

Identify Elements of the VTA Projecting RPE Circuit Neurons in Mouse *Mus musculus* Prefrontal Cortex

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Abstract

The Reward Prediction Error (RPE) model was presented decades ago but still has elements adding to the theory as the mystery of neuron signaling pathways unravels. Following the projections of the dopaminergic neurons, the RPE circuit originates from the ventral tegmental area (VTA), goes to the nucleus accumbens (NAc) reaches a higher-order processing center, which is the prefrontal cortex (PFC). Furthermore, downstream GABAergic neurons will be activated according to the “error,” which indicates the value difference between the anticipated and actual rewards. This study aims to investigate the intermediate steps of the circuit, if any, and the role these intermediate neurons played in stimulating the GABAergic neurons after the sensory inputs are integrated and processed at the PFC. Numerous studies have investigated the function and mechanism of the VTA-NAc-PFC connections, whereas this experiment focuses on the second half of the circuit. At least one, and possibly many, PFC residing, VTA projecting neurons with long axons can be identified.

Keywords: Dopaminergic neurons, Prefrontal cortex, Reward prediction error

1. Introduction

Learning behavior can be observed across the animal kingdom and the ability to learn, or reinforced memory per se, is a critical component of both adaptation and survival. In 1999, Schultz et al. developed the model of Reward Prediction Error (RPE), the difference between predicted and received reward [1]. The ventral tegmental area (VTA) is an evolutionarily conserved structure that has roles in reward-seeking, learning, and recent literature shows that the VTA neurons are also involved in addiction and depression [2-3]. Dopaminergic (DA) neurons in the VTA region encode for the difference in value between the expected value of the reward with the reward received by comparing previous and present experiences. In addition to reward coding dopaminergic neurons, the VTA region is highly abundant with non-overlapping GABAergic neurons [4]. Different than DA neurons, GABA neurons are inhibitory neurons that suppress the activities of VTA. When lower than predicted reward received, mice will have a suppression in the DA neurons activities. The relationship between structure and function has always been a central theme in the field of biology. It is widely believed that deconstruction of the brain structure will give fundamental understanding of the neuroscience. Connectomics generates simplified circuit diagrams at different resolutions (i.e. macroscopic or mesoscopic) that classify neurons in terms of their connectivity to each other. As the RPE model suggests, the neuron circuit consists of three distinct regions in

the brain. Along with GABA neurons, DA neurons can be found in the nucleus accumbens (NAc) and the prefrontal cortex (PFC) [5]. Upstream neurons are well-studied compared to downstream neurons in the circuit, especially the second half of the circuit where the long-range projections originate from the PFC to VTA GABA neurons. Long range neurons, like the ones coming out of the PFC and going back to VTA, have not been identified in previous studies and their functional properties remain unknown. By using viral tracing and tagging assays, the experiments are aiming to discover more intermediate neurons that reside in the PFC and identify connectivity patterns between components of neural circuits. To further elucidate the component of the RPE model neurons, a series of experiments are design to identify the targeted neurons.

The neuroanatomy technologies are likely to take, in this particular experiment, are pseudorabies viral tracing and transgenic mice with further genetic recombination techniques. Furthermore, the behaviors of the neurons are hypothesized to be following the pattern of the associated learning, similar to the GABA neurons. In addition to the well-characterized VTA DA neuron projection to the PFC, the VTA also sends a substantial GABA projection to the mesocortical area, comprising 58% of all mesoprefrontal neurons [6]. Within the PFC, VTA GABA terminals synapse on both other excitatory neurons and GABA local circuit neurons and are thus demonstrating facilitation to mediate both direct inhibitory and indirect excitatory influences. In particular, PFC terminals synapsed on

mesoprefrontal DA neurons, despite the fact that these cells make up a minority of the projection population [7]. Future studies are necessary to understand the sources of afferent input that drive activity in the newly discovered GABA mesocortical pathway.

