Neural Response Patterns to Novel Spatial & Social Environments in the Poison Frog *Dendrobates auratus*

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Abstract

Poison frogs are diurnal, territorial and express complex parental care that requires parents to relocate resources in the environment. For example, green and black poison frogs, *Dendrobates auratus,* have exceptional spatial and social memory allowing them to map large areas of their habitat and to recognize their mates and territorial neighbors. To better understand the neural mechanisms of spatial and social cognition in poison frogs, we set out to identify neural activity patterns that differentiate responses to spatial and social environments. To do so, we first validated the use of pS6, a neural activity marker, in poison frogs. We utilized immunocytochemistry (ICC) with varying reagent concentrations to examine pS6 staining in subjects in two experiments. Frogs in the first experiment were placed in a novel environment or left unstimulated in their home cage as controls. We found that exposure to an unknown environment caused significantly higher levels of pS6 in the medial pallium (MP, homolog of the hippocampus), indicating that pS6 has the potential to act as a neural activity marker in poison frog brains [1]. The results from the first experiment encouraged us to use specific primary antibody concentrations (1:1000) for the second pS6 experiment, which looked to differentiate pS6 stimulation from novel spatial and social environments in the MP and the POA, a brain region important for social behavior [2]. We observed noticeably higher levels of pS6 in both spatially and socially stimulated organisms in the MP. In the POA, there were distinctly higher levels of stimulation from social stimulation compared to the equally stimulated control and spatial groups. With this information, we conclude that pS6 is able to accurately differentiate neural activity in response to spatial and social information. With an increased same size and the

sampling of more brain regions, future studies using this approach will enable us to identify the neural circuits for social and spatial cognition in poison frogs. Identifying neural mechanisms of complex spatial and social cognition in frogs can help us understand how these mechanisms have evolved in vertebrates.

Introduction

The ability to integrate spatial and social information underlies many complex behaviors in vertebrates. For example, in poison frogs, parental care requires careful attention to social information like the number of developing tadpoles and their rate of maturation. In a spatial view, parents need to keep track of where they place each tadpole as they're dispersed across the forest canopy. In addition, poison frogs are territorial, a trait that depends on remembering the spatial boundaries of their territories and their territorial neighbors. Finally, female poison frogs show mate guarding and deceptive courtship. However, the mechanisms of social and spatial cognition are poorly understood in frogs. Understanding these mechanisms of frogs is important for a variety of invertebrate organisms, including humans, due to their use as a comparison group in the evolution of tetrapods. Thus, a more insightful view of the complex neural mechanisms in frogs will provide detail into similar components of many other vertebrates.

A characteristic feature of poison frogs is parental care and tadpole transport. This begins with the mother, who lays her eggs in the reproductive territory of the father for him to fertilize. The father then maintains the quality of the egg clutches by attentively guarding them and hydrating them with urine until they hatch. At this point,

he will transport each of his offspring, one or two at a time, to known pools of water in trees or phytotelmata across the forest [3][4]. Here tadpoles finish their growth and are sometimes provided unfertilized eggs as a food source. Such devotion to the care of their young requires not only well-developed social cognition but also demands a strong spatial memory to navigate the complex physical environment of the rainforest [5]. With the goal of finding out which brain regions are most activated during these processes, a previous study analyzed the brain of successful dendrobatid parental caregivers/tadpole transporters [1]. Their work concluded that the brain regions with the most neural induction were the MP, a homolog of the mammalian hippocampus vital to memory and learning, and POA, a modulator of social behaviors. [1][6]

Our primary goal was to identify the neural activity patterns that mediate responses to spatial and social environments. Immediate early genes (IEGs), such as c-fos and Egr-1 [7][8], are commonly used as neuronal activity markers; however, we wanted to utilize pS6, a ribosomal protein that highlights sights of phosphorylation in response to numerous stimuli [6]. To validate the use of pS6 as a neural activity marker in our species, we first focused on the pS6 primary antibody concentrations needed for reliable detection of changes in pS6 in response to environmental stimulation. Thanks to earlier studies, we know that pS6 at a concentration of 1:500 is sufficient in the immunohistochemistry (IHC) analysis of other poison frog species [1][10]. However, we do not know if that is the optimal concentration for an ICC *Dendrobates auratus*, so we also ran the assay with a 1:1000 ratio as well.

