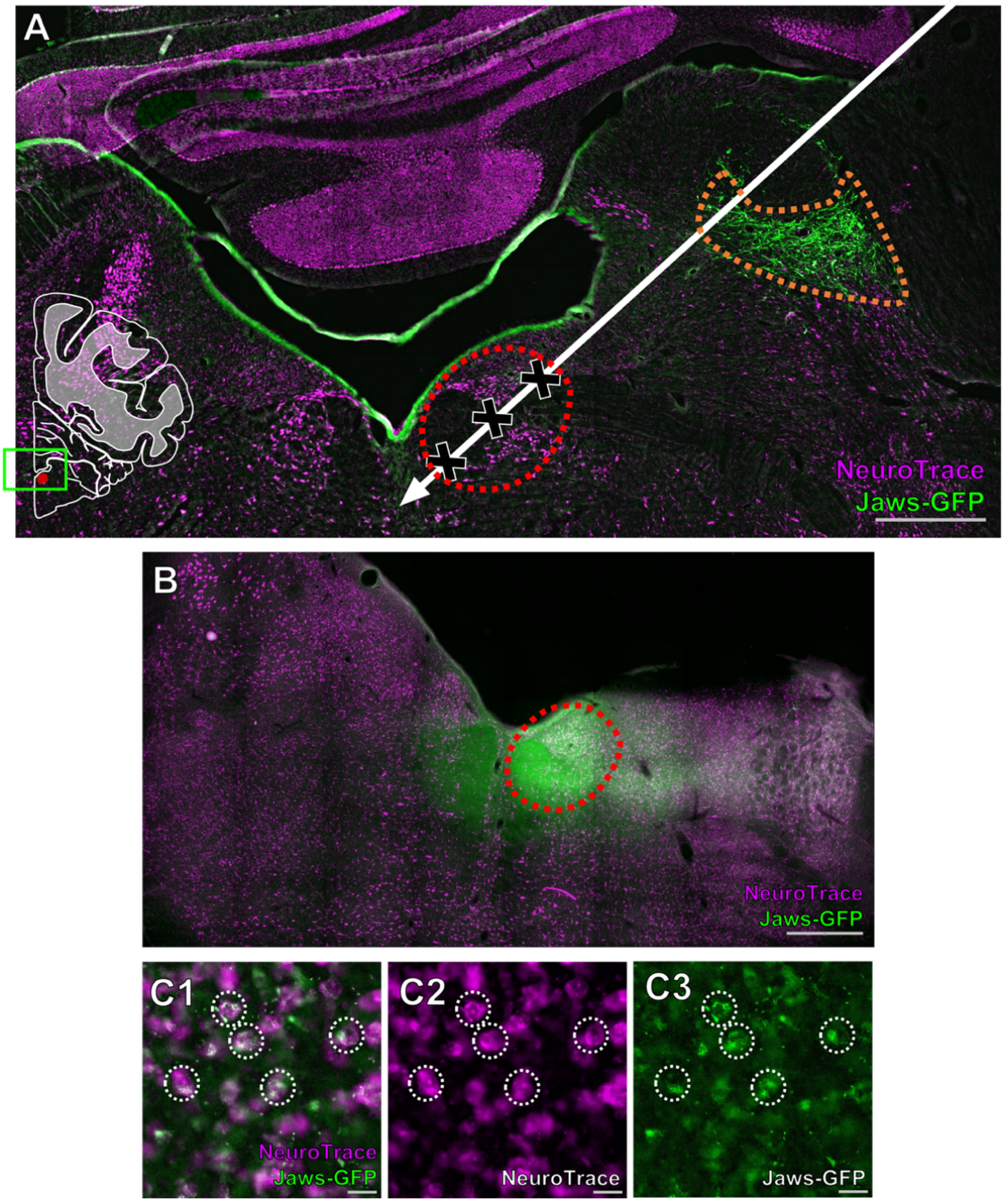

Figure 1. **Expression of Jaws-GFP in cynomolgus macaque brainstem vestibular areas abducens nucleus (ABD) and vestibular nucleus (VN)** *with fluorescent Nissl stain NeuroTrace pseudo-colored in magenta and Jaws-GFP expression pseudo-colored in green.*

