IS THE AREA POSTREMA RESISTANT TO THE ANESTHETIC EFFECTS OF MS-222?

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Abstract

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MS-222 is a common fish anesthetic that dampens neural activity by inhibiting neuronal voltage-gated sodium channels (Na_v) required for action potentials. Previous experiments conducted by our lab's primary investigator using fluorescence calcium imaging in zebrafish neurons resulted in the observation that neurons in the area postrema (AP), a circumventricular organ located in the dorsal hindbrain, displayed prominent fluorescence intensity during MS-222 induced sedation. I used the genetically encoded calcium indicator GCaMP6s to conduct fluorescence calcium imaging with the goal of recording neural activity in the larval zebrafish area postrema and optic tectum to compare the anesthetic effects of MS-222 across brain regions. A 2 x 2 analysis of variance (ANOVA) was used to calculate the differences in fluorescence intensity before and after MS-222 in both brain regions. Contrary to our hypothesis, the results of the experiment indicate that MS-222 had an insignificant effect on the change in fluorescence intensity in each brain region and although insignificant, appears to slightly increase area postrema and optic tectum mean standard deviations. These results are theoretically inconsistent with the literary understanding of how voltage-gated sodium channel antagonism affects cellular behavior. A small sample size with high variance is the most likely explanation for these results.

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Introduction

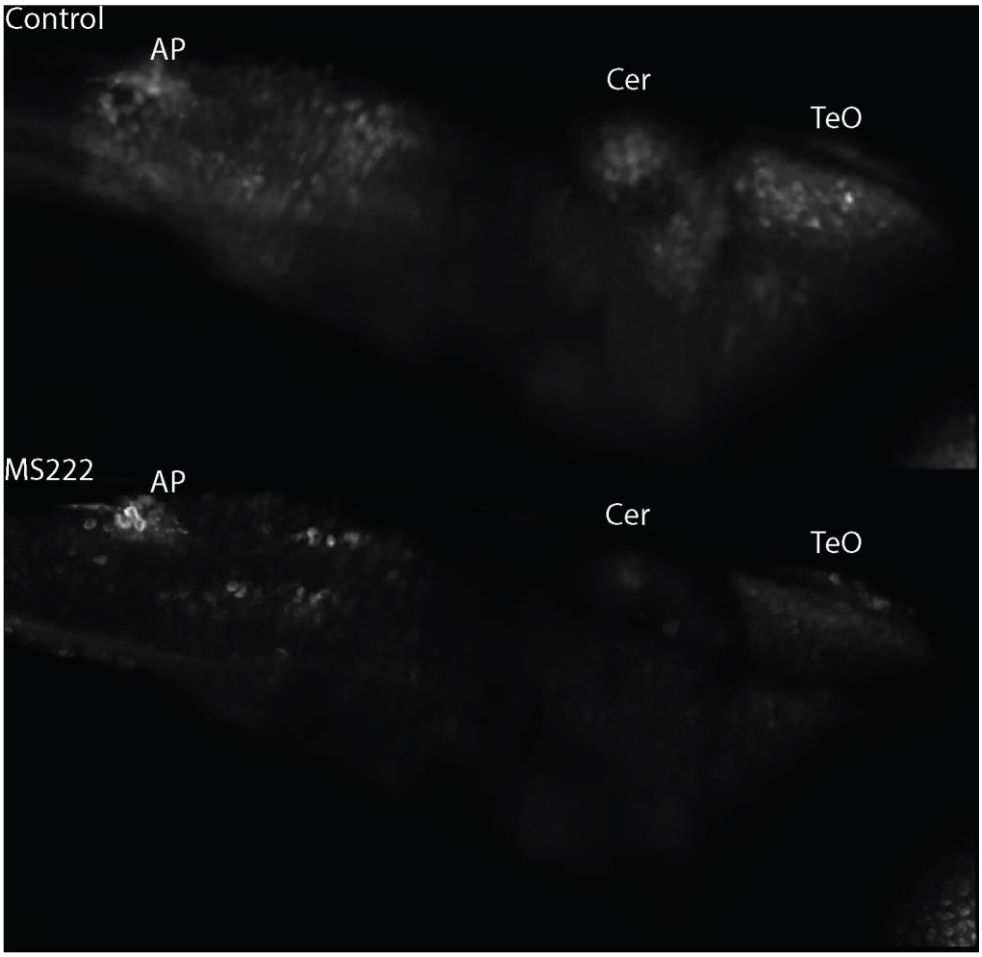
The Area Postrema

Similar to how organisms evolved brain regions dedicated to sensing their external environment, the brain must also monitor an organism's internal environment for visceral malaise, ingested toxins or noxious stimuli, and to track hormone concentrations in the bloodstream to make advantageous homeostatic decisions. To achieve this task, one strategy the brain deploys is having specialized areas called circumventricular organs (CVO) that use their unique advantage of having projections located outside the blood brain barrier to monitor the bloodstream for hormones and toxins (Zhang et al., 2021). The CVO system is mostly conserved between teleost, like the zebrafish, and humans suggesting the functionality of these structures to be evolutionarily beneficial to an organism's survival (Korzh & Kondrychyn, 2020). Our research focuses on the area postrema (AP), a sensory CVO located in the dorsal hindbrain of the zebrafish. The AP is a chemoreceptor that monitors for ingested toxins and promotes nausea and emesis to expel toxins and to warn of aversive environments (Miller & Leslie, 1994; Wee et al., 2022; Wong et al., 2014; Zhang et al., 2021, 2022). The AP accomplishes this task by expressing a large variety of receptor types capable of detecting the presence of many different hormones and toxins (Edwards & Ritter, 1981; Fry & Ferguson, 2009; Jeong et al., 2021; Smith et al., 2016; Zhang et al., 2021, 2022). The literature also details many other zebrafish behaviors the area postrema is active during. These behaviors include aversion behaviors, food intake, and prey capture (Edwards & Ritter, 1981; Fry & Ferguson, 2009; Smith et al., 2016). During previously conducted calcium imaging