Next, we used our optimized staining procedure to compare the activation of the MP and POA when frogs were exposed to either a novel spatial or social environment.

We predicted that the social stimulation would evoke a response from the POA but not the MP. In contrast, we predicted that both forms of simulation would produce a significant neural response in the MP, compared to controls, due to the MP's involvement in both memory and spatial cognition [6]. This study will set the stage for more detailed studies that will help us understand the mechanisms and evolution of complex cognition.

Experiment 1: pS6 as neural activity marker in poison frogs.

Methods

Husbandry

We acquired frogs from Indoor Ecosystems LLC (Whitehouse OH) where they were bred in captivity. We housed pairs of frogs in terraria with plants and leaf litter and maintained animal enclosures at about 27°C and 75% humidity with a light:dark cycle of 12:12 h. We fed the frogs, fruit flies with vitamin supplements three times per week. All procedures were approved by The University of North Carolina Institute of Animal Use Care and Committee (protocol 19-285).

Tissue Collection

To provide a novel source of stimulation, we placed subjects individually in a large opaque plastic cup which served as a novel environment. Each frog remained in the cup for approximately 1 hour before being anesthetized with a topical application of benzocaine. Subjects were then promptly decapitated, and their brain cases were dissected. Control frogs remained in their home cage until collection time.

Once collected, the brains were fixed for 2 hours in 4% paraformaldehyde before dehydration in 30% sucrose overnight. After dehydration, brains were embedded in medium (Tissue-Tek OCT) and frozen in liquid nitrogen before storage at -80° C until sectioning.

We sectioned brains, anterior to posterior, using a cryostat at 16 µm in four series. When a brain was completely sectioned, all associated slides were placed in a slide container and returned to the -80° C freezer. They remained there until it was time to carry out the ICCs.

Immunocytochemistry

To validate the primary antibody and assess the effectiveness of different concentrations, we used the following conditions: no primary antibody, a concentration of 1:500, or a concentration of 1:1000.

Running the immunocytochemistry was a two-day process. On the first day, we first bought the slides out of the freezer and allowed them to air dry at room temperature for 30 minutes. We then placed them in slide dishes, followed by a 10-minute 0.3% H_2O_2 quench in PBS. Slides then experienced three 5-minute washes in PBS. After the third wash, we applied Immedge Pen on all glass edges of the slide. Next was the addition of 250 µL of blocking solution per slide (for ten slides: 10 ml PBS, 0.02 g BSA, 30 µL Triton-X, & 500 µL NGS) to reduce background staining. Then we let the slides incubate overnight, with 250 µL of the 1° antibody, at 4° C. The 1° antibody solution (pS6 (S235/236) rabbit mAb (Cell Signaling,4858s) was diluted with 2.5 mL blocking solution and either 5 or 2.5 µL of 1° antibody.

The second day started with another three washes in PBS for 5 minutes, followed by 1 hr in 250 µL of 1:200 2° antibody solution (2.5 mL blocking solution and 12.5 µL biotinylated goat anti-rabbit IgG (vector Labs, BA-1000). This led to three more 5-minute PBS washes (3x5), then 1 hour in 250 µL ABC solution (for 10 slides: 2.5 ml PBS, 1 drop of Reagent A, 1 drop of Reagent B, mixed for 15-30 minutes.) Reagents came from ABC elite kit, Vector Labs, PK-6100. After another round of 3x5 PBS washes, the DAB (DAB substrate kit, Vector Labs, SK-4100) solution (for 10 slides: 2.5 mL DI water, 1 drop of Reagent 1, 2 drops of Reagent 2, 1 drop of Reagent 3) was made right before use. Slides were exposed to DAB for 10 minutes. A final 3x5 PBS wash was run then the slides were dehydrated in ethanol baths of 50%, 70%, 95%, 100%, and 100% for 5 minutes each. Lastly, they went into 2 separate xylene baths for 5 minutes, where they were then coverslipped and allowed to dry.