*(**A**) Representative coronal section of a brainstem slice indicating the approximate target area ABD, outlined by a red circle, relative to pictured neighbouring connected area VN, outlined in orange. Clear Jaws-GFP expression is seen only in VN. The theoretical approach of the injection cannula is indicated by the white arrow, with targeted injection sites indicated by black Xs. The section pictured does not represent a section containing the exact plane of the injection cannula. The location of the taken image relative to coronal hemisection is indicated in the inset and outlined in the small green box. Scale bar = 1000 μm. (**B**) Representative coronal sections of a more caudal slice relative to the slice image taken in (A). Diffuse Jaws-GFP expression is outlined with red dashed lines, seen closer to the relative area of ABD. Scale bar = 1000 μm. (**C**) High-magnification images of diffuse expression seen in (B), with white dashed line circles for ease of comparison between channel images. Scale bars = 30 μm. (**C1**) Merged image containing: (**C2**) neurons marked with NeuroTrace Nissl stain and (**C3**) Jaws-GFP.*

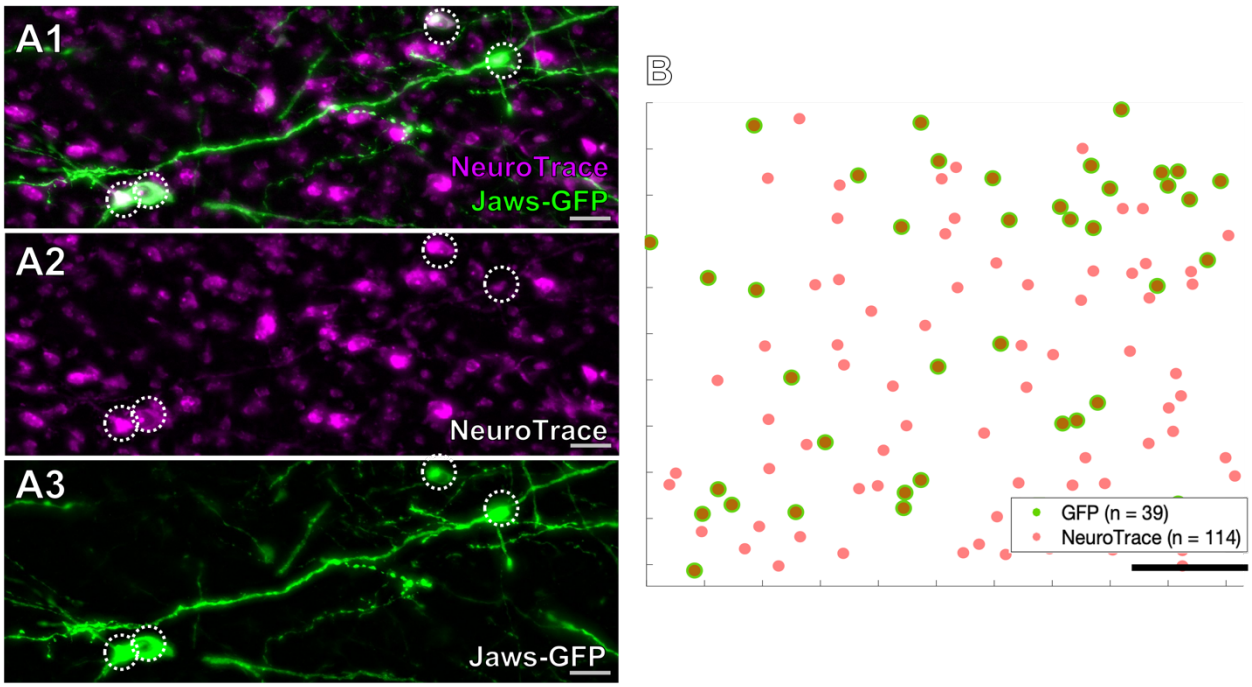

Figure 2. **Jaws-GFP expression quantification in VN** *with fluorescent Nissl stain NeuroTrace pseudo-colored in magenta and Jaws-GFP expression pseudo-colored in green. (**A**) High magnification representative images of expression in VN neurons, with white dashed line circles indicating colocalization between channel image signals. Scale bars = 30 μm. (**A1**) Merged image containing: (**A2**) neurons marked with NeuroTrace Nissl stain and (**A3**) Jaws-GFP. (**B**) Locations of cell bodies in a representative area of VN expressing Jaws-GFP in green and NeuroTrace in magenta. Scale bar = 100 μm. Of 114 NeuroTrace-positive neurons, 39 were positive with GFP, having a sensitivity of 34.5%.*

