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Optogenetic Rescue of Locomotor Dysfunction and Dopaminergic Degeneration Caused by Alpha-Synuclein and EKO Genes

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α-Synuclein (α-Syn) is a small presynaptic protein and its mutant forms (e.g. A53T) are known to be directly associated with Parkinson's disease (PD). Pathophysiological mechanisms underlying α-Syn-mediated neurodegeneration in PD still remain to be explored. However, several studies strongly support that overexpression of mutant α-Syn causes reduced release of dopamine (DA) in the brain, and contributes to motor deficits in PD. Using a favorable genetic model *Drosophila* larva, we examined whether reduced DA release is enough to induce key PD symptoms (i.e. locomotion deficiency and DA neurodegeneration), mimicking a PD gene α-Syn. In order to reduce DA release, we expressed electrical knockout (EKO) gene in DA neurons, which is known to make neurons hypo-excitable. EKO led to a decrease in a DA neuronal marker signal (i.e., TH – tyrosine hydroxylase) and locomotion deficits in *Drosophila* larva. In contrast, acute and prolonged exposure to blue light (BL, 470 nm) was sufficient to activate channelrhodopsin 2 (ChR2) and rescue PD symptoms caused by both α-Syn and EKO. We believe this is for the first time to confirm that locomotion defects by a genetic PD factor such as α-Syn can be rescued by increasing DA neuronal excitability with an optogenetic approach. Our findings strongly support that PD is a failure of DA synaptic transmission, which can be rescued by optogenetic activation of ChR2.

Key words: α-Synuclein, EKO, optogenetics, Parkinson's disease, Dopaminergic neurons, *Drosophila melanogaster*

INTRODUCTION

A presynaptic protein α-Synuclein (α-Syn) is known to play an important role in neuronal plasticity [1, 2, 3, 4], but its physiological role is not completely clear yet. Ironically, the pathophysiological role of α-Syn drew intense public attention since studies revealed that mutated α-Syn genes are directly associated with PD [5]. α-Syn is also known to be a major component of cytoplasmic inclusion Lewy body, a histological hallmark of PD [5, 6].

Pathophysiological mechanisms underlying α-Syn-mediated neurodegeneration in Parkinson's disease (PD) remain to be further explored. In the meanwhile, several studies strongly support that overexpression of mutant α-Syn causes reduced release of dopamine (DA) in striatum, and thus contributes to motor deficits in PD [7]. For example, overexpression of a mutant human α-Syn in mice showed reduced locomotor activity and DA release [8]. This is consistent to the fact that drugs currently used to ameliorate PD symptoms increase DA levels in the brain. A gold

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standard PD drug, L-DOPA is a precursor of DA [9], bypassing an enzyme tyrosine hydroxylase (TH), a limiting factor in DA synthesis. MAO-B inhibitors are also used to increase DA levels as they inhibit MAO-B, an enzyme metabolizing DA. In addition, several DA receptor agonists (e.g., bromocriptine, pramipexole) have been used to reduce PD symptoms by increasing DA signal tone [10]. Drug treatments are easy and most popular interventions for PD, but also cause severe side effects due to non-specific binding and/or off-target effects. Therefore it is necessary to develop alternative strategies to treat PD symptoms without such complications.

A popular approach to target specific neural circuits is called 'optogenetics'. The technique involves the insertion into the genome of genes (e.g., ChR2 - channelrhodopsin 2) encoding a light-sensitive channel protein [11, 12]. When expressed in the membrane of a neuron and then exposed to light of a certain wavelength (e.g. blue light - 470 nm), these channels open, letting ions pass through them. Depending on the type of channel, this can activate (e.g. ChR2) or suppress a neuron (e.g. NpHR – a halorhodopsin). Genetic tools allow for the expression of these channels in specific groups of neurons – including DA neurons involved in PD. Therefore, optogenetics can be used to selectively manipulate excitability of DA neural circuits regulating motor behavior.

In this study, we wanted to examine whether reduced DA release is enough to cause PD-like symptoms. If so, can it be rescued by non-pharmacological approach? The fruit fly *Drosophila melanogaster* larva expressing a human disease gene has been used as a genetic PD model largely due to its sophisticated genetic tools available and genetic similarity to mammals [13, 14]. Recently, we have developed *Drosophila* larval model for PD which shows two cardinal PD symptoms (i.e. locomotion deficiency and DA neurodegeneration) by a mutant human α-Syn [15].

In order to decrease DA release, electrical knockout (EKO; 16) gene has been expressed specifically in DA neurons of fly larval brain. *Drosophila* larva is an excellent model for optogenetic experiments because of semi-transparent skin [17], which allows direct passage of light into neurons in the brain. After observing reduced locomotion in EKO larvae, therefore, optogenetic approach was used to rescue the locomotion defects. ChR2 has been used to excite DA neurons. In this study, we also examined the number of DA neurons in the brain from transgenic flies expressing α-Syn, EKO, or EKO+ChR2.

MATERIALS AND METHODS

Fly strains

Flies were grown in standard cornmeal/agar media with 0.4% propionic acid on a 12-hour light/dark cycle at 25°C. Fly strains used in this study were: wild-type (Canton-S), TH-Gal4 (a gift from Dr. J. Hirsh, University of Virginia), UAS-α-Syn (A53T); TH-GAL4 (TH-A53T, a gift from Dr. L. Pallanck, University of Washington), UAS-EKO (Bloomington *Drosophila* Stock Center), and UAS-ChR2 (a gift from Dr. B. Condron, University of Virginia). A standard UAS x GAL4 binary system [18] was used to drive expression of transgenes (e.g., UAS-EKO).

