Immediate Early Gene Expression in D1-SPNs and D2-SPNs During a Striatum-dependent Reinforcement Learning Task

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Abstract

Dopamine signaling is thought to promote movement by differentially altering the excitability of the striatum's principal neurons (D1- and D2-SPNs). Here, we used immunohistochemistry to quantify the expression of Fos, a marker of neural activity, in mice trained to run in a head-fixed fear conditioning task that requires dopamine signaling in the striatum. Training in the task increased the number of Fos-expressing neurons, and a greater proportion of these neurons were D1-SPNs than D2-SPNs. However, this relative increase in D1-SPN Fos expression was not specific for learning the task, as a similar increase was observed in animals that underwent training, but did not learn to perform the motor response. In those animals, D1-SPN activation may encode something other than the learned response. Although further experiments are necessary to determine what the Fos-active populations encode in learners and non-learners, the training-dependent changes we observed in the levels of Fos expression in D1- and D2-SPNs may correspond to fluctuations in neural plasticity that may contribute to the changes in neural calcium activity previously observed by others in our laboratory. Our findings have implications for understanding disease processes that affect the dopamine system, such as Parkinson's disease and schizophrenia.

Introduction

The striatum is the main input to the basal ganglia, a collection of interconnected brain nuclei involved in motor control (Albin et al. 1989, DeLong 1990, Smith et al. 1998). Aberrant striatal and basal ganglia function plays a crucial role in many neurological disorders characterized by deficits in motor function. For instance, in Parkinson's Disease, dopamine-releasing cells that project to the striatum and modulate the direct and indirect basal ganglia pathways degenerate to impair movement. 95% of the cells located within the striatum are spiny projection neurons (SPNs), of which there are two types: one that expresses D1 dopamine receptors (D1-SPNs) and the other that expresses D2 dopamine receptors (D2-SPNs) (Al-Muhtasib et al., 2018). D1- and D2-SPN activity changes due to phasic fluctuations in dopamine that regulate the intracellular signaling and gene expression cascades that modify their excitatory synaptic strength (Shen et al., 2008). In the classical view, dopamine is thought to increase D1-SPN and decrease D2-SPN activity to promote movement and motor learning (Reynolds et al. 2001, Kravitz et al. 2010).

Previous work in the lab found that D1- and D2-SPNs exhibit differential calcium activity during learned movement in the head-fixed, conditioned avoidance task. At the early stages of training, both D1- and D2-SPNs activated during cued motion onset. However, later in training, D1-SPNs became preferentially activated during cued movement. By contrast, D2-SPNs are activated during these learned movements at lower levels and later in time. With these initial findings, our experiment sought to determine whether these different patterns of activity also correspond to differences in the expression of immediate early genes in D1- and D2-SPNs. We hypothesized that learning causes increases in D1-SPN and decreases in D2-SPN Fos expression, a canonical immediate early gene that has increased expression in active neurons.

D1 and D2 receptor signaling is thought to differentially modulate the activity of SPNs to drive motor learning. One way to assay these changes is by monitoring immediate early gene activation and expression in these cells. Specifically, we quantified Fos expression in D1- and D2-SPNs in mice trained in a head-fixed, striatum-dependent fear conditioning task. By establishing how immediate early gene expression maps onto the two principal cell types of the striatum and validating a transgenic tool for monitoring immediate early gene expression *in vivo*, our studies lay the groundwork for future investigations to pinpoint the mechanisms by which striatal neural activity is altered to drive reinforcement learning and how these neuromodulatory processes may go awry in striatum-associated diseases. These findings will help to develop a deeper understanding of these processes and inform potential therapeutic strategies for neurological and psychiatric diseases.

Methods

Training

The mice used in these experiments were genetically engineered to express the red fluorophore tdTomato selectively in D1-SPNs. A headbar was implanted onto a mouse's cranium, which was used to head-fix the mouse onto the training wheel (Figure 1A). Its tail was placed into a cylindrical plastic holder with electrical leads through which a mild electric shock could be administered to the mouse's tail as a negative reinforcer during training. While on the training wheel, the mouse was exposed to a sweeping tone of 2kHz to 8kHz over a period of 4 seconds and delivered a 0.5-s tail shock when the mouse did not exceed a specific running speed within 3.5 s. Each training session lasted 30 minutes, which consisted of 50-55 trials (Figure 1B).