Image analysis

We used a Leica DM 5000 B microscope and SPOT Imaging software to examine our sections and take photos of our focus areas in the MP. For each brain region, we would photograph three sections per subject as close to one another as possible. We attempted to remain in one brain hemisphere (right or left) during MP analysis for consistency, but if it were impossible to get three good pictures in one hemisphere, we would collect what we needed from the other. Sections were skipped over if they had tears through the brain region of focus, along with most sections that folded over themselves.

Once three images for the brain region and subject were collected, we opened the files in ImageJ. This allowed us to use the multi-select tool and hand-count the

number of distinctly stained cells. ImageJ then provided the total number of cells counted, which was then recorded in a datasheet. The three images per subject were then averaged, giving the mean number of stimulated cells per frog, and all subject means in a treatment group were averaged to provide the mean cell count per group.

Results

When comparing stimulated individuals to their unstimulated controls, they showed significantly higher levels of pS6 activation. In the 1:500 1° antibody treatment, we found a 7.7-fold increase in stimulated individuals compared to the unstimulated control (Figure 1). In the 1:1000 1° antibody treatment, we found a 13.8-fold increase in the stimulated individuals compared to the unstimulated control (Figure 1) Though lower in the number of labeled cells, the 1:1000 1° antibody treatment appeared to be more sensitive to group differences than the 1:500 group. This selectivity produced less staining of both activated cells and relatively no staining of background material which allowed for easier analysis. For subsequent ICCs, only the 1:1000 ratio was used following these findings.

We also included a no-antibody control to validate the antibody staining using alternate sections. These slides were run through an identical ICC procedure as above, with the caveat being that they experienced no incubation with the 1° antibody. The outcome of this resulted in seemingly 0 activated cells in a section that was stimulated. Thanks to the additional experiment, we can conclude that the staining was done by the antibody

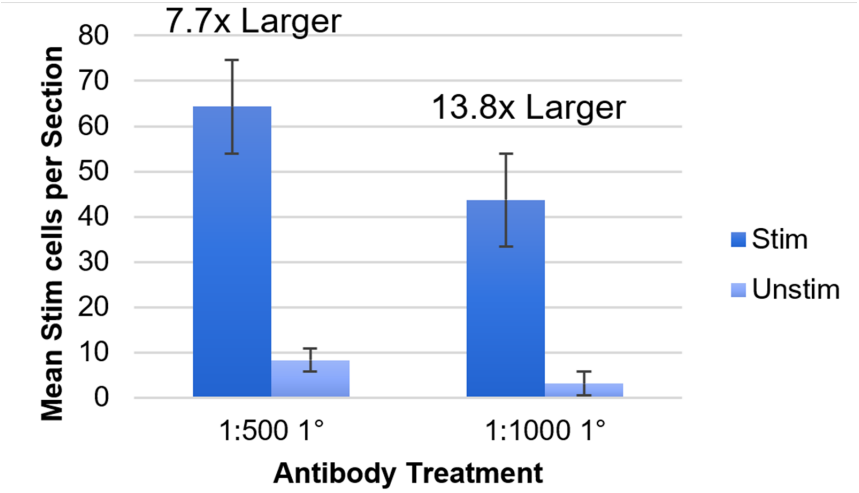

Figure 1. Effect of antibody concentration and behavioral stimulation on pS6 labeling in the medial pallium. Two different antibody concentrations were used to assess the use of an anti-pS6 antibody as a neural activity marker in individuals exposed to a novel environment (Stim) and compared to unstimulated controls (Unstim). Neural activity was assessed as the mean number of pS6-labeled cells.

Experiment 2: Neural activity patterns in responses to novel spatial and social environments

Methods

In order to determine whether social and spatial environments evoked similar neural responses in the MP and distinctly different ones in the POA, we created three treatment groups. Each treatment group had four male subjects for a total of 12 for the experiment. In the spatial treatment group, we exposed subjects individually to a uniquely arranged physical environment with colorful unnatural objects placed around the habit (Figure 2); we introduced a unique combination of novel objects for each subject. All four subjects were released in the novel physical environment for 1 hour,

free to move around or hide as they pleased. After the 1-hour exposure, each subject was removed, anesthetized, and sacrificed.