Locomotion test

Individual larvae (90~94 hours after egg-laying) were separated from the food using a 15% glucose solution and rinsed with distilled water. They were then placed on the surface of a plate of 2.5% agar mixed with 1mL India ink (to have a black background). The larvae were allowed to acclimate for 1 minute and a video was then recorded for 30 seconds at approximately 10 frames per second using a Moticam3 digital camera (Motic) and Motic Images Plus 2.0 software. The video was analyzed using the MTrack2 plug-in (from http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html) for ImageJ (from http://rsb.info.nih.gov/ij/). The path length was recorded; scores were quantified as the distance traveled per minute.

Whole brain staining

For staining the *Drosophila* larval brains, they were dissected using sharp forceps in ice cold dissecting saline solution. The brains were then fixed in 4% paraformaldehyde for 30 minutes on ice and then washed thoroughly with PBS. After 1 hour in blocking/permeabilization solution (2.5% normal goat serum and 0.15% Triton-X 100 in 1x PBS), brains were stained overnight at 4°C with a mouse monoclonal antibody to tyrosine hydroxylase (TH, 1:750) (Immunostar). They were then incubated for two hours in goat anti-mouse IgG labeled with Alexa Fluor 546 (diluted 1:750 in 1xPBS). Brains were mounted on 50×22 mm glass coverslips and images were taken on a Zeiss LSM510 confocal microscope. The total number of TH-positive cells in each fly larval brain was counted manually, as previously described [15, 19].

Optogenetics

Blue light (BL, 470 nm) for ChR2 activation was shed using a custom-built optogenetics rig [20]. A GW Laboratory DC power supply was connected to a 700 mA Buckpuck constant current generator (Luxdrive). The Buckpuck was connected via a switch to a blue LED (Luxdrive Endor Star). To prevent overheating, the LED was connected to a heat sink (Thorlabs). Larvae were exposed to blue light (BL; intensity, 1 mW/mm^2), either during testing or prior to testing. For optogenetic experiments, larvae were fed with food containing 1 mM all-trans-retinal (Sigma-Aldrich). Light intensity was measured using a Sanwa Mobiken laser power meter.

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RESULTS

Locomotion defects by reducing the excitability of dopaminergic (DA) neurons in Drosophila larval brain

It is known that overexpression of mutant α-Syn causes reduced release of dopamine (DA) in striatum [7, 8], and thus contributes to motor deficits in Parkinson's disease (PD). We proposed a hypothesis that reduced excitability of DA neurons may mimic α-Syn toxicity by reducing DA release. Therefore, we chose to utilize the electrical knock-out (EKO) transgene encoding a constitutively active K^+ channel [16] to suppress the excitability of *Drosophila* DA neurons. A standard UAS×Gal4 binary system (Fig. 1A) [18] was used to express EKO specifically in DA neurons [15, 21]. Subsequently, the locomotion was measured from the larvae expressing EKO in DA neurons (TH-EKO). The third instar TH-EKO larvae showed noticeably slow locomotion speed (Fig. 1) and also patterns similar to a fly larva expressing a mutant human PD gene α-Syn (TH-A53T) (refer to Fig. 1 in 15). The results showed that reduced DA release can be a key alteration underlying locomotion deficits in *Drosophila* larvae.

Number of DA neurons is reduced by EKO gene

In addition to locomotion deficits, the loss of DA neurons is a key cellular hallmark of PD. Therefore, we examined whether reduced locomotion in TH-EKO is associated with reduction of tyrosine hydroxylase (TH) expression using whole-brain mounting technique [15, 19]. TH is a key enzyme in DA synthesis and thus widely used as a DA marker. Our quantification of DA neurons

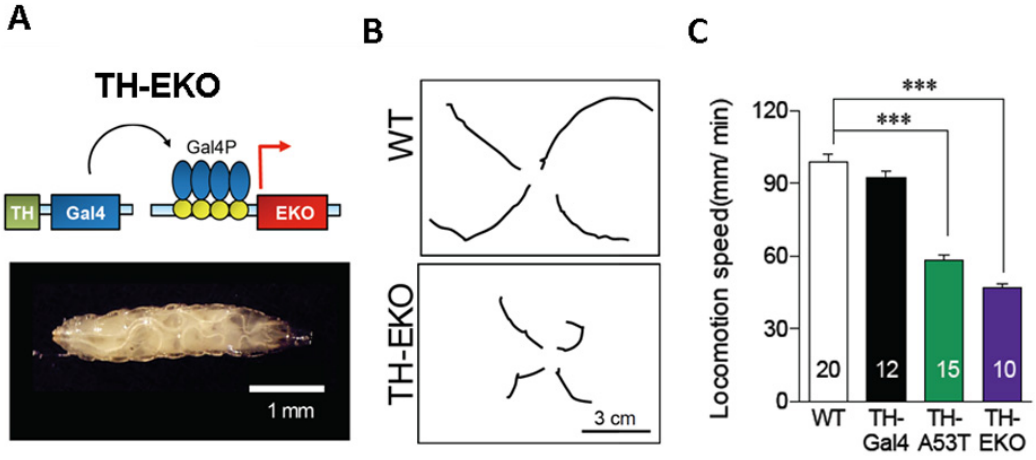

Fig. 1. Locomotion defects by reducing the excitability of dopaminergic (DA) neurons in *Drosophila* larval brain. (A