Expression in the PIVC

To evaluate if we can adequately drive optogenetic expression in the parietoinsular vestibular cortex (PIVC), we injected the viral construct AAV9-mDlx-ChR2-mCherry-Fishell-3 into the PIVC of a female cynomolgus macaque using an automated injection system (see Wetzel et al. 2019 for details) and examined the resulting optogenetic expression histologically, (**Figure 3**).

We saw the expression of ChR2-mCherry, seen in red, as far as 2.5 mm from the cannula track, encompassing our area of interest, PIVC, wholly (**Figure 3A**). As the expression of ChR2-mCherry was driven by a promoter specific to GABAergic interneurons, slices were also stained for Parvalbumin, a protein found in most GABAergic interneurons, seen in cyan. At a higher magnification, ChR2-mCherry is expressed throughout cell membranes and dendrites of Parvalbumin-positive neurons (**Figure 3B**). Within the region of PIVC in a stained slice, 684 neurons were positive with Parvalbumin expression, 281 neurons were positive with ChR2-mCherry expression, with 182 of these ChR2-mCherry positive neurons were also positive for Parvalbumin, (**Figure 3C**). When evaluating the sensitivity of our virus to interneurons in general, we find that the ratio of ChR2-mCherry+ neurons to Parvalbumin+ neurons is about 41%. When evaluating the specificity of our virus to PV-positive neurons, we find that 64% of expression+ neurons also are positive with PV.

As ChR2-mCherry expression was widespread through cortical slices, we looked at the expression pattern of ChR2-mCherry in the cortex moving in the more caudal direction, (**Figure 4A**). We quantified the transduction area in both the Sylvian fissure or lateral sulcus that contains PIVC and in other cortical areas superior PIVC, to gauge the expression intensity in

non-targeted areas of the brain. The white dashed lines mark out the Sylvian fissure or lateral sulcus containing PIVC. Using the MRI from this animal, we estimated the distances between slices and the expression area in mm^2 relative to the coronal axis, **Figure 4B**. Asterisks signify approximately where injection tracks were made. Overall, results show that transduction along the cannula track in the cortex with this promoter is inevitable and should be considered when conducting optogenetic electrophysiological study.