experiments in our lab, the observation was made that AP neurons appeared to produce prominent fluorescence intensity during MS-222 sedation (Fig. 1). Perhaps this observation demonstrates another unique behavior of the area postrema: resistance to the anesthetic drug MS-222.

Figure 1.

Zebrafish brain z-projections of standard deviation before and after MS-222



Note. Standard deviation z-projections created in ImageJ of a 6-day post fertilization larvae zebrafish brain before (control) and after being anesthetized in 0.01% MS-222.

Labelled are the optic tectum (TeO), cerebellum (Cer), and area postrema (AP). The fish is positioned laterally, with retinal ganglion cells of the eye visible in the bottom right corner of each image. Motion correction as described in the methods was performed before each z-projection was created.

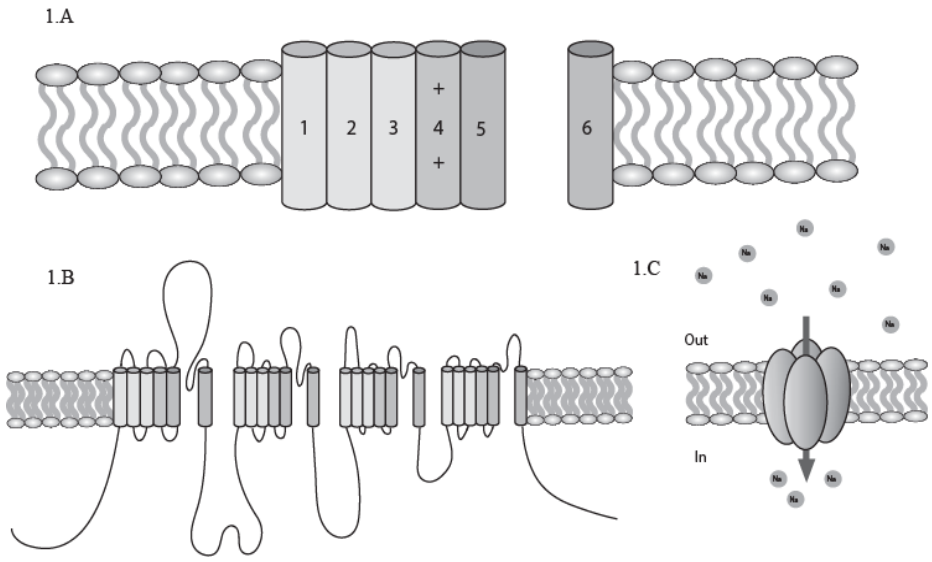
MS-222 and Voltage-Gated Sodium Channels

MS-222 is the one of the most popular fish and amphibian anesthetic drugs being used today, with its range of use spanning from clinical veterinarian practices to field and laboratory research (Leyden et al., 2022). Once dissolved, MS-222 quickly enters a fish's blood circulation through the gills and crosses the blood brain barrier (Topic Popovic et al., 2012). Upon uptake MS-222 is a fast and effective anesthetic capable of knocking out reflexes such as postural righting and eye tracking within a matter of seconds (Leyden et al., 2022). Although MS-222 works rapidly and is easy to administer, there are some areas of the literature that discuss MS-222's potential aversive effects on zebrafish. There is evidence coming from light/dark preference testing in zebrafish that suggests the presences of MS-222 is aversive, causing zebrafish to forgo their preferred environment for one that does not contain MS-222 (Readman et al., 2013, 2017; Wong et al, 2014). The mechanism of action giving MS-222 anesthetic properties is that it acts as an antagonist of voltage-gated sodium channels (Na_v), inhibiting action potentials (Arnolds et al., 2002; Attili & Hughes, 2014; Butterworth & Strichartz, 1990; Musk, 2020). Enough inhibition of voltage-gated sodium channels across the organism results in a state known as sedation, which can progress to anesthesia and eventually euthanasia if the dose and exposure time are sufficient.