To provide a novel social stimulus, we introduced an unfamiliar male frog into the cage of the focal frog. Each focal frog was exposed to a different stimulus frog. Similar to the spatial treatment, each focal frog was exposed to the new frog for a total of 1 hour, after which the focal frog was sacrificed the same way as the spatial and experiment 1 subjects. The control group was conducted the same way as the first experiment, by collecting the subject from their home cage. There were a total of 4 days of sample collection. We applied the manipulations in pseudorandom order, so that time of day was not confounded with treatment. To do this, we staggered the stimulation and collection times for each treatment per day (Table 1).

Immunocytochemistry

The ICC followed the same procedure as Experiment 1, and there were only a few distinctions in the amounts of reagents used. For the 1° antibody overnight step, only a 1:1000 concentration (2.5 ml blocking solution and 2.5 µL 1° antibody) was used. On day 2, the 2° antibody concentration was 1:500 (2.5 mL blocking solution and 5 µL biotinylated goat anti-rabbit IgG (vector Labs, BA-1000), and 750 µL of ABC and DAB were used per slide.

Analysis

Data analysis was the same as Experiment 1 with the same equipment and software. The only significant difference was the number of subjects being assessed, and we also looked at the POA. To examine the POA, we prioritized the anterior region where the POA was first coming into view and took three photos moving posteriorly

through the brain. Due to tissue loss during processing, we were unable to collect data from all four subjects in each group, resulting in sample sizes of 2-3 per group per brain region. We used ANOVA to test whether the number of pS6 labeled cells varied with the treatment group in the MP and POA separately.

Figure 2. Novel physical environment for spatially stimulated subjects. The first novel environment tank housing the normal substrate and flora for their cages. Four distinctly unfamiliar items were placed, going clockwise; (1) a red solo cup, (2) a shiny silver tape measure, (3) a brick painted white, and (4) a large roll of blue tape. Dimensions of the cage gave a length (left to right) of 60 cm, a width (front to back) was 45 cm, and a height (soil to roof) was 37 cm.

Wednesday			
Time	Environment	Social	Control
9:00-9:15			
9:15-9:30			
9:30-9:45	Stim		
9:45-10:00	Stim	Stim	
10:00-10:15	Stim	Stim	
10:15-10:30	Stim	Stim	
10:30-10:45	Dissect	Stim	
10:45-11:00	Fix	Dissect	
11:00-11:15	Fix	Fix	Dissect
11:15-11:30	Fix	Fix	Fix
11:30-11:45	Fix	Fix	Fix
11:45-12:00	Dissect	Fix	Fix
12:00-12:15		Dissect	Fix
12:15-12:30			Dissect

Table 1. Schedule for one day of stimulation and tissue collection. Experimental schedule for one subject from each group of Environment, Social, or Control treatments. The schedule included brain dissection times from the body, fixation length in paraformaldehyde, and dissection from the brain case.

Results

We found that the number of pS6 labeled cells in the MP varied with treatment ($F(2,6) = 6.23$, $P = 0.03$; Figure 3). The control group expressed the lowest level of pS6 labeling, while the social and spatial groups showed similarly high levels of labeling. This pattern suggests that novel social and spatial environments were similarly salient for the MP. In the POA, we observed a clear distinction in the amount of cell activation present in the social group compared to the spatial and control groups (Figure 4). However, these differences were not statistically significant ($F(2,4) = 4.4$, $P = 0.098$).