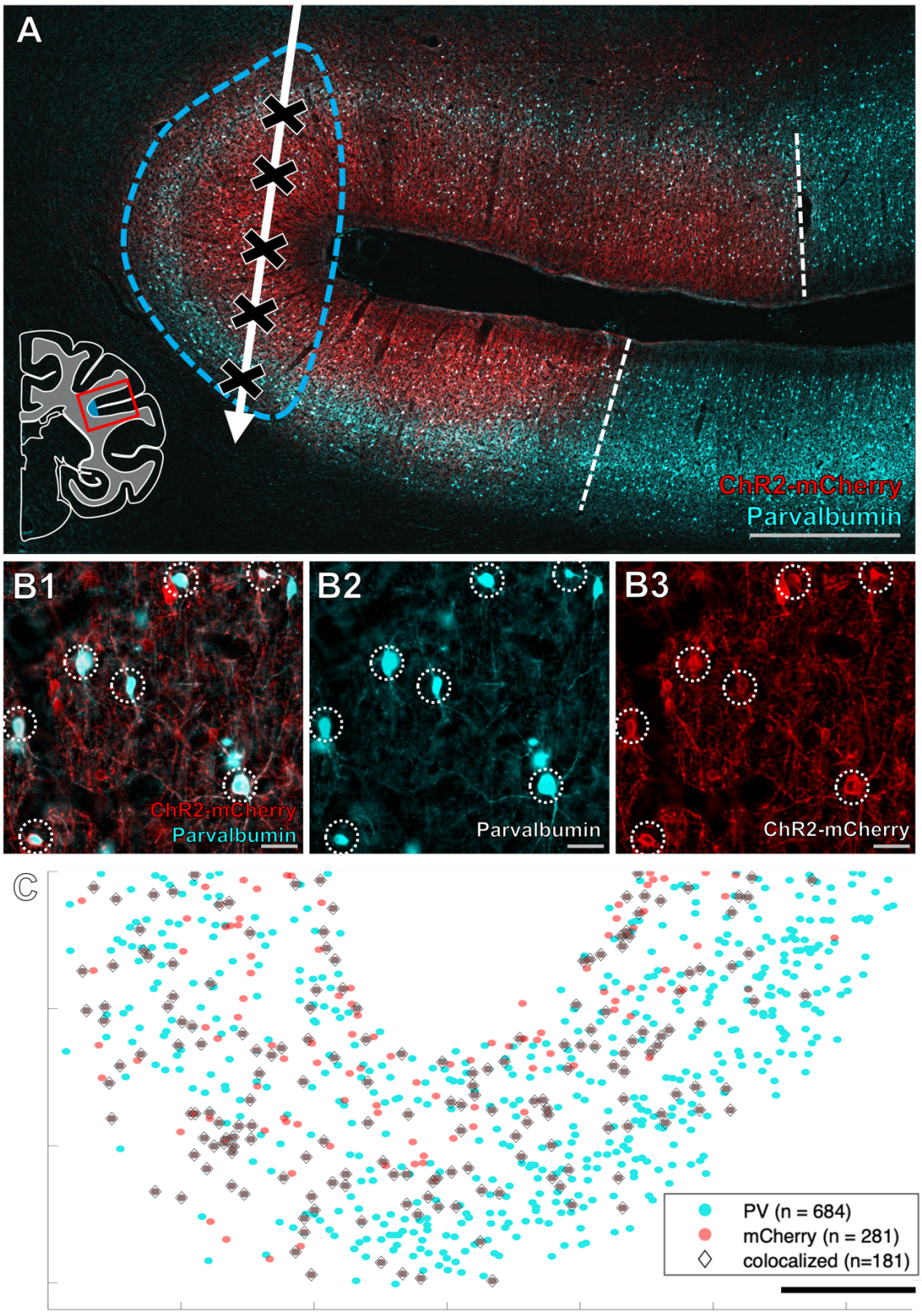

Figure 3. **Expression of ChR2-mCherry in cynomolgus macaque PIVC** *with ChR2-mCherry expression pseudo-colored in red, and Parvalbumin in pseudo-colored in cyan. (**A**)*

*Representative coronal section of a cortical brain slice indicating the approximate target area PIVC, situated in Sylvian fissure or lateral sulcus, outlined in blue. The theoretical approach of the injection cannula is indicated by the white arrow, with targeted injection sites indicated by black Xs. The section pictured does not represent a section containing the exact plane of the injection cannula. Optogenetic expression in red extends up to 2.5 mm away from cannula track, as indicated by white dashed lines. The location of the taken image relative to coronal hemisection is indicated in the inset and outlined by a red rectangle. Scale bar = 1000 μm. (**B**) High magnification representative images of expression in PIVC neurons, with white dashed line circles indicating colocalization between channel image signals. Scale bars = 30 μm. (**B1**) Merged image containing: (**B2**) Parvalbumin and (**B3**) ChR2-mCherry. (**C**) Locations of cell bodies in PIVC expressing ChR2-mCherry in red and Parvalbumin in cyan. Scale bar = 500 μm. The ratio of ChR2-mChrry positive neurons to Parvalbumin-positive neurons was 281:684, or an overall sensitivity to GABAergic neurons of 41.1%. Of 281 Parvalbumin-positive neurons, 181 were positive with ChR2-mCherry, having a specificity to Parvalbumin-positive GABAergic neurons of 64.4%.*