Sodium voltage-gated channels are critical to the maintenance of homeostasis and normal biological functioning because they allow for cellular communication and tissue excitation by generating the rising phase of action potentials through a rapid influx of sodium ions (Ahern et al., 2016; Attili & Hughes 2014; Catterall 2000, Isaac et al., 2020). In zebrafish there are 8 members of the synuclein alpha (SCNA) gene family responsible for the transcription of different subtypes of voltage-gated sodium channels (Novak et al., 2006). Although similar in structure, the functionality, distribution, and expression of these different subtypes appear to be unique. There is evidence of critical differences amongst Na_v subtypes regarding drug selectivity, susceptibility, or resilience linking back to changes in Na_v alpha subunits (Attili & Hughes, 2014; Isaac et al., 2020) (Fig. 2). All pharmacological agents that act on Na_v channels have receptor complexes on the alpha subunits (Cattarall et al., 2005). Na_v channels are known to have considerable diversity in zebrafish, for example, genes for different Na_v channel subunits are expressed in different action-potential generating tissues, including cardiac muscle (which express $Na_v1.1$ type channels), skeletal muscle (which express $Na_v1.4$ type channels) and nervous system (which express $Na_v1.6$ type channels) (Attili & Hughes, 2014; Novak et al., 2006). Additionally, there is evidence demonstrating that $Na_v1.4$ channels might be resistant to the effects of MS-222 (Attili & Hughes, 2014). If our observation is true and the AP is resistant to the anesthetic effects of MS-222, that could suggest that the AP is expressing MS-222 resistant Na_v channel subtypes.

Figure 2.

Voltage-gated sodium channel structure



Note. Section 1.A depicts a single domain of the voltage-gated sodium channel alpha subunit. The alpha subunit is comprised of 4 domains each with 6 membrane-spanning segments. The 4th segment acts as a voltage sensor. A gap between segments 5 and 6 forms the ion pore. 1.B shows an unraveled perspective of all 4 membrane-spanning domains of the voltage-gated sodium channel. 1.C demonstrates a full voltage-gated sodium channel embedded in a cellular membrane during the influx of sodium ions. Functional differences in voltage-gated sodium channel subtypes like voltage sensing, resistance, or susceptibility to certain pharmacological compounds occur because of differences within the alpha subunit.

Statement of the Problem

Despite its popularity as an anesthetic drug, the literature regarding how MS-222 interacts with circumventricular organs, particularly the area postrema is limited. If our observations are correct and the area postrema is resistant to MS-222, that could imply several different possible explanations worthy of further investigation. One possible explanation these findings could suggest is that the AP is expressing a different type of voltage-gated sodium channel than other brain regions, bringing into question Na_v channel diversity within the zebrafish central nervous system. Perhaps there is an evolutionary advantage to expressing an MS-222 resistant Na_v subtype in the area postrema during development. Another possibility is that MS-222 sedation is leading to disinhibition of the area postrema. Recent DNA sequencing research has detailed area postrema neural circuitry governing the promotion of nausea and vomiting in rodents (Zhang et al., 2022). The research found that nausea promoting excitatory neurons of the area postrema are receiving constant inhibitory signaling from other area postrema neurons during normal functioning. However, if the inhibitory signaling is disrupted or agonists bind to the nausea promoting neurons, this can lead to disinhibition of the excitatory AP neurons resulting in nausea and vomiting in animals capable of doing so and flavor avoidance (Zhang et al., 2022). MS-222 sedation could be interrupting the inhibitory signaling to the area postrema. This could suggest that the brain perceives the early stages of anesthesia onset similar to how it perceives toxins or other nausea producing stimuli. That would align with the documented stages of anesthesia in humans based on Guedel's classifications (Siddiqui & Kim, 2023). Stage two is described as the

excitement or delirium stage characterized by spastic movements, vomiting, and rapid, irregular respirations (Siddiqui & Kim, 2023). Vomiting would most likely involve area postrema activity in the human brain (Miller & Leslie, 1994). Further promoting the idea of a hypothesized anesthetic and area postrema connection. Research into the above speculations could prove to be very impactful and fill gaps within the literature, but before research exploring the potential implications of MS-222 resistance in the AP can proceed, objective evidence proving area postrema resistance to the anesthetic effects of MS-222 must be demonstrated.

Current Research

The research conducted was designed to provide data of MS-222 and AP interactions by measuring spontaneous neural activity in two brain areas, the area postrema and the optic tectum, using fluorescence calcium imaging in which brightness changes over time in a neuron indicates the degree of action potential firing. Fluorescence images of the brains of living 6-day old zebrafish were collected at a rate of 1 image per second for 10 minutes, generating a 600-frame video for each recording epoch. The brains were viewed laterally and focused on the area postrema but had other brain areas visible in the field of view, enabling simultaneous measurements. The standard deviation of brightness values across video frames within each brain area was the index for neural activity for that brain area. For each zebrafish (N = 20), two recording epochs were run, the first in control conditions and the second after 30min of incubation in 0.01% MS-222.

Hypothesis

The hypothesis being tested in this experiment is that MS-222 would suppress neural activity in the optic tectum at a greater magnitude than it would suppress activity in the area postrema. The rationale behind this hypothesis is that if the AP expressed a resistance to MS-222, activity in the AP would be unaffected by the drug condition changes, but activity in the optic tectum would decrease as Na_v channels present on neurons in the optic tectum are being inhibited by MS-222 in the central nervous system.

Methods

Subjects

All protocols and procedures were performed in compliance with the institutional animal care and use committee guidelines at Cal Poly Humboldt (2022P82). Transgenic Elavl3:GCaMP6s adult fish raised in facility water and maintained on a 14-hour light 10-hour dark cycle at 28°C were bred to produce the larval clutch for this experiment. In these zebrafish, all neurons have expressed green fluorescence that continuously changes in brightness based on intracellular calcium levels related to neural activity. Six-day old larvae zebrafish (N=20) underwent imaging procedures to record brain activity both in control conditions and during MS-222 exposure in a within subject's experimental design. 12 out of these 20 experiments could not be analyzed due to problems during image acquisition. In three experiments the imaging focal plane (z-plane) drifted, resulting in recorded areas of interest being lost to view during the 10-minute acquisition period. In four experiments, area postrema neurons were recorded with saturated brightness or with insufficient brightness signals to reliably measure, meaning neural activity-induced brightness increases could not be detected. In five experiments, inconsistencies in image size settings during the recording process made them incomparable to other experiments.