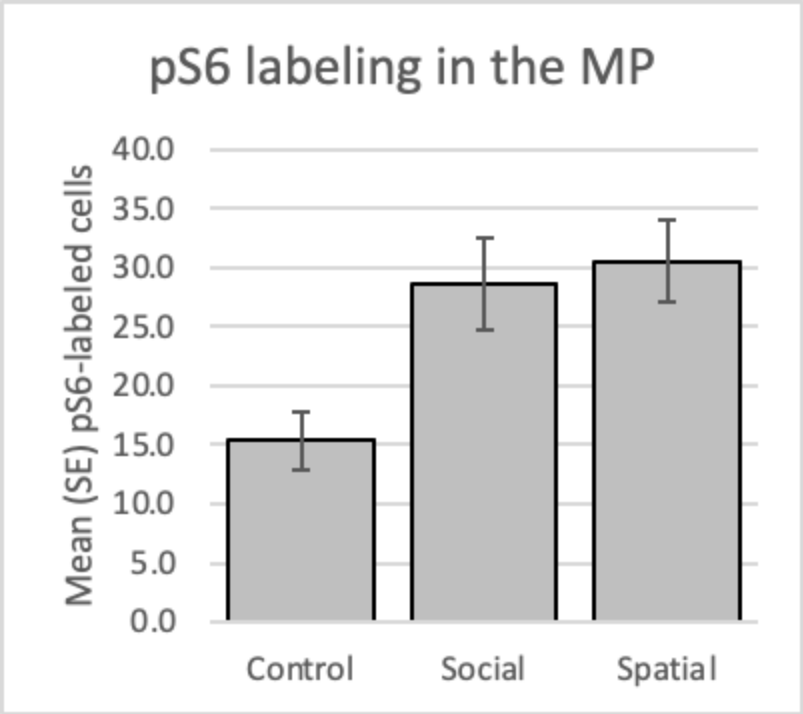

Figure 3. Bar chart showing the relationships between the mean (SE) number of stimulated cells in the medial pallium (MP) of frogs exposed to a novel physical environment (Spatial), a novel cage mate (Social), or no change in either (control). There is a significant difference amongst the groups ($p = 0.03$), with the controls showing the least amount of stimulation.

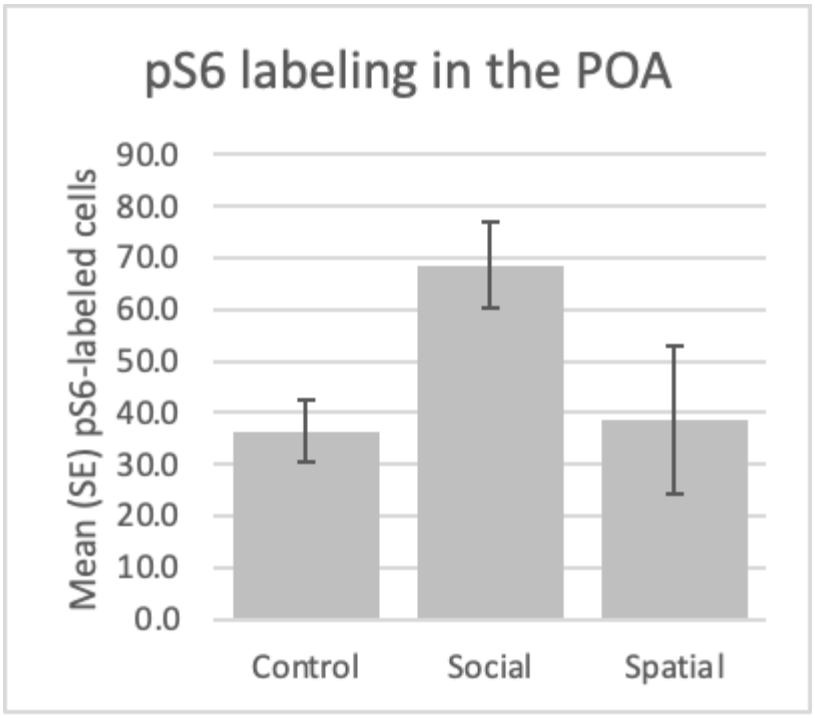

Figure 4. Bar chart showing the relationships between the mean (SE) number of stimulated cells in the preoptic area (POA) of frogs exposed to a novel physical (Spatial) and social (Social) environment. These are compared to frogs who experienced no environmental change (Control). The social group showed a much higher mean stimulation (68.56) than the control (36.33) and spatial group (38.67), but the data was not significant enough to reject the null hypothesis (p=0.098).

Discussion

With our study, we wanted to develop a greater understanding of the activity patterns important in mediating the neural responses to spatial and social environments. To do this, we needed to develop the best assay techniques with pS6 in order to accurately analyze the reactions of the MP and POA in novel situations. Therefore, we first validated the ICC conditions for using pS6 as a neural activity marker in *D. auratus.* Next, we manipulated the spatial and social environments of male poison frogs and measured neural activity in the MP and POA using pS6 labeling. We found that exposure to novel spatial and social stimuli will produce similarly elevated levels of activation in the MP. However, only novel social stimulation was able to elevate the

activity of the POA. This suggests that activity in the MP is correlated with both spatial and social cognition while activity in the POA is only correlated with social cognition.