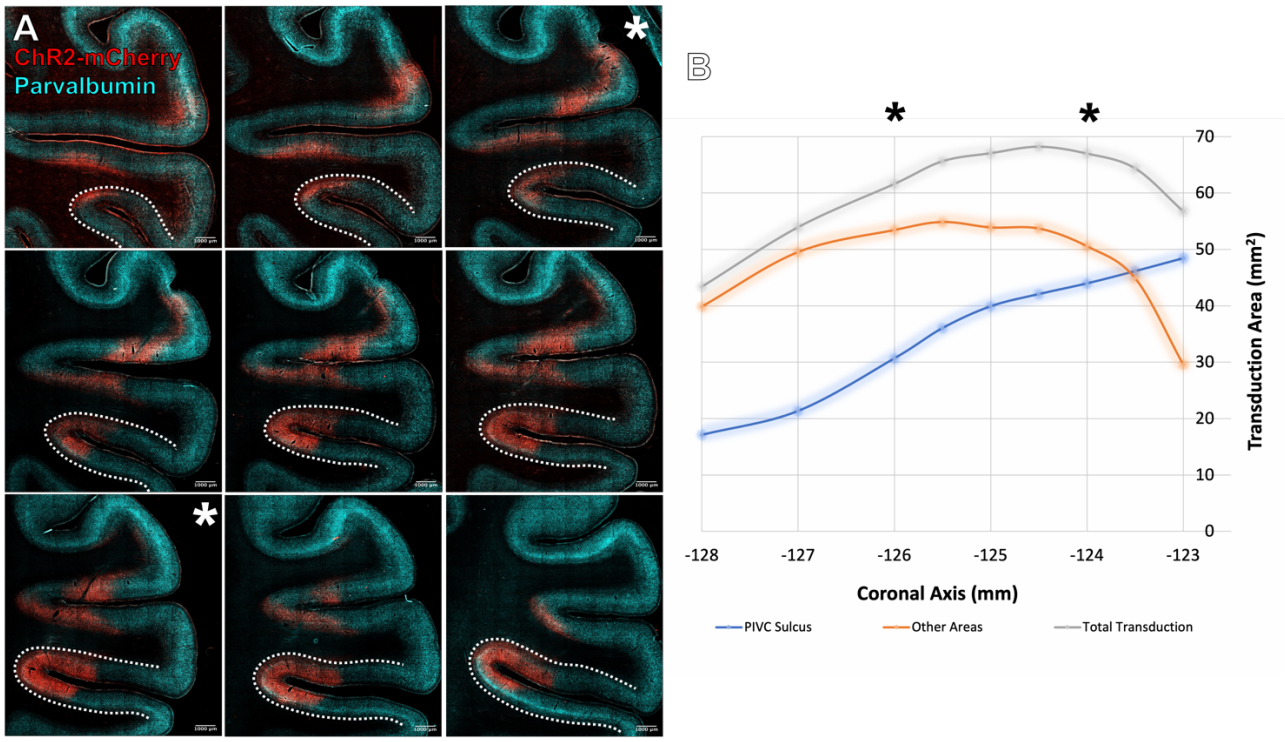

Figure 4. **Expression of ChR2-mCherry throughout the cynomolgus macaque cortex** *with ChR2-mCherry expression pseudo-colored in red, and Parvalbumin in pseudo-colored in cyan. (**A**) Virus expression was widespread and found throughout areas adjacent to the injection cannula track. Approximate slices where one of two tracks could be identified are indicated with asterisks. The Sylvian fissure or lateral sulcus containing PIVC is outlined with white dashed lines. Scale bars = 1000 μm. (**B**) Line graph of transduction area in mm^2 was measured in 9 slices, with the approximate coronal axis chosen per slice after comparison to MRI images of the given animal's brain. Transduction area within the sulcus containing PIVC is in blue and transduction areas outside of the sulcus in orange. Total transduction area is indicated in grey. Transduction area in non-targeted areas is comparable to targeted sulcus.*

Autofluorescence observed in green fluorescence channel

To illustrate the importance of robust immunohistochemistry and fluorescent image acquisition techniques, we imaged autofluorescence from lipofuscin in the brainstem in a sample without virus expression, **Figure 5**. *Lipofuscin* is a lipid molecule that accumulates in cell cytoplasm with age that has broad excitation and emission spectra (Brizzee et al., 1969). Without proper consideration, lipofuscin autofluorescence can be falsely viewed as optogenetic expression. Lipofuscin accumulations can be seen in the brainstem neuron cytoplasm.