2. Methods

2.1 Identify the targeting neurons

A transgenic line of mice (*Mus musculus*) is created with the expression of channelrhodopsin and green fluorescence protein. As shown in Figure 1, a transcriptional stop factor is encoded between the LoxP of which are all downstream a general promoter region. Introns expressing channelrhodopsin is encoded in the gene as well as a green fluorescence protein (GFP) transcriptional factor. The glycoprotein-deleted pseudotyping rabies with the envelope protein of a virus that is unable to infect mammalian neurons thus has limited access and toxicity to the tissue. Infection by pseudotyped SADΔG, can be controlled by selective expression of its cognate receptor, TVA, on target neurons, which is superior to traditional neurotrophic viruses. [8-9]. The rabies glycoprotein will infect the neurons and exclusively retrograde trans-synaptic spread the virus. The genetically modified SADΔG variant rabies virus is administrated at the VTA region on GABAergic neurons. The CRE box is engineered in the rabies virus genome and followed by a stop codon aiming to slice out the transcriptional stop factor in the mouse's genome.

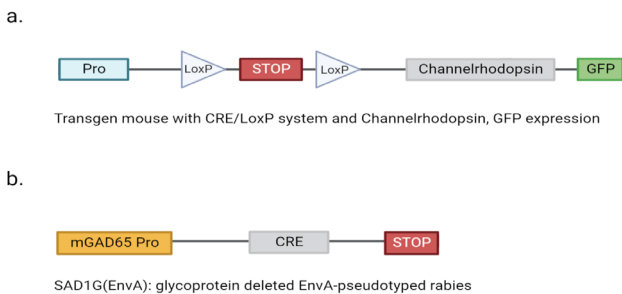


Figure 1. Cre and lox system is expressed in the mice and rabies virus.

A. transgenic mice line has both Channelrhodopsin and GFP embedded thus fluorescence will be observed once expressed. b. GAD2 promoter is incorporated here to selectively targeting the GABA neurons and then go retrograde to activate the Channelrhodopsin/GFP coupling in the targeted neurons. Created with BioRender.com. The starting point for monosynaptic retrograde tracing strategies that use SAD1G(EnvA) can be defined by co-expression of the genes that encode TVA and the rabies glycoprotein in starter neurons. Direct CNS injection

of SADΔG selectively infects seed neurons, which are GABA neurons in this case, will carry the virus to the upstream neurons projecting from the PFC. Inhibitory neurons release the inhibitory neurotransmitter, GABA, which is synthesized from glutamate by glutamic acid decarboxylase (GAD). Gad promoter regions were screened for inhibitory neuron-specific promoters and GAD65 promoter has been proved to work exclusively at inhibitory neurons [10]. Therefore, GFP will be expressed at the neurons of which have channelrhodopsin expression. 20 mice of age 100 days are selected for the injection under general anesthesia[11].

2.2 Functional analysis of the fluorescence labelled neuron using RPE

The targeted neurons project onto GABA neurons in the VTA region therefore they must have long axons. Due to their unique anatomical feature, a more advanced imaging technique is needed to visualize the change and expression of GFP. Thus a more sophisticated observation technique is required. Thus two-photon microscopy provides a wide range of visual field and enough resolution to identify the neuron of interest [12]. After placing the microscope at the right field, fiberoptics are installed and angled to the neuron as well. Then the mice are trained to associate two odors with different rewards and the possibility of getting the reward.

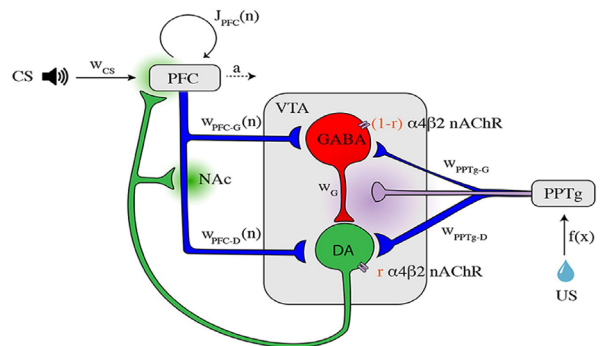


Figure 2. A simplified schematic diagram showing the mechanism of RPE system.