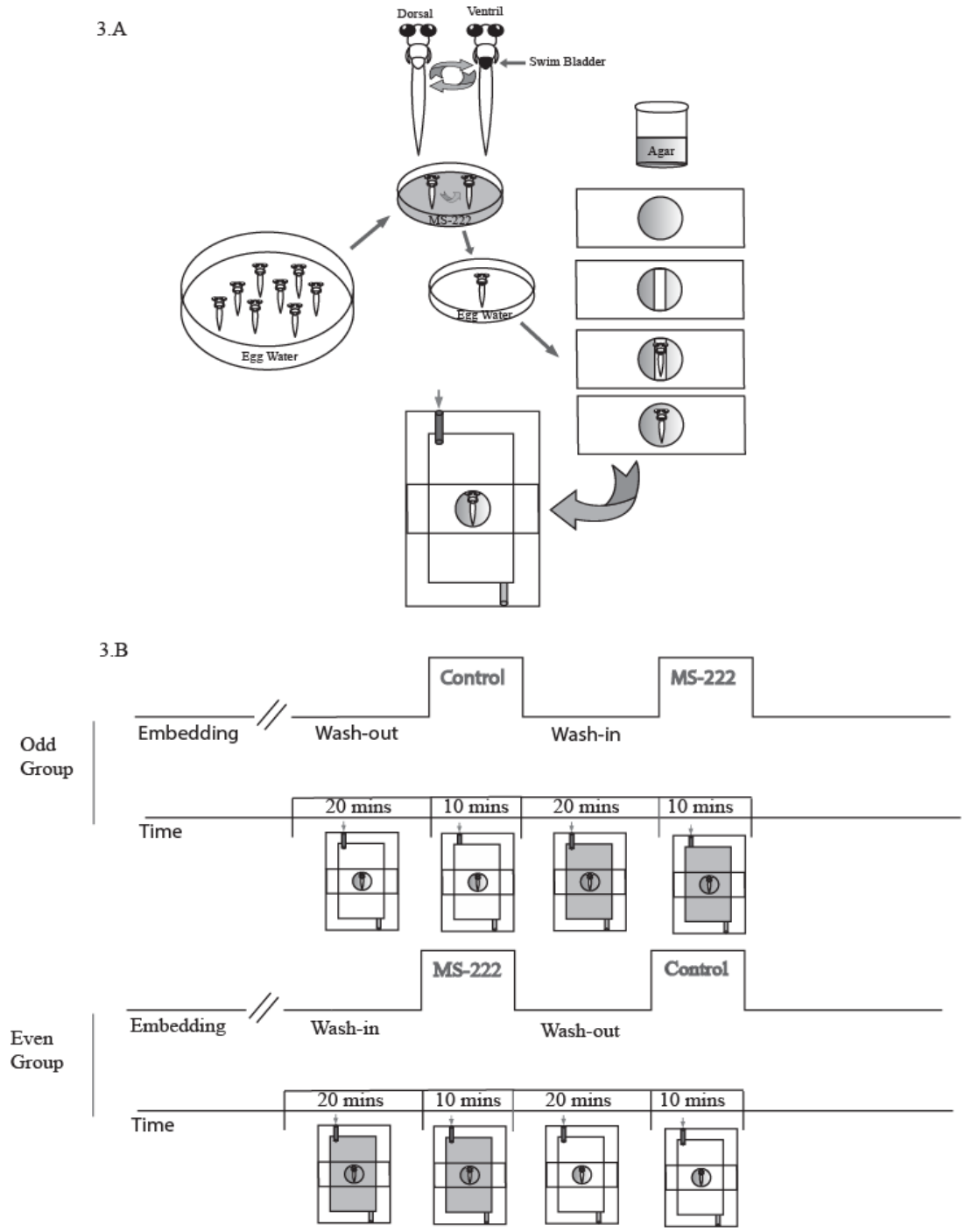
Drug Delivery Sequence

Larvae were divided evenly into two groups to counterbalance the order of recordings by randomly selecting larvae and alternating between control-first (odd numbers) and MS-222 first (even numbers) recordings (Fig. 3.B). Unfortunately, due to

data exclusion an unequal number of control-first (five subjects) and MS-222 first (three subjects) experiments were available for analysis, and the overall sample size was insufficient to include trial order as a factor in the analysis.

Figure 3.

Embedding, imaging, and drug delivery sequence



Note. Two-part graphic detailing the embedding protocol, drug delivery sequence, and recording epochs each fish underwent during this experiment. 3.A details the step-by-step process of embedding larvae zebrafish and preparing them for imaging. Fish are anesthetized in MS-222 then washed-out, embedded in 1.2% agar, and left to acclimate in the imaging reservoir. 3.B details the drug delivery sequence and recording epochs immediately following each embedding. The first fish was randomly selected and followed the "odd group" drug delivery sequence, receiving the control egg water as the first drug condition. The group and drug delivery sequence were alternated for each subsequent fish following the first fish. For each group section of figure 3.B, the top line depicts when recording epochs begin following each wash-out or wash-in period. The upstroke in the line indicates when the camera is recording and the drug condition during each recording period is printed within the upstroke. The bottom line of each group depicts the imaging reservoir with different colored water referring to the present drug condition at each stage in the experiment. MS-222 positive water is stylized in grey in the graphic and control egg water is white.