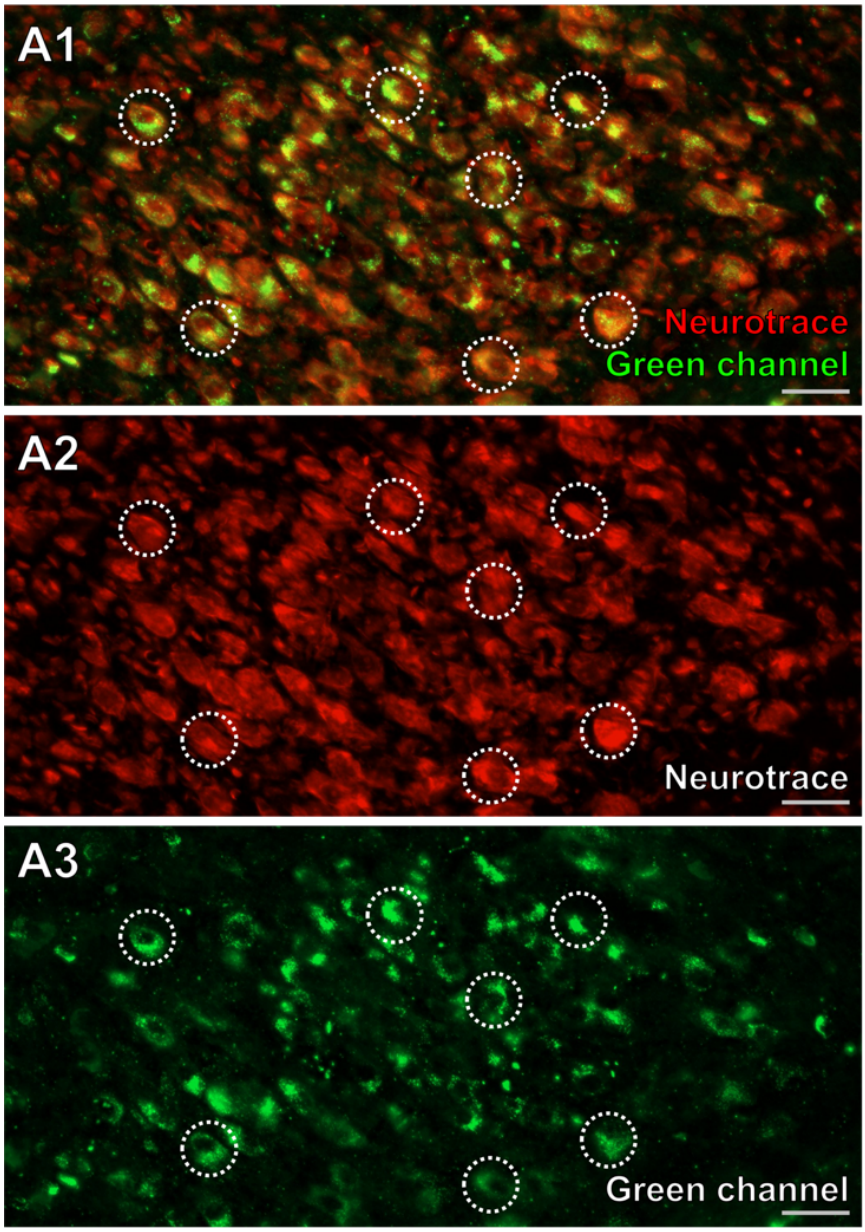

Figure 5. **Autofluorescence found in green fluorescence microscope channel in the cynomolgus macaque brainstem** *with the green channel pseudo-colored in green and fluorescent Nissl stain NeuroTrace pseudo-colored in red. Scale bars = 50 μm. (**A1**) Merged image containing: (**A2**) neurons marked with NeuroTrace Nissl stain and (**A3**) GFP-channel emission of lipofuscin autofluorescence.* (**A1**)

Discussion

Summary of findings

This project aimed to histologically evaluate induced optogenetic expression in the NHP central vestibular system in preparation for the future application of optogenetics to vestibular electrophysiological studies in NHPs. To target two vestibular brain areas of interest, the brainstem vestibular nuclei, and the PIVC, we chose vector constructs based on anatomical and molecular differences between cortical and brainstem tissue.