Three main components of the RPE system are the GABA neurons, DA neurons, and the interneurons that connect them. These neurons reside mainly in the VTA region but critical elements can also be found at the NAc and PPC. As shown here, a neutral stimulus is given prior to the training will not generate any feedback in the system. However, when the stimulation is paired with a reward (i.e. an reward input, designated as $f(x)$ here) the mouse will gradually learn that there is correspondence between the stimulus and the reward. Therefore, the neutral stimulus becomes a conditioned stimulus and will be processed at the PFC. By disabling the targeted neuron, the RPE

circuit is predicted to be interrupted as the neuron is an intermediate step. Figures adapted from Deperrois et al [13].

Odor A is associated with 80% chance of getting a small reward (i.e. 0.5 ml of sugar water). Odor B is associated with 20% chance of getting a big reward (i.e. 2 ml of sugar water). After training the mice for two weeks, electrodes are placed at the VTA region of all the mice to examine the field action potential. As shown in Figure 2, the mice should have alterations in the feedforward and feedback loop thus developing different behavioral outcomes based on the probabilities and the sizes of the rewards. Then the mice are divided into 2 groups. The FPSP of individual mouse in each group is recorded when they are getting the either the big or small reward. The probability of getting reward is the same for both groups. With all the conditions being equal, one group (ten mice) has their laser light turned on during the experiment and recording process.

3. Conclusion

The anticipated results are: 1) When the laser light is on, there will be no significant difference in recordings between the big and small reward. Furthermore, the targeted neurons are responsible for the activation of GABA neuron so when they are silenced the GABA neurons are less likely to be activated thus the VTA recording will display a higher firing rate. 2) With laser light turned off, the learning association ability of the mice will remain intact. In other words, the activation level of the VTA DA neurons will be lower when getting small reward comparing to big reward. Upon successful identification of the connection between PFC and VTA GABA neurons this study also investigated the functional connection between the two regions. Thus proved there is not only such long range signal transduction pathway exist but also its role in the RPE learning system. However, less than ideal circumstances could occur. In theory, this approach could be used to identify monosynaptic inputs to any population in which it is possible to selectively drive TVA and the rabies glycoprotein, but the technique has limitations: first, the proportion of monosynaptic input neurons labeled by SADΔG is low and subject to variability. A second weakness of the SADΔG system is its neurotoxicity, which is kept at minimal compares to other traditional staining and labelling techniques. The experiments are to be finished in no more than 3 week and the injection will hinder the use of functional tools. The glycoprotein-deleted rabies variants lies in uncertainty regarding potential biases in its tropism. The potential for differential selectivity according to proximity or

phenotype has been raised by a number of investigators [14]. The GAD promoter infused virus should have the ability to precisely targeting the inhibitory GABA neurons in this case as it is expressed specifically in the inhibitory neurons tested by Hoshino et al. The brain no where similar to a binary machine. The experiments aimed to investigate the function of the neuron in the RPE model as it is part of the circuitry. More factors could play a role in the electrophysiology recordings. The PFC integrates information from numerous neurons which means co-release of dopamine and GABA are weighted by the system to make its final decision. The idea is that instead of all dopamine neurons encoding a similar mean prediction error, each neuron might encode either a more “optimistic” or more “pessimistic” prediction, distributed around the mean. Using many cells to represent a range of predictions allows the brain to capture a full probability distribution for future rewards and in theory improves reinforcement learning [15]. There is currently no lateral inhibition discovered in the RPE learning process and the evaluation procedure sure plays a role in decision making and reward perception. Furthermore, there is a “belief state” which has be proved to exist in between the decision, action and learning reinforcement step in the RPE loop [16]. In belief state the mouse evaluates the previous trials and “name” a value to the reward given by these trials. If the mouse received a train of low probability rewards, which denotes the probability of getting it is actually not low but high, the mouse will bring the activation level of the DA neurons down and less excited about the in fact less seen reward. Also there are neurotransmitters beyond the two types of neurons mentioned in the RPE model. Glutamate can also be found at the VTA region and co-release of classical neurotransmitters and neuropeptides increases the cellular heterogeneity of VTA neurons [17]. Despite the co-release of these neurotransmitters and the facilitation between glutamate neurons and GABA neurons, the glutamate neurons activates when mice feel threatened. Avoidance behaviors can be triggered by glutamate neurons and its influences on the GABA neurons is unknown. The intrinsic neurons were not silenced during the experiments so there is a possibility that GABA activation was buffed by glutamate neurons as the mice were transferred the mice to a new environment for following procedures. Given the nature of long-ranged projections like GABA neurons and the localizations of these neurons, co-existing glutamate neurons could have antagonistic effect on the GABA neurons thus further affect the RPE system. Application of glutamate receptor agonists into the PFC suggests that PFC terminals synapse onto the dendrites of DA neurons elevates extracellular DA within mesocortical

