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Acute Aversive Stimuli Rapidly Increase the Activity of Ventral Tegmental Area Dopamine Neurons in Awake Mice

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Abstract—The ventral tegmental area (VTA) is one of the origins of the brain dopaminergic system and is involved in regulating various physiological functions such as pain processing and motivation. In this study, we utilized a fiber photometry system to specifically investigate the activity of dopamine neurons in the VTA using dopamine transporter promoter-driven Cre recombinase-expressing mice and site-specific infection of adeno-associated virus carrying the FLEX G-CaMP6 gene. As expected, expression of G-CaMP6 was restricted to VTA dopamine neurons. We recorded G-CaMP6 green fluorescent signal, which reflected dopaminergic neuronal activity, in awake mice exposed to tail pinch, ultrasonic sound, predator odor, and a male intruder mouse. These stimuli resulted in a rapid and short-lasting increase in the activity of VTA dopamine neurons while the control stimuli of a gentle tail touch and appearance of empty box did not induce any changes. In addition, fluorescence intensity was not changed by any of these stimuli in the control animals expressing hrGFP instead of G-CaMP6 in VTA dopamine neurons. Our data clearly show that acute aversive stimuli rapidly increase the activity of VTA dopamine neurons and thus suggest a salience-processing role. **© 2018 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: G-CaMP6, fiber photometry, tail pinch, ultrasonic sound, predator odor, intruder mouse.

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INTRODUCTION

Physical and mental activity depends on the state of various neurotransmitters in the central nervous system. Dopamine (DA), one of the monoamine neurotransmitters containing a monoamino-group, plays important roles in the regulation of motor, sensory, and mental functions (Horvitz et al., 1997; Heinz and Schlagenhauf, 2010; Hagino et al., 2015). In the central nervous system, there are several dopaminergic pathways, such as the mesolimbic pathway, the mesocortical pathway, the nigrostriatal pathway, and the hypothalamo–hypophyseal pathway (Ikemoto, 2007). The nucleus where the mesolimbic and mesocortical pathways originate is the ventral tegmental area (VTA) in the mesencephalon (Ikemoto, 2007), and the origin of the nigrostriatal pathway is the substantia nigra pars compacta. Malfunction of these systems is related to several mental disorders and some of their respective symptoms. Specifically, the mesolimbic–cortical pathways that arise from VTA are relevant to the positive and negative symptoms of schizophrenia (Laruelle et al., 1996), reward (Datla et al., 2002; Berridge, 2009), mood symptoms (Tidey and Miczek, 1996; Tye et al., 2013), fear (Abraham et al., 2014), learning (Brown et al., 2012), and nociception (Kalivas and Duffy, 1995; Ungless et al., 2004). Although VTA neuronal activity has been studied extensively using traditional electrophysiological methods, use of newly developed fiber photometry method (see below) is limited to a study of rewards and reward-associated activity changes (Gunaydin et al., 2014).

For approximately the last ten years, calcium-imaging experiments have been used to record the activity of specific neuronal populations (Kerr et al., 2000; Ohki et al., 2005; Huber et al., 2012). Cui et al. developed a fiber photometry system in 2013 (Cui et al., 2013), and for the last several years the system has become an increasingly popular tool for detecting Ca^{2+} signal in specific neuronal populations *in vivo* (Cui et al., 2013; Gunaydin et al., 2014; Natsubori et al., 2017; Muir et al., 2018). The fiber photometry system adopts the GCaMP protein as a detector of Ca^{2+} concentration in the intracellular space, which is closely related to neuronal activity. GCaMP is a genetically encoded fluorescent calcium indicator protein developed by Nakai et al. (2001) and is composed of monomolecular green fluorescent protein (GFP) and calmodulin (CaM) at the C terminus and an M13 peptide from the myosin light chain. After its initial

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Abbreviations: AAV, adeno associated virus; CaM, calmodulin; DA, dopamine; DAT, dopamine transporter; GFP, green fluorescent protein; NAc, nucleus accumbens; PBS, phosphate-buffered saline; PFC, prefrontal cortex; TMT, 2,4,5-trimethyl thiazoline; VTA, ventral tegmental area.

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development, GCaMP has been repeatedly refined and improved (Tian et al., 2009; Akerboom et al., 2012) with one of the latest versions, G-CaMP6, being much more sensitive to fluctuations in Ca^{2+} signal (Ohkura et al., 2012). GCaMP is superior to traditional calcium indicators such as Fura-2 because the probe can be incorporated into specific cells and subcellular components via genetic-engineering techniques. Cell-specific analysis could be performed via histological methods such as examining c-Fos immunoreactivity but the time resolution is not as precise as fiber photometry (hours vs. seconds). By taking advantage of these features, the fiber photometry system can detect real-time *in vivo* Ca^{2+} signal from targeted cell populations with high specificity and sensitivity.

