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Connectomics Investigation of Role of Parabrachial Nucleus Aversion using Optogenetics: A Experiment Proposal

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Abstract:

In recent decades, there has been a notable increase in research activity focused on the aversive and reward circuits of the brain. The evidence indicates that the parabrachial nucleus (PBn) is a crucial element in the generation of aversion responses in mice. Following successful stimulation of the PBn, mice display increased aversive and stress-related behaviours. The study employs Fos TRAP2-ChR2 mice, opioids such as oxycodone, and a range of imaging technologies to monitor and manipulate neural pathways. Behavioral tests and optogenetic manipulation are used to assess real-time behavioral responses in mice, with the aim of uncovering the neural pathways and mechanisms involved in PBn-mediated aversion. Nevertheless, the underlying mechanisms of these behaviours remain unclear. The objective of this study is to investigate the connectomics of the parabrachial nucleus (PBn) in order to determine its association with regions involved in motivation, such as the nucleus ambiguus or the amygdala. It is hypothesised that this association may lead to aversion by affecting these regions. An alternative hypothesis is that if the parabrachial nucleus is connected to areas that are not directly related to it, it may be considered a motivational area in its own right. The identification of these connections will elucidate the underlying mechanisms of aversion mediated by PBn, thereby providing a deeper understanding of the neural basis of aversive behaviour and its regulation.

Keywords: Parabrachial Nucleus, Aversion, Fiber Optic Sensor, GCamp

1. Introduction

Parabrachial Nucleus(PBn) is a nucleus located in the pons and the auditory cortex of the brain, specifically in the superior temporal gyrus, lateral to the primary auditory cortex (A1)(Trairatphisan et al., 2013; Yujun Deng et al., 2020.). It is a major node for cerebral output, sensory input, and it also controls appetites, pain and alarm. (Gauriau & Bernard, 2002; Palmiter, 2018). There are numerous papers out there on PBn: Up to the time of this presentation, there are more than 150,000 articles related to this topic(Cheng et al., 2024However, currently there are very few of them that look into the role of PBn in aversion from a circutial level. The most prominent example is a recent article on opioids and the connection of PBn(Smith et al., 2024). The paper discusses the circuits between the Dorsal Parabrachial Nucleus (DPn) and PBn areas and how opioids affect them. The study found that aversive and stressful behaviours in mice were significantly higher in behavioural tests like Conditioned Place Preference (CPP), Open Field Test (OFT), and Elevated Plus Maze (EPM) when the DPn successfully exited the PBn. Yet, even this major paper published in the prestigious Science journal failed to explain why the PBn leads to such behaviours. Hence, this is a gap in our current understanding of neuroscience. Therefore, we propose to study the mechanism behind this by investigating the connectomics of the PBn. By identifying these connections, we aim to elucidate the underlying mechanisms of aversion mediated by the PBn, potentially paving the way for new insights into the neural basis of aversive behaviours and their modulation.

2. Method

2.1 Raw materials and reagents

FosTRAP2-ChR2 mice
Opioids (like oxycodone)

4% paraformaldehyde (PFA)
4-hydroxytamoxifen
c-Fos antibody and fluorescent secondary antibody
Adeno-associated virus (AAV) vector, including GFP, GCaMP6, and ChR2
Confocal microscope
Two-photon microscope
Optical fibre probe and implant device
Laser light source and control system

2.2 Experimental Procedure

We chose to use FosTRAP2-ChR2 mice because the FosTRAP technique can specifically label these mice. With increased expression of light-sensitive proteins, we can directly manipulate neuronal activity through optogenetics. Then, PFA would be used to perfuse subsequent sections. 4-hydroxytamoxifen is used to capture target neurons in the Fos technique. Afterwards, antibodies are used for imaging in the fos technique, and the AAV virus has the following three categories to help us reveal the neural pathway in the second experiment.

For the first experiment, we first prepare to use Fos technology to map the neural pathways associated with PBn to help us accurately inject the virus in Gcamp.

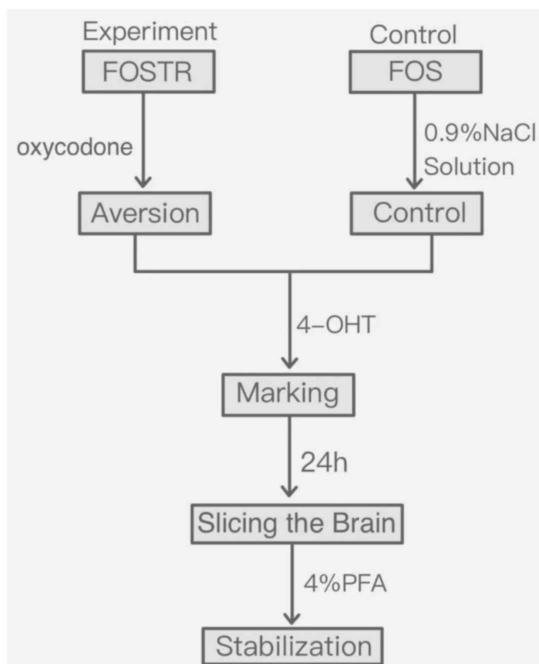

Figure 1. Flow chart of the first experiment.