We assessed each mouse's instantaneous running speed by polling the running wheel's rotation every 0.25 s using a rotary encoder. During each trial, the mice must begin stationary, and then have 3.5 seconds to exceed the running

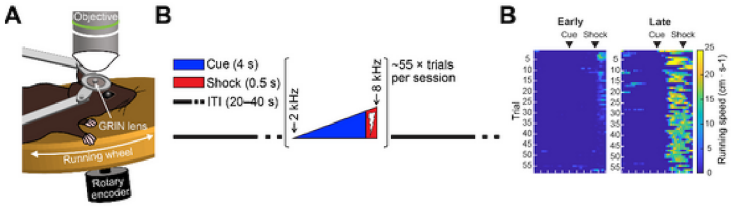


Figure 1. (A) Visual for head-fixed mouse behavior. A headbar is implanted onto a mouse's cranium. (B) Mice are exposed to a sweeping tone of 2kHz to 8kHz for 4 seconds and given a mild electric shock when the mice do not meet a specific running speed. The mouse is given ~50 trials.

threshold of 10cm/s after receiving a cue. Failure to cross this threshold of running speed in response to the cue resulted in a 0.15 mA tail shock. After either avoiding or receiving the shock, the mice were given an intertrial interval (ITI) period of 20-40 seconds during which time no cues or shocks were delivered. The random nature of the ITI prevented the mouse from predicting the next trial, allowing us to specifically assess the mouse's association between the cue and motion onset. After the ITI period expires and the animal becomes stationary, it is given a sweeping cue once again. The criterion for progressing a mouse onto the next phase was two consecutive sessions with greater than 70% avoidance, thus qualifying as a learner.

Aside from the mice that had learned the task, we included two other experimental groups: mice that failed to learn in the task (non-learners) and mice that were exposed to the task but never received a tail shock (untrained). Mice were classified as non-learners when they did not meet the 70% avoidance criterion across training. For the control group, the mice were not given the tail shock when they failed to complete the task. All other aspects of the training were kept the same.

Perfusion

After training completion, mice from all groups were given a 10-min session of probe trials in which they received cue presentations but no tail shocks. They were euthanized and transcardially perfused after one hour with a phosphate-buffered saline solution followed by the same solution containing 4% paraformaldehyde. Unlike learners, non-learners were perfused one hour after a final session of training in phase 1 with no evidence of learning. The brains were then harvested and stored in a solution containing 4% paraformaldehyde.

Immunostaining

The brains were suspended in solidified agar and sliced at a thickness of 70 microns using a vibratome. The sliced brains were washed and permeabilized by rinsing them 3 times for 5 minutes each session in phosphate buffered saline (PBS) + Triton-X-100 (0.3%) in net wells. The brains were blocked by incubating them for 60 minutes in a solution of PBS + Triton-X-100 (0.3%) and 10% normal serum from the secondary antibody host species (donkey) in net wells. The incubated brain slices were then immunostained with the primary antibody (rabbit anti c-Fos) (1/1000 dilution) in PBS + Triton-X-100 (0.3%) and 1% normal donkey serum in a 24-well plate. The imimmunostained brains were washed again 3 for 5 minutes each session in PBS + Triton-X-100 (0.3%) in net wells. To visualize immunostaining, the brains were incubated for 1-2 hours in a solution of secondary antibody (donkey anti-rabbit IgG conjugated to alexa fluor 488 [green]) at a 1/500 dilution in PBS + Triton-X-100 (0.3%) with 1% normal donkey serum in a 24-well plate. The brain slices were then finally washed 3 times for 5 minutes each session in PBS in net wells.

Mount & Analyze

The immunostained brain slices were mounted onto glass slides and imaged using a two-photon microscope. The magni- fication was 16x objective with 1.69 optical zoom (overall effective magnification of 27x). The software used was Prairie View. The images were analyzed using a cell counter in the Image J program and MatLab to determine the immunofluorescence intensity of the entire cell. Two pictures of the same region of cells were taken, one showing the Fos-expression in immunostained green fluorescence and the other showing the red tdTomato markers of D1-SPNs. We overlaid the two images to determine the differences in Fos activation between D1-SPNs (red) and D2-SPNs (unlabeled). Activated D1-SPNs were both green and red, displaying an overlaid color of yellow. D2-SPNs were solely colored green by the activated Fos staining (Figure 2).