Chemicals

MS-222 was obtained from the Cal Poly Humboldt Fish Hatchery and used within its expiration date. A 0.03% stock solution was made by dissolving 0.03mg MS-222 in 100ml of egg water and adding sodium bicarbonate to achieve neutral pH. The stock solution was stored in a refrigerator, used within 1 month, and diluted 1:3 in egg water on the day of each experiment for a working concentration of 0.01%. The literature shows that 0.01% MS-222 produces sedation in larval zebrafish appropriate for performing light

surgical procedures (Félix, 2018). As a control, “egg water”, as described in THE ZEBRAFISH BOOK (Westerfield, 2007)., was used.

Procedure

Larvae were embedded in agarose (1.2% in egg water) laterally to allow sagittal imaging of the brain (Fig. 3.A). After embedding, larvae were acclimated for approximately 20 minutes to the imaging chamber, which included a heated, recirculating perfusion pump carrying drug or control solution across the larva’s body. Twenty-minute solution wash-in or wash-out periods preceded each recording to allow for the effects of MS-222 to wear off or set in and to deter carryover effects (Fig. 3.B).

Imaging

An Olympus FV1000 confocal microscope, coupled to an Olympus BX61 microscope and 10x .45NA objective lens, was used for imaging. Images were collected at a rate of one frame per second for 10 minutes. The image of the brain was centered on a single depth plane in which the area postrema and optic tectum periventricular gray zone neurons were both visible in the sagittal section.

Analysis of Calcium Imaging

All calcium imaging data generated in the experiment was analyzed using Fiji/ImageJ and followed this general procedure:

1. Motion correction and image stabilization using the “Image Stabilizer” plugin in ImageJ.

2. Extraction of fluorescence brightness values from manually drawn regions of interest over the whole area postrema and periventricular gray zone neurons of the optic tectum.

3. Calculating standard deviation of brightness intensity across time for all fish, ROIs, and conditions. The standard deviation of fluorescence brightness captures the degree of brightness changes over time and therefore the degree of neural activity.

Statistics

SPSS software was used to conduct a 2 x 2, fully within subjects repeated measures analysis of variance (ANOVA) to compare neural activity of the area postrema and optic tectum in both control and MS-222 conditions. The primary hypothesis that area postrema neurons are resistant to the anesthetic effects of MS-222 relative to other brain areas predicts a statistical interaction effect between drug treatment and brain area on neural activity, specifically, that the optic tectum would show greater inhibition by MS-222 than the area postrema.

Results

Contrary to our hypothesis the results of the 2 x 2 ANOVA revealed the drug condition had no significant main effect on the change in fluorescence intensity, $F(1, 4) = 3.68$, $p = .128$, the brain area had no significant main effect on the change in fluorescence intensity, $F(1,4) = 1.25$, $p = .327$, and no significant interaction effect was found between the drug condition and the brain area, $F(1,4) = 0.83$, $p = .414$ (Table 1). Although no results were significant, MS-222 did appear to increase the area postrema mean change in fluorescence intensity observed between the control condition ($M = 72.96$, $SD = 55.93$) and the MS-222 condition ($M = 130.07$, $SD = 85.37$). A similar increase in mean change in fluorescence intensity was also seen between the optic tectum control condition ($M = 142.86$, $SD = 121.35$) and optic tectum MS-222 condition ($M = 315.13$, $SD = 364.71$) (Fig. 4).

Table 1.