We would divide the mice into two groups: the experimental group and the control group, each with ten mice. One group would be given oxycodone, and the other saline as a control. Both groups would be injected intraperitoneally with 4-OHT immediately after the drug was administered to capture and label activated neurons. 24-48 hours after 4-OHT injection, waiting for FOS-labeled neurons would express the Cre enzyme and trigger reporter gene expression.

First, we will make brain slices from two groups of mice. Brain tissue would be cleaned with PBS and immobilized overnight with 4% PFA. Then, it would be put on the cryotome. The purpose of this step is to use Fos techniques to determine which brain regions are associated with PBn activation

We will then perform immunohistochemical staining and incubate the sections with C-FOS antibodies at 4°C overnight. During the incubation of the primary antibody, it should be diluted in PBS containing 0.1% Triton X-100 and 1% normal goat serum. Thereafter, during the incubation of the secondary antibodies, the slices were incubated at room temperature with fluorescently labelled secondary antibodies for 2 hours. The procedure as described above would be used for Immunohistochemical Staining to verify the activation of neurons.

Finally, the stained slices are mounted on a slide. The sections would be imaged using confocal microscopy or fluorescence microscopy to analyze the location and distribution of c-Fos positive cells. In addition, the data of the experimental group and the control group were compared, and statistical software was used for statistical analysis to determine the activation of PBn neurons under aversive stimuli. The end goal of the final step of the first experiment is to use confocal microscopy to analyze the sample and then to determine which brain regions were associated with PBn activation

In the next experiment, we will use these three markers for the purpose of Real-time monitoring and manipulation of PBn and its associated neural pathways.

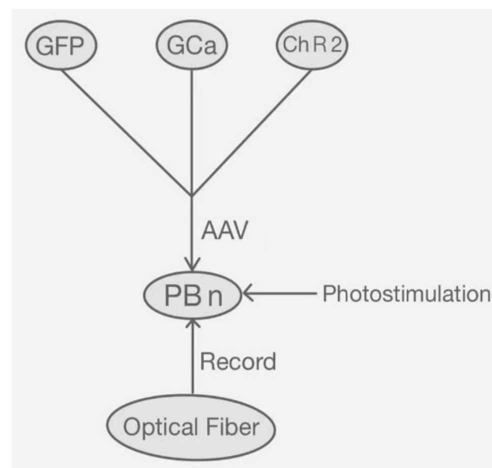

Figure 2. Flowchart showing the experimental procedure for the second experiment.

We randomly choose another group of FosTRAP2-ChR2 mice.

The first step is the preparation of the virus, which constructs a Cre-dependent gonadal-associated virus (AAV) vector containing GFP, calcium indicator protein (GCaMP6), and light-sensitive ion channel (ChR2). Henceforth, we treat mice and anaesthetize them (such as with isoflurane). The AAV virus was later slowly injected into the mouse PBn based on the exact location obtained from experiment 1. After allowing the mice to rest for a few days, the same procedure would be performed, with fibre-optic probes slowly implanted into the PBn.

Subsequently, we would use a two-photon microscope for in vivo calcium imaging. GCaMP6 was stimulated by a laser to record calcium signal changes in PBn neurons. Calcium signals reflect the activity level of neurons. At the same time, we use a laser light source control system to activate or suppress ChR2 using specific wavelengths of light. The effects of light stimulation on the activity of PBn neurons and the behaviour of mice were observed.

Finally, we plan to conduct Real-Time Place Preference (RTPP) experiments to evaluate real-time behavioural responses in mice under optogenetic manipulation. The graph below is a model for our experiment. It consists of an experimental chamber with two distinct regions, one associated with a reward or aversive stimulus (such as a light stimulus) and the other as a blank control.

We used the mice in experiment 2 and divided them into a control group and an experimental group. The control group will not undergo the light stimulation test.

Next, we will operate the experimental mice. First, it is best to let the experimental mice adapt to the experimental box for some time before starting the experiment. For the formal experiment, the mice were placed in the middle of the RTPP chamber, allowing them to explore both areas freely. In the experimental group, optogenetic manipulation is performed to light stimulate an area for a specific period (for example, light stimulation is initiated each time you stay in the area for more than 2 seconds).

During the experiment, a two-photon microscope or other imaging device was used to monitor GCAMP6-labeled neuron activity in real time and record calcium signal changes. The residence time and behavioural changes of the mice in both regions were recorded simultaneously.

In the behavioural data analysis, the total residence time of the mice in the light-stimulated region and the control region was calculated, and the differences between the experimental and control groups were compared. In addition, the number of times the mice entered the two regions was counted, and their exploratory behaviour was assessed. Behavioural changes in the light-stimulated areas were observed and recorded.