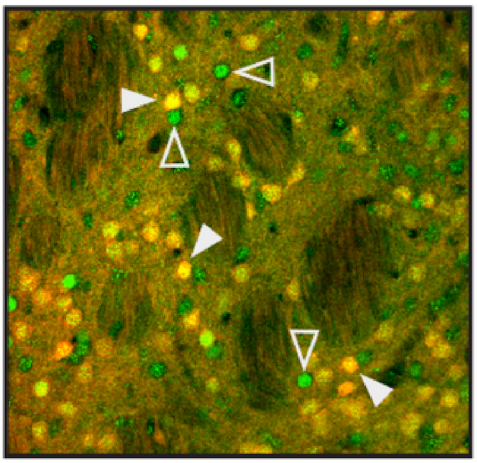


Figure 2. Captured image of Tdt expression overlaid with Fos expression of the striatal region of a mouse. Cells with green Fos+ expression and tdT+ markers are activated D1-SPN, highlighted in the filled arrows. Cells with only green Fos+ expression are activated D2-SPN, shown in the lined arrows.

As shown in Figure 3, Fos positive, Tdt positive cells were manually annotated (labeled '1') as activated D1-SPNs, Fos positive, Tdt negative cells (labeled '2') as activated D2-SPNs, and areas of background fluorescence (labeled '3'). Background fluorescence intensity was used to normalize all cellular fluorescence intensities to isolate the Fos specific immunofluorescence.

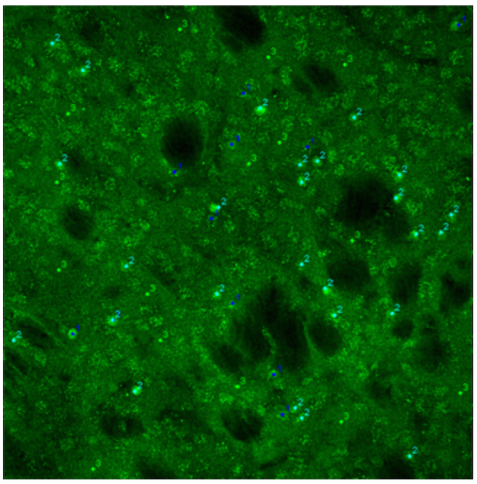
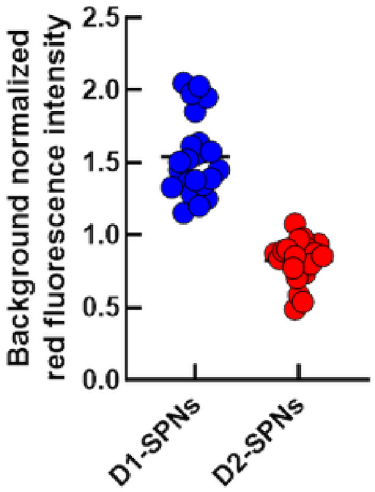


Figure 3. Image of the ImageJ cell counter program in which we marked Fos+ D1 cells with a 1, Fos+ D2 cells with a 2, and points on the background with a 3.

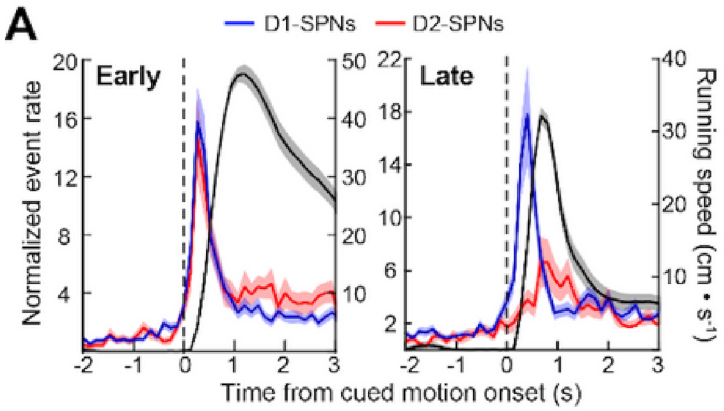


Graph 2: This graph shows the red fluorescence intensity that differentiate the D1 and D2-SPNs.

Data

Preliminary Data

Previous experiments using calcium imaging to monitor D1- and D2-SPN activity during learning in this task (in vivo) showed that D1- and D2-SPNs coactivate when mice run in response to the auditory stimulus early in training (Graph 1). By contrast, after mice had learned to perform this task, a disparity in cue-evoked, movement-related activity became apparent, with D1-SPNs activating earlier and to a greater extent that D2-SPNs.