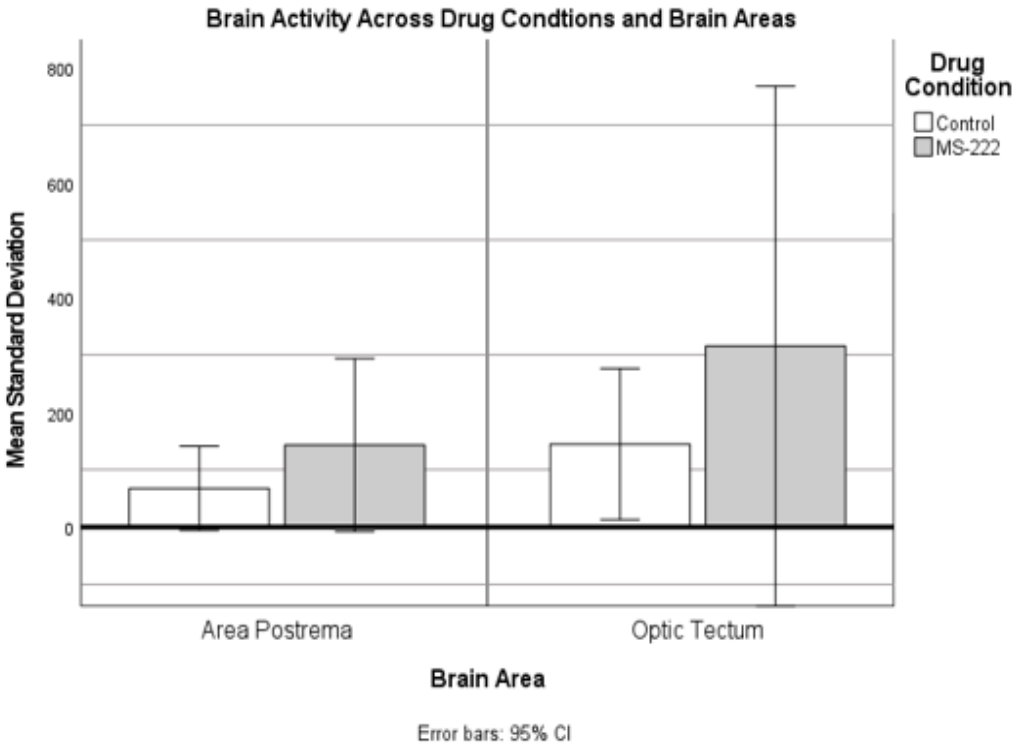
2x2 ANOVA

Effect	*SS*	*df*	*MS*	*F*	*p*
Drug Condition	77690.08	1	77690.08	3.68	.128
Brain Area	75741.89	1	75741.89	1.25	.327
Drug Condition: Brain Area	11338.66	1	11338.66	0.83	.414

Note. The table shows the main effects and drug condition x brain area interaction. There were no significant effects in drug condition, no significant effects in brain area, and no significant interaction effect found.

Figure 4.

Mean differences before and after MS-222 across brain areas.



Note. The mean difference in standard deviation before and after MS-222 for each brain group was displayed in a bar graphed to demonstrate the mean increase in standard deviation across both brain areas after MS-222 exposure. The mean standard deviation of fluorescence intensity in the area postrema was found to increase significantly from M = 72.96 in the control settings to M = 130.07 in MS-222 conditions, p = .035. The mean standard deviation of fluorescence intensity in the optic tectum insignificantly increased from M = 142.86 in the control settings to M = 315.13 in MS-222 conditions, p = .206.

Post hoc paired t-tests were conducted to see if the mean increase in fluorescence intensity was significant for each brain area before and after MS-222 (Fig. 4). The results revealed a significant increase in the change of fluorescence intensity in the area postrema after MS-222 exposure, $t(7) = -2.62$, $p = .035$, $d = -.925$ and an insignificant increase in change of fluorescence intensity in the optic tectum after MS-222 exposure, $t(4) = -1.51$, $p = .206$, $d = -.674$ (Table 2).

Table 2.

Paired t-test results

Region of Interest	Control *M*	Control *SD*	MS-222 *M*	MS-222 *SD*	*t*	*p*	Cohen's d
Area Postrema	72.96	55.93	130.07	85.37	-2.62	.035	-0.925
Optic Tectum	142.86	121.35	315.13	364.71	-1.51	.206	-0.674

Note. Table showing the results of the post hoc paired t-tests. Sample size was different amongst area postrema ($n = 8$) and optic tectum ($n = 5$). Area postrema mean standard deviation increases significantly following MS-222 exposure, $p = .035$. Optic tectum mean standard deviation increases but not in a significant manner, $p = .206$.

Discussion

Interpreting the Results

The results of the 2 x 2 ANOVA demonstrate that the anesthetic drug MS-222 used in this study has no significant effects on larvae zebrafish brain activity and that any observations made, such as the increasing mean and standard deviation after MS-222 exposure, are most likely due to a small sample size with high variance rather than MS-222 resistance in the area postrema. Cleaning and preparing the dataset for analysis by removing recordings with z-drift, oversaturated ROIs, and unequal pixel dimensions, yielded less than half of the original recordings suitable for data analysis ($n = 8$). Which greatly reduced the power of these statistical tests to find significant effects and was a strong limitation of this study. The recordings that did meet the criteria for the final dataset still had high variance in many different imaging factors amongst the subjects such as: laser intensity required for imaging, magnitude of GCaMP expression, slight tilt variations in embedding, and the potential occurrence of whole brain Ca^{2+} waves.

Although the observed increase in mean standard deviation is insignificant and most likely just a chance result given the sample size, MS-222 increasing activity in the observed brain areas would seem counterintuitive to how inhibition of voltage-gated sodium channels affects cellular behavior and GCaMP signaling (Fosque et al., 2015). Meaningful interpretation of the data can cease upon understanding that the sample size lacked the power to provide significant results and any new findings in the results will also be insignificant. However, exploration into what limitations impacted the

experimental results could be valuable to learn so better protocols for future research can be prepared.

Limitations of the Study and Recommendations for Future Research

The presented research had several limitations which impacted the results. First the small sample size that resulted from cleaning the data is one of the greatest limitations of this study as it critically impacted the ability for statistical tests to find significant results. Future research should deploy imaging protocols that focus on producing uniform and easily comparable recordings to limit the need for data exclusion. The oversaturation and pixel size differences that required some data to be removed are easily avoided if proper imaging protocols are followed and without these limitations impacting the research the sample size would be much greater.

Another limitation of this experiment was testing only a single concentration of MS-222. The literature delineates potential differences in development stages of zebrafish and their susceptibility to MS-222 (Rombough, 2007). Expanding the experiment conducted to also include the additional factor of varying MS-222 concentrations could help better explain how the AP reacts to MS-222. Additionally, using varying MS-222 concentrations could also correct for the possibility that the MS-222 used in this experiment was too small to induce anesthetic effects as expected and instead was perceived as a noxious stimulus leading to irritation with no sedation.