Results from Experiment 1 showed us that there was significant potential for using pS6 labeling as a neural activity marker but we also found greater sensitivity in the 1:1000 1° group. Comparing this group to the 1:500 showed nearly twice as much selectivity with minimal background staining, making it much less challenging to count the cells during image analysis. This conclusion is supported by another study that sought to optimize ICC protocols and concluded that there is a strong correlation between false positives and antibody concentrations [11]. As with our higher antibody concentration of 1:500, there was distinctly more nonspecific background increasing the likelihood of false positives. This is the most probable reason why there was about 1.5 times more mean staining for both the stimulated and unstimulated 1:500 groups as compared to their 1:1000 counterparts.

Because of the high sensitivity seen in the 1:1000 antibody group compared to 1:500, we decided to use the 1:1000 concentration for our second experiment's ICCs. Using this ICC method aided us in discovering the significantly higher levels of MP stimulation in subjects exposed to novel social or spatial environments contrasted against controls. These results support our prediction that the MP would be responsive to both social and spatial stimuli.

The other part of the study that focused on the POA, however, did not provide enough significance to support our predictions completely. This was most likely due to small sample sizes due to poor section quality; there were only two (control and spatial) or three (social) usable subjects per group. Such few subjects resulted in greater

standard deviations for all groups than what would be seen in a much larger data set. Perhaps, a larger study with significantly more data points would reduce the amount of variance and provide the values necessary to deem the experiment significant and support our original hypothesis about the POA. We know that the POA is an important hub for the organization of social behaviors [2][12], so there is a strong possibility that our group size is a major factor in our high P-value.

Previous work found that both the MP and POA showed increased activity during tadpole transport in poison frogs [1]. Our results point to distinct contributions of the MP and POA to the social and spatial aspects of tadpole transport. In vertebrates, the POA is part of the social behavior network and the MP is part of the mesolimbic reward system [13]. Together these two systems are part of the social decision-making network that allows vertebrates to produce appropriate social behaviors in the right contexts. Parental care in poison frogs likely requires the coordinated activity of the POA and MP to support the interaction of the parent with offspring and navigation to tadpole deposition sites in the forest canopy. Future studies that separate elements of spatial cognition from the expression of parental care will allow us to more fully appreciate the differential contributions of these two brain regions to this complex behavior.

As a whole, our studies have produced valuable information on pS6 and its uses in analyses of the brain of *D. auratus.* Firstly, we can confidently say that pS6 can effectively be used as an activity marker and alternative to immediate early genes (IEGs). We know there are a plethora of future directions this research can go [14][15]. A next step for our lab is to understand the time course of pS6 activation. Knowing when this activity marker shows peak labeling will enable more precise timing of tissue

collection. It will also give more insight into the cellular timing of protein phosphorylation and neuronal activation [16]. In addition, future studies that determine the range of social and spatial stimuli necessary for activation of the POA and MP will contribute to a more complete picture of the neural mechanisms of social and spatial cognition in poison frogs. Because frog brains are considered the best extant representatives of ancestral tetrapods [17], understanding the neural mechanisms of social and spatial cognition in frogs can inform our understanding of how these mechanisms evolved.

Acknowledgments and author contributions

Acknowledgments here. I acknowledge Sabrina Burmeister and Maddie Rader and Stacy Schenkei

In Experiment 1, Sabrina Burmeister (SB) manipulated the animals and collected the tissue; Maddie Rader and SB sectioned the tissue and performed the ICC; Justice Sexton (JS) counted cells, summarized and graphed the data. In Experiment 2, JS and SB manipulated the animals; SB dissected the tissue; JS sectioned the tissue, performed the ICC, counted the cells, summarized and graphed the data; SB performed the statistical analyses. JS wrote the paper, SB provided feedback and suggested edits, JS revised the paper. Stacy Schenkei was the frog caretaker.

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