In the brainstem, our target area, the ABD, had only diffuse and non-specific expression. However, robust and sensitive expression was found in the superior vestibular nucleus (VN), a vestibular area of interest directly connected to ABD. We observed robust, sensitive, and specific opsin expression in PIVC, completely covering our area of interest. The virus was also expressed in cortical areas along the cannula track, demonstrating widespread viral efficacy.

Optogenetic Expression in the Brainstem

Our study has demonstrated optogenetic expression in the primate VN after injection of AAV9-hSyn-Jaws-KGC-GFP in the neighbouring ABD. Adenoviruses carrying the gene for the red-shifted halorhodopsin Jaws with expression driven by hSyn were first shown to successfully transduce rodent motor cortex neurons in 2014 (Chuong et al., 2014). They were previously found to successfully inhibit the activities of primate eye-movement-sensitive neurons in the brainstem (Krauss, 2020).

The transfection mechanisms of adenoviruses are not well understood. One hypothesis is that the expression shown in VN does not necessarily indicate us missing our target, the ABD. Expression seen in VN may result from vector backflowing through the cannula track. However, no cannula-like expression pattern was seen. Additionally, as seen in more rostral and caudal slices, GFP expression is shown in areas more medial than VN, similar in localization to ABD. The lack of distinct expression in ABD and robust expression in neighbouring VN may be due to our choice of adenovirus serotype (AAV9), a serotype known to undergo retrograde and anterograde transport, (Manfredsson et al., 2009; Green et al., 2016), and the connections between VOR-mediating neurons within ABD and VN, (Cullen & Taube, 2017; Scudder & Fuchs, 1992). That being said, if axon transport is responsible for the expression we see in VN, most transduced neurons would be PVP and FTN neurons that do not contribute directly to perception like VO neurons do. Further study and application of optogenetics to VN vestibular-only neurons will require a direct injection of viral vector into VN.

Optogenetic Expression in the Cortex

Our study has demonstrated robust optogenetic expression in primate PIVC using AAV9-mDlx-ChR2-mCherry-Fishell-3. Similarly, previous studies have shown that adenoviruses carrying the gene for Channelrhodopsin-2 with expression driven by mDlx can successfully transduce GABAergic neurons in non-human primate area V1 (Dimidschstein et al., 2016; De et al., 2020).

Expression of ChR2-mCherry was seen throughout cortical areas superior to PIVC along the injection cannula track. It is possible to keep expression transduction within the targeted

site with a longer wait before cannula removal or by using a smaller cannula gauge to inject a viral vector. This information could be valuable to researchers intending to record from multiple sites in different cortical areas or cortex sulci. In our case, excess transduction is not an issue, as guide tubes will block any incident light from reaching non-target areas. In future experiments, we will not be recording in all areas the virus may transduce; optogenetic activation will not be an issue as we will record with an electrode directly attached to an optical fibre.

Considerations for Fixed-Tissue Imaging of NHP Brains

As a histology project focusing on primate brain samples, we encountered several problems requiring troubleshooting that were not immediately obvious to histology experts that investigate brain samples from smaller mammals such as mice. For experiments involving optogenetic expression evaluation, several considerations must be made for successful fixed-tissue imaging of NHP brains.

One particular problem that was seen in all primate brain samples was the presence of autofluorescence from lipofuscin, a lipid-protein molecule that increases in concentration in neuronal cell bodies as a function of age. (Brizzee et al., 1969) Lipofuscin autofluorescence quencher such as TrueBlack Plus (Biotium) was essential to producing images with clear expression patterns.