The final substantial limitation in this experiment was the lack of visibility of all ROIs during the recording sessions. The original goal was to image from dorsally embedded zebrafish, which would provide a better field of view containing the optic

tectum, cerebellum, and AP. Unfortunately, many of the larvae zebrafish hatched for this experiment expressed heavy dorsal pigmentation, limiting the dorsal view of ROIs and forcing lateral embedding to view the area postrema. Beyond the direct limitation of narrowing the field of view and observable ROIs in each recording, there is also a possibility that the lateral embedding combined with one photon microscopy provided unwanted optic tectum stimulation during the recording periods. The reasoning behind this speculation is that GCaMP is a fluorescence chromophore and needs light excitation to emit fluorescence when binding to calcium (Barnett et al., 2017). For these experiments we used an argon laser which produces a visible blue-green beam at around 488 – 514nm to excite GCaMP molecules. The wavelength of the argon laser is well within the visible spectrum that zebrafish tetrachromatic retina photoreceptors are capable of detecting (Guggiana-Nilo & Engert, 2016; Hartmann, 2018). Opening the possibility that the fish being imaged is having its visual perceptual network stimulated by the laser. Retinal stimulation in zebrafish does lead to optic tectum activation (Heap et al., 2018). Additionally, light sheet fluorescent microscopy (LSFM) has also been shown in the literature to increase GCaMP signaling in the optic tectum of zebrafish and caused unwanted stimulation of the visual network (de Vito, 2022; Liu et al., 2021).

I would highly recommend future research corrects these limitations and further investigates the area postrema response to MS-222 as conclusive findings could prove to be very impactful on our understandings of anesthetic drugs and how the brain senses them, as well as serving as preliminary research for a plethora of follow up studies into the implications of MS-222 resistance in the area postrema.

References

Ahern C.A., Payandeh, J., Bosmans, F., & Chanda, B. (2016). The hitchhiker's guide to the voltage-gated sodium channel galaxy. *Journal of General Physiology*, 147(1), 1-24. doi: 10.1085/jgp.201511492

Arnolds, D. E. W., Zottoli, S. J., Adams, C. E., Dineen, S. M., Fevrier, S., Guo, Y., & Pascal, A. J. (2002). Physiological effects of tricaine on the supramedullary/dorsal neurons of the Cunner, Tautogolabrus adspersus. *The Biological Bulletin*, *203*(2), 188–189. https://doi.org/10.2307/1543388

Attili, S., & Hughes, S. M. (2014). Anaesthetic tricaine acts preferentially on neural voltage-gated sodium channels and fails to block directly evoked muscle contraction. *PloS one*, *9*(8), e103751. https://doi.org/10.1371/journal.pone.0103751

Barnett, L. M., Hughes, T. E., & Drobizhev, M. (2017). Deciphering the molecular mechanism responsible for GCaMP6m's Ca2+-dependent change in fluorescence. *PloS one*, *12*(2), e0170934. https://doi.org/10.1371/journal.pone.0170934

Butterworth, J., & Strichartz, G. (1990). Molecular Mechanisms of Local Anesthesia. *Anesthesiology*, *72*(4), 711–734. https://doi.org/10.1097/00000542-199004000-00022

Catterall, W. A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron*, *26*(1), 13-25.

de Vito, G., Turrini, L., Müllenbroich, C., Ricci, P., Sancataldo, G., Mazzamuto, G., Tiso, N., Sacconi, L., Fanelli, D., Silvestri, L., Vanzi, F., & Pavone, F. S. (2022). Fast whole-brain imaging of seizures in zebrafish larvae by two-photon light-sheet microscopy. *Biomedical optics express*, *13*(3), 1516–1536. https://doi.org/10.1364/BOE.434146

Edwards G.L., & Ritter R.C., (1981). Ablation of the area postrema causes exaggerated consumption of preferred foods in the rat. *Brain Research*, *216*(2), 265-76. https://doi.org/10.1016/0006-8993(81)90129-3

Félix L.M., Luzio A., Themudo M., Antunes L., Matos M., Coimbra A.M., Valentim A.M., (2018). MS-222 short exposure induces developmental and behavioural alterations in zebrafish embryos. *Reproductive Toxicology*, 81, 122-131. https://doi.org/10.1016/j.reprotox.2018.07.086

Fosque, B. F., Sun, Y., Dana, H., Yang, C. T., Ohyama, T., Tadross, M. R., Patel, R., Zlatic, M., Kim, D. S., Ahrens, M. B., Jayaraman, V., Looger, L. L., & Schreiter, E. R. (2015). Neural circuits. Labeling of active neural circuits in vivo with designed calcium integrators. *Science (New York, N.Y.)*, *347*(6223), 755–760. https://doi.org/10.1126/science.1260922

Fry M., & Ferguson A.V. (2009). Ghrelin modulates electrical activity of area postrema neurons. *The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *296*(3), 485-492. https://doi.org/10.1152/ajpregu.90555.2008

Guggiana-Nilo, D. A., & Engert, F. (2016). Properties of the visible light phototaxis and UV avoidance behaviors in the larval zebrafish. *Frontiers in behavioral neuroscience*, *10*, 160. https://doi.org/10.3389/fnbeh.2016.00160