3. Discussion

The objective of the experiment is to elucidate the mechanisms by which the parabrachial nucleus (PBn) contributes to the expression of aversive behaviours in mice. The results would indicate that the PBn is intricately involved in the neural circuitry underlying aversion, as evidenced by the behavioural and physiological responses observed following its stimulation. The use of Fos TRAP2-ChR2 mice and advanced imaging techniques would facilitate the mapping and manipulation of these neural pathways with precision, thereby providing insights into the functional connectivity of the parabrachial nucleus. The results of the behavioural tests, including those measuring conditioned place preference, open field and elevated maze, would demonstrate a significant increase in aversive and stress-related behaviours in mice upon PBn activation. This would be consistent with the findings of previous studies, which have indicated that the parabrachial nucleus plays a crucial role in the processing of aversive stimuli. However, our research contributes to this understanding by proposing potential pathways through which the PBn exerts its influence. In particular, our connectomics study would indicate that the PBn is associated with key regions involved in motivation and emotional regulation, including the nucleus ambiguus and the amygdala. This would suggest that the PBn may contribute to the generation of aversion by modulating the activity of these regions. The use of optogenetic manipulation provided further evidence to support the role of the parabrachial nucleus (PBn) in aversive behaviour. The ability to activate or inhibit PBn neurons in real time would permit the observation of direct alterations in behaviour, thereby reinforcing the hypothesis that PBn constitutes a pivotal element of the aversion circuit. The data obtained from calcium imaging demonstrated a correlation between neuronal activity in PBn, as gauged by GCaMP6 signals, and aversive stimuli. This would provide further evidence of the involvement of PBn in the processing of such stimuli.

Notwithstanding these findings, a number of questions remain to be addressed. A significant area of investigation is the nature of the interactions between PBn and other brain regions. While our study provides a foundational map of PBn's connections, further research is required to elucidate the functional roles of these connections. For instance, it is currently unclear whether PBn exerts a direct influence on aversion or whether this effect is mediated by intermediary regions. Moreover, the specific neurotransmitter systems involved in PBn-mediated aversion require further investigation. Additionally, future studies should examine the role of PBn in aversion across different species and in response to a variety of aversive stimuli, with

the aim of generalising the findings of this study. Moreover, the long-term consequences of PBn manipulation on behaviour and neural circuitry have yet to be elucidated.

In conclusion, the results of the experimental proposal would provide compelling evidence that PBn plays a central role in aversive behaviour in mice, likely through its connections with key motivational and emotional brain regions. By mapping these connections and modulating PBn activity, the experiment would make a substantial contribution to our understanding of the neural basis of aversion. These findings have the potential to inform the development of novel therapeutic strategies for conditions characterised by dysregulated aversion, such as anxiety and depression. Further research is required to elucidate the intricacies of PBn's neural circuitry and its broader implications for behaviour and mental health.

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Yule Zheng, Lingyue Huang, Yuxi Li, and Kechen Liu contributed equally to this work and should be considered co-first authors

References

Cheng, Z., Wang, Y., Zheng, R., & Mu, W. (2024). The prediction of two-dimensional PbN: Opened bandgap in heterostructure with CdO. *Frontiers in Chemistry*, *12*, 1382850. https://doi.org/10.3389/fchem.2024.1382850

Freeman, J., Vladimirov, N., Kawashima, T., Mu, Y., Sofroniew, N.J., Bennett, D.V., Rosen, J., Yang, C.-T., Looger, L.L., Ahrens, M.B. (2014). Mapping brain activity at scale with cluster computing. Nat. Methods, 11, 941–950.

Gauriau, C., & Bernard, J. (2002). Pain Pathways and Parabrachial Circuits in the Rat. *Experimental Physiology*, *87*(2), 251–258. https://doi.org/10.1113/eph8702357

Guzowski, J.F., Timlin, J.A., Roysam, B., McNaughton, B.L., Worley, P.F., Barnes, C.A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. Curr. Opin. Neurobiol., 15, 599–606.

Palmiter, R. D. (2018). The Parabrachial Nucleus: CGRP Neurons Function as a General Alarm. *Trends in Neurosciences*, *41*(5), 280–293. https://doi.org/10.1016/j.tins.2018.03.007

Smith, A. C. W., Ghoshal, S., Centanni, S. W., Heyer, M. P., Corona, A., Wills, L., Andraka, E., Lei, Y., O'Connor, R. M., Caligiuri, S. P. B., Khan, S., Beaumont, K., Sebra, R. P., Kieffer, B. L., Winder, D. G., Ishikawa, M., & Kenny, P. J. (2024). A master regulator of opioid reward in the ventral prefrontal cortex. *Science*, *384*(6700), eadn0886. https://doi.org/10.1126/science.adn0886

Saleeba, C., Dempsey, B., Le, S., Goodchild, A., & McMullan, S. (2019). A student's guide to neural circuit tracing.

Trairatphisan, P., Mizera, A., Pang, J., Tantar, A., Schneider, J., & Sauter, T. (2013). Recent development and biomedical applications of probabilistic Boolean networks. *Cell Communication and Signaling*, *11*(1), 46. https://doi.org/10.1186/1478-811X-11-46

Yujun Deng, Song Da, Ni Junjun, Qing Hong, & Quan Zhenzhen. (n.d.). *Reward prediction error in learning-related behaviors*. https://doi.org/10.3389/fnins.2023.1171612