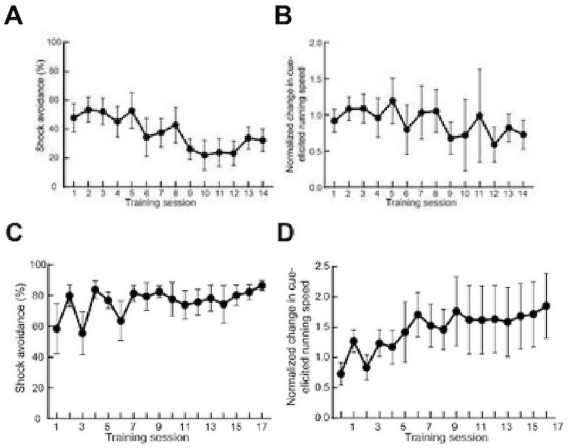
Graph 1: This graph shows that D1 and D2-SPNs exhibit differential calcium activity during a movement-dependent task.

Positive Control

As a positive control for our experimental analysis pipeline, we measured the red-fluorescence intensities of manually annotated tdt positive (D1-SPNs) and tdt negative cells (D2-SPNs). As shown in Graph 2, there were substantially greater levels of red-fluorescence in cells that were deemed tdt positive. The average red-fluorescence intensity for tdTomato was over 1.5, and that of D2-SPNs was near 0.8. These values were normalized to background red-fluorescence intensities to control for any difference in baseline fluorescence between different brain slices and images.

Behavioral Performance

Mice were classified as learners in the task if they passed a threshold of 70% successful trials in a session, as defined by exceeding the running speed threshold required to cancel the tail shock following the auditory cue. Mice were deemed non-learners if they did not achieve a 70% success rate at the end of the trials. Furthermore, there was no significant improvement in running speed for non-learners. Learners, on the other hand, had a high shock avoidance percentage and increased normalized change in cued running speed (Graph 3).

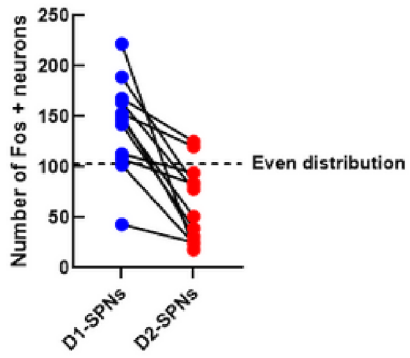


Graph 3. This graph shows the average fraction of avoidance of the mild electric shock between the mice that learned the task (A and B) versus the ones that did not (C and D). A 70% avoidance rate by the last trial was needed for the task to be deemed “learned.”

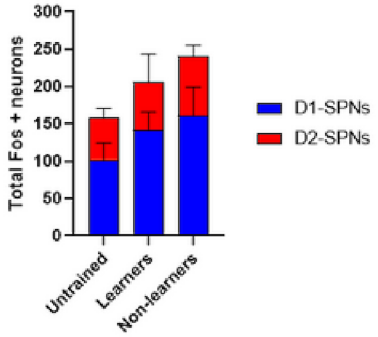
Cell Quantification

In mice that had learned in the task, there were more Fos-expressing D1-SPNs than D2-SPNs, suggesting there is higher neural activity in D1-SPNs in mice that had learned in the task (Graph 4).

In Graph 5, the number of Fos+ neurons for the untrained control, the learner, and the non-learners are shown. This graph clearly shows that the untrained group has an overall lower number of Fos+ cells. However, all three experimental groups had similarly increased proportions of Fos expressing D1- to D2-SPNs.

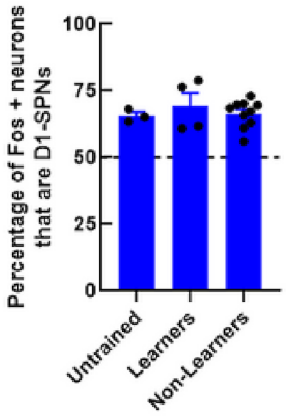


Graph 4. Graph showing the number of cells that were considered D1 and D2 cells in learners.



Graph 5. Graph showing the number of cells that were considered activated D1 and D2 cells in the untrained group, learners, non-learners.