Hartmann, S., Vogt, R., Kunze, J., Rauschert, A., Kuhnert, K. D., Wanzenböck, J., Lamatsch, D. K., & Witte, K. (2018). Zebrafish larvae show negative phototaxis to near-infrared light. *PloS one*, *13*(11), e0207264. https://doi.org/10.1371/journal.pone.0207264

Heap, L. A., Vanwalleghem, G. C., Thompson, A. W., Favre-Bulle, I., Rubinsztein-Dunlop, H., & Scott, E. K. (2018). Hypothalamic projections to the optic

tectum in larval zebrafish. *Frontiers in neuroanatomy*, *11*, 135. https://doi.org/10.3389/fnana.2017.00135

Korzh, V., &; Kondrychyn, I. (2020). Origin and development of circumventricular organs in living vertebrate. Seminars in Cell & Developmental Biology, *102*, 13–20. https://doi.org/10.1016/j.semcdb.2019.10.010

Isaac, E., Cooper, S. M., Jones, S. A., & Loubani, M. (2020). Do age-associated changes of voltage-gated sodium channel isoforms expressed in the mammalian heart predispose the elderly to atrial fibrillation? World journal of cardiology, *12*(4), 123–135. https://doi.org/10.4330/wjc.v12.i4.123

Leyden C, Brüggemann T, Debinski F, Simacek CA, Dehmelt FA and Arrenberg AB (2022) Efficacy of Tricaine (MS-222) and hypothermia as anesthetic agents for blocking sensorimotor responses in larval zebrafish. *Front. Vet. Sci.* 9:864573. https://doi.org/10.3389/fvets.2022.864573

Liu, J., Salvati, K. A., & Baraban, S. C. (2021). In vivo calcium imaging reveals disordered interictal network dynamics in epileptic *stxbp1b* zebrafish. *iScience*, *24*(6), 102558. https://doi.org/10.1016/j.isci.2021.102558

Miller, A. D., & Leslie, R. A. (1994). The area postrema and vomiting. *Frontiers in neuroendocrinology*, *15*(4), 301–320. https://doi.org/10.1006/frne.1994.1012

Musk, G. C., Ezzy, B. J., Kenchington, L. M., Hopper, W. A., & Callahan, L. M. (2020). A comparison of buffered tricaine methanesulfonate (MS-222) and isoeugenol anesthesia for caudal fin clipping in zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science*: JAALAS, *59*(6), 732–736. https://doi.org/10.30802/AALAS-JAALAS-20-000027

Novak, A. E., Taylor, A. D., Pineda, R. H., Lasda, E. L., Wright, M. A., & Ribera, B. (2006). Embryonic and larval expression of zebrafish voltage-gated sodium channel α-subunit genes. Developmental Dynamics, 235(7), 1962–1973. https://doi.org/10.1002/dvdy.20811

Readman, G. D., Owen, S. F., Murrell, J. C., & Knowles, T. G. (2013). Do fish perceive anaesthetics as aversive? *PloS one*, *8*(9), e73773. https://doi.org/10.1371/journal.pone.0073773

Rombough P. J. (2007). Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (Danio rerio) larvae. *Comparative biochemistry and physiology. Part A, Molecular &*

integrative physiology, *148*(2), 463–469. https://doi.org/10.1016/j.cbpa.2007.06.415

Siddiqui BA, Kim PY. Anesthesia Stages. [Updated 2023 Jan 29]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557596/

Smith, P. M., Brzezinska, P., Hubert, F., Mimee, A., Maurice, D. H., & Ferguson, A. V. (2016). Leptin influences the excitability of area postrema neurons. *American journal of physiology. Regulatory, integrative and comparative physiology*, *310*(5), R440–R448. https://doi.org/10.1152/ajpregu.00326.2015

Topic Popovic, N., Strunjak-Perovic, I., Coz-Rakovac, R., Barisic, J., Jadan, M., Persin Berakovic, A., & Sauerborn Klobucar, R. (2012). Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. Journal Of Applied Ichthyology, *28*(4), 553-564. https://doi.org/10.1111/j.1439-0426.2012.01950.x

Wee, C. L., Song, E., Nikitchenko, M., Herrera, K. J., Wong, S., Engert, F., & Kunes, S. (2022). Social isolation modulates appetite and avoidance behavior via a

common oxytocinergic circuit in larval zebrafish. *Nature Communications*, *13*(1). https://doi.org/10.1038/s41467-022-29765-9

Westerfield, M. (2007) THE ZEBRAFISH BOOK, 5th Edition; A guide for the laboratory use of zebrafish (Danio rerio), Eugene, University of Oregon Press. Paperback. (4th Edition available online)

Wong D, von Keyserlingk MAG, Richards JG, Weary DM (2014) Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia and anaesthesia. PLOS ONE *9*(2): e88030. https://doi.org/10.1371/journal.pone.0088030

Zhang, C., Kaye, J.A., Cai, Z., Wang, Y., Prescott, S.L., & Liberles, S.D. (2021). Area Postrema cell types that mediate nausea-associated behaviors. *Neuron*, *109*, 461-472.e5. https://doi.org/10.1016/j.neuron.2020.11.010

Zhang, C., Vincelette, L. K., Reimann, F., & Liberles, S. D. (2022). A brainstem circuit for nausea suppression. *Cell reports*, *39*(11), 110953. https://doi.org/10.1016/j.celrep.2022.110953