area and further suggests that a potential mechanism is excitatory PFC cells that synapse onto mesoprefrontal DA neurons, an inhibitory feedback loop created by reciprocal projections may mediate such mechanism. Most *in vivo* studies report that DA suppresses PFC cell activity [18,19]. As stated earlier, the pathway may also play a role in the unique responses to stress [20], because both PFC cells and mesocortical DA neurons are activated by stressful stimuli. The finding of synaptic input from prefrontal cortex to mesocortical DA neurons has important significance for understanding the role of DA in facilitating learning by the communication of future expectations [21-23]. All in all, this particular experiment was aiming to identify a component of the RPE circuit and investigating its primary function during the learning process. The functional properties of the targeted neurons are under no influences of other neurons regardless. There might be more connections downstream that affect the outcome of the learning or responding behavior but that is beyond the scope of the original intention. Further experiments can be conducted on primates to see how silencing an upstream neuron would cause the learning pattern to change in the RPE circuit. Influences of rewarding and aversive outcomes on activity in monkey Lateral PFC has shown to influence reward expectation and visuospatial processing [24-25].

Intensive and rigorous studies have been done regarding the RPE system not only in animal models but also in human trials. Research done by Cohen et al. showed that human adolescents may be hypersensitive to rewards. Although the aspect of reward processing responsible is currently unknown but the RPE circuit is highly likely to be involved. They found that prediction error signals in some of the adolescence showed higher than anticipated peaks compared to such regions in an adult brain, namely the striatum. This suggests that heightened dopaminergic prediction error responsivity contributes to adolescent reward seeking [26]. Similar cases can also be seen in adults, especially patients with neurodegenerative diseases. Cognitive scientists utilized neurophysiological and functional magnetic resonance imaging (fMRI) results addressing the processing of reward prediction errors and how they might be altered in drug addiction and Parkinson's disease [27]. Drugs that are able to alter phasic dopaminergic responses, which in this case, accentuate RPE signals can cause increases in fMRI activity in mesolimbic areas in response to drugs. On the other hand, long term drug intake has been shown to have correlation with compromised dopamine neuron functions. In Parkinson's disease, dopamine replacement therapies seem to induce impairments in learning from negative outcomes [27]. As previously discussed, RPE learning

process are important elements for reinforcement learning and flexible decision-making processes. Alterations, whether naturally or artificially, in these signals has been proved to cause a range of neuropsychiatric disorders. Overdosed reward caused addictions has putative alterations in reward prediction errors which could lead to serious drug abuse. Intake of inhibitors or controlled consumption of the addicted drug seem to be associated with lower dopamine availability, potentially affecting reward perception and thus promoting drug use. In Parkinson's disease, conversely, therapies might induce impairments in learning from negative outcomes that increase the likelihood of developing behavioral addictions [27]. In an neuroanatomy view, the distinct characteristics of these neurons can be categorized and analyzed using techniques like RNA sequencing and other genetic tools. Promoters in such long axon neurons can be identified and labelled to see if there are any neighboring neurons that might be connect to it. The grouping of neurons will lead to further functional analysis and connectomic studies in the future.

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