Actually, the VTA region has heterogeneity in its neurochemical profile and the neuronal population is comprised of about sixty percent dopaminergic neurons, thirty percent GABAergic neurons, and less than three percent glutamatergic neurons (Nair-Roberts et al., 2008; Sesack and Grace, 2010). Due to this heterogeneity, being able to specifically target the dopaminergic portion of the neuronal population and examining it with high sensitivity via a fiber photometry system is advantageous in appraising its activity and possible function. We have recently established and thoroughly-tested a fiber photometry system using G-CaMP6 and successfully examined orexin neuronal activity in the hypothalamus, which is another heterogeneous structure in the brain (Inutsuka et al., 2016; Futatsuki et al., 2018).

In this study using fiber photometry, we aim to appraise the real-time activity of VTA DA neurons in awake mice in response to aversive stimuli because the VTA region is sensitive to not only well-known rewards-associated stimuli but also various types of stressors (Overton et al., 2014; Holly and Miczek, 2016). We first introduced the G-CaMP6 protein in VTA DA neurons using transgenic mice with dopamine transporter (DAT) promoter-dependent expression of cre recombinase in DA neurons and site-specific injection of adeno-associated virus (AAV-CAG-FLEX-G-CaMP6-WPRE) into VTA. We confirmed the specific expression of G-CaMP6 in VTA DA neurons via an immunohistochemical method. Next, we used fiber photometry to record the Ca^{2+} signal of G-CaMP6 expressed in VTA DA neurons of awake mice while they were exposed to aversive stress stimuli.

EXPERIMENTAL PROCEDURES

Ethical approval

All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of laboratory Animals and approved by the Institutional Animal Use Committee of Kagoshima University (MD15092).

Animals

We used transgenic mice carrying a DAT promoter-regulated Cre recombinase transgene (DAT-Cre mouse, Slc6a3$^{tm1(cre)Xz}$/J, Jackson Laboratory, Stock#020080). The genotype of DAT-Cre mice was identified by PCR of DNA extracted from the tail according to the protocols at Jackson's home page (https://www.jax.org/strain/020080). Eight- to fourteen- week-old male mice were used in this experiment. Mice were maintained in the laboratory at the standard conditions, which included a 12/12-h light/dark cycle (lights on at 7:00 AM and off at 7:00 PM), a temperature of 24 ± 1 °C, and food and water *ad libitum*. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used.

Stereotaxic AAV injection

AAV vectors were produced using the AAV Helper-Free system (Agilent Technologies, Inc., Santa Clara, CA, USA) and purified as has been described (Inutsuka et al., 2016) (Fig. 1A). Surgeries for AAV injections were carried out under 2–3% isoflurane anesthesia using stereotaxic instrument (ST-7, Narishige, Tokyo, Japan). AAV-CAG-FLEX-G-CaMP6-WPRE (serotype DJ; 1 μl/injection, 2×10^{13} copies/ml) or AAV-CMV-FLEX-hrGFP (serotype DJ; 1 μl/injection, 6×10^{12} copies/ml) were slowly drawn up into a glass micropipette fabricated from microtube (1B150F-3, World Precision Instruments, Inc., Sarasota, FL, USA), which was connected to a Nitrogen pressure source through polyethylene tubing and an injection manipulator (I-200J, Narishige) (Fig. 1B). AAV was unilaterally injected into the VTA (Injection site was from bregma −3.15 mm, lateral +0.5 mm, and ventral −4.25 mm from the surface of the brain). After AAV injection, the micropipette was left in place for ten minutes before being slowly withdrawn. Experiments were carried out after at least fourteen days (two weeks) because that is the approximate time it takes for fluorescent proteins (G-CaMP6, hrGFP) to express.

Immunohistochemistry

Mice were transcardially perfused with 20 ml of phosphate-buffered saline (PBS) and 20 ml of 4% paraformaldehyde solution under anesthesia with urethane (1.6 g/kg, i.p.) over two weeks after AAV injection. The brain was removed and post-fixed with 4% paraformaldehyde and immersed in 30% sucrose in PBS for two days. Sequential 30-μm slices were sectioned with a cryostat (Cryotome FSE, Thermo Scientific, Yokohama, Japan). The brain sections were immersed in blocking solution (1% normal horse serum and 0.3% Triton-X in PBS) for one hour at room temperature. The sections were incubated with primary antibody for two hours. The primary antibody used was anti-Tyrosine Hydroxylase raised in rabbit (AB152, EMD Millipore Corp., Temecula, CA, USA) which was diluted in blocking solution at 1:500. Next, the sections were rinsed with PBS three times, and then incubated with secondary antibody for two hours. The secondary antibody used was NL637-conjugated anti-rabbit raised in donkey (NL005, R&D Systems, Inc., Minneapolis, MN, USA) which was diluted in blocking solution at 1:200. The sections were rinsed, mounted upon microscope slides (PRO-02, Matsunami, Osaka, Japan),

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