Thus, despite an increase in the overall number of Fos+ neurons following exposure to the task, Graph 6 shows that the proportion of Fos+ D1-SPNs compared to D2-SPNs was similar in all three experimental groups.



Graph 6. Graph showing the percentages of cells that were considered activated D1 and D2 cells within each of the untrained group, learners, non-learners.

Analysis and Discussion:

From the preliminary data utilizing calcium imaging *in vivo*, we saw that there was a difference between D1- and D2-SPN activity detected within mice from the early stages of training compared to after they learned in the task.

To confirm this finding, we utilized Fos expression to measure the activation of D1- vs. D2-SPNs. We saw a greater number of Fos+ D1-SPNs in learners compared to D2-SPNs, therefore aligning with our initial hypothesis (Graph 4).

However, one caveat is that both learners and non-learners show an increase in D1-SPN activity. In other words, even though non-learners failed to acquire the learned stimulus-motor response, D1-SPNs still showed a relative increase in activity. Because both non-learners and learners exhibit a higher level of D1-SPN activity when compared to the control group (Graph 5), we posit that the activated D1-SPNs in non-learners may encode something other than the learned motor response. This change in the levels of Fos expression in D1-SPNs may have occurred due to the aversive experience of the tail shock, although this cannot be proven using our current experimental approach. However, these changes in Fos expression may correspond to changes in neural plasticity that may contribute to the changes in neural calcium activity observed in the lab. Adjudicating this idea will require further experiments and different experimental tools to monitor Fos expression and neural activity in vivo.

Potential Future Research

The lab is implementing a new type of cell detection called FosTRAP. In this method, the tdTomato markers, or red fluorescence, "TRAP" activated, Fos+ cells, permanently labeling them red *in vivo*. In Figure 4 below, we are comparing the number of Fos+ cells from Fos immunostaining to the red TRAPed cells to confirm that this approach works within the striatum. As seen in this initial test, around 80% of the activated Fos+ cells were successfully trapped, which shows that tdTomato accurately reports the Fos expression. Moving forward, this could help directly compare Fos and calcium activity simultaneously *in vivo* in order to determine if Fos active cells encode different task parameters in the learners and non-learners.

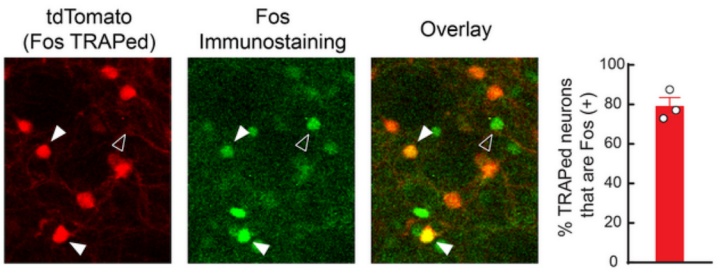


Figure 4. Images of the same cell region showing FosTRAPed cells (1), Fos immunostaining (2), and the overlay of the pictures. The graph on the right shows the percentage of TRAPed neurons that are Fos+.

In all, our findings concerning D1- and D2-SPNs in a conditioned avoidance task lay the groundwork for future investigations to pinpoint the mechanisms by which striatal neural activity is altered to drive reinforcement learning, which could later be developed into therapies that more precisely target specific domains of dysfunction in diseases associated with the striatum. Waiting for our work to validate the Fos-TRAP mice, the lab plans to use this genetic tool to simultaneously image D1- or D2-SPN calcium activity and Fos expression in vivo to link immediate early gene expression to neural coding of learned movement in the striatum.

Conclusions

Better understanding how this process is orchestrated will further our understanding of these neural circuits and how they may function in brain diseases. However, we do acknowledge that the conclusion thus far will require further testing to bolster the number of learner and non-learner mice to evaluate statistical significance. This information lays the groundwork for future investigations to pinpoint the mechanisms by which striatal neural activity is altered to drive reinforcement learning, which could later be developed into therapies that more precisely target specific domains of dysfunction in diseases associated with the striatum.

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Acknowledgements

We would like to thank Dr. Jones Parker from Northwestern University Feinberg School of Medicine for allowing us to work in his facilities.

We would also like to thank Madison Martin, Niki Moya, Andrew Hunter, Ben Yang, and Seongsik Yun for helping us along the way.

Finally, we would like to thank the SIR department at IMSA for giving us the opportunity to expand our knowledge and experiences in research.