To further minimize confusion or to verify results from images, we recommend amplifying the signals of fluorescent proteins commonly conjugated to opsins with primary antibodies, and staining with a secondary antibody with excitation and emission spectra that

significantly differs from lipofuscin, such as far-red secondary antibodies. In literature, Fortuna and colleagues (2020) describe what appears to be autofluorescence artifacts in tested viruses using opsins conjugated with mCherry as “intracellular accumulations,” arguing that the involved viral constructs are not as effective as others used (Fortuna et al., 2020). The possibility of lipofuscin autofluorescence confusing results continues to hinder the evaluation of optogenetic expression results and encourages the use of lipofuscin quencher.

Potential Avenues for the Application of Optogenetics to the NHP Vestibular System

Feedback between cortical and subcortical areas of the NHP vestibular system

We chose to target ABD to evaluate our optogenetic methods' efficacy as it is directly responsible for a relatively simple target behavioural effect. However, as we could not induce optogenetic expression in ABD, we can only fully contemplate the feasibility of further experiments if our hypothesis about our abducens injection transducing FTN and PVP neurons through axonal transport is correct. Our initial proposal involved evaluating the effect of optogenetic inhibition on the vestibuloocular reflex (VOR) by training an animal to fixate at a single spot during rotations of the body and head.

Despite the lack of optogenetic expression in abducens, we could observe robust expression in the connected vestibular nucleus, meaning our chosen virus was still compatible with the neuronal anatomy of vestibular nuclei neurons. As VN encodes for both translational and rotational motion, upstream perception and processing by VPL in the thalamus and PIVC, optogenetic experiments in VN could allow for the investigation of feedback introduced by VN sent to the higher-order areas that synapse to VN.

In an article by Inoue and colleagues, published in 2015, pathway-selective optogenetic stimulation in the oculomotor system was achieved by injecting their virus into the FEF; inducing optogenetic expression in the upstream superior colliculus. They were able to evoke saccades towards the response fields that correspond to the superior colliculus stimulation site. This study demonstrates the feasibility of investigating neural pathways between connected cortical and subcortical areas (Inoue et al., 2015). When applied to the study of information transmission and the encoding of vestibular sensation, studies could reveal insights previously unknown about feedback between VN and higher-order structures.

Complex vestibular perceptual and behavioural effects

We were able to induce robust optogenetic expression throughout and beyond the parieto-insular vestibular cortex with our second chosen virus, leaving us confident about applying optogenetics in this area and making way for temporally precise experiments involving perception, as well as feedback from PIVC to downstream structures in the Thalamus and brain stem.

A study by Chen and colleagues, published in 2016, investigated the contribution of PIVC to a heading perception task. Heading involves the judgment of the direction of linear self-motion, and monkeys were trained to indicate perceived direction after being moved on a platform at different degrees away from the midline. Muscimol was injected bilaterally to understand the influence of long-term neuronal inhibition on perception. Psychophysical thresholds indicated that the heading degree threshold increased from 1.4 degrees prior to inhibition, to 2.8 degrees immediately post-inhibition, to 10.2 degrees 12 hours later, and

finally recovery about 36 hours later (Chen et al., 2016). Applying a similar experimental design with optogenetic inhibition would result in more temporally precise conditions, allowing for mixed trials for perceptual tasks and allowing us to understand better how PIVC contributes to the perception of self-motion. Most importantly, it would significantly reduce the time an animal will spend with disrupted vestibular function, making optogenetics the more ethical approach versus using muscimol.

Future Directions: Anatomical Aims

In future vestibular optogenetic expression studies, there are many avenues to consider. For example, to address a central vestibular brain region not targeted in the present study, we could target and inject the ventral posterolateral nucleus (VPL), situated in the thalamus, to tackle the coding of vestibular perception between coding in the vestibular nuclei and PIVC.

To address the issues encountered with generating optogenetic expression in the abducens nucleus, we could attempt the same injection with a neuron-specific promoter other than human synapsin 1, such as CaMK. Additionally, the vestibular nucleus can be targeted directly to transfect all VN cell types, instead of solely PVP and FTN neurons, if the expression was due to transport from the abducens to VN.

Finally, as we are inducing optogenetic expression with adenovirus, we must consider adaptive immunity if injecting a viral vector serotype in an animal that has been injected with a given viral vector serotype before. As discussed by Mendoza and colleagues in 2017, it will be necessary to use another serotype, such as AAV1 or AAV2 (Mendoza et al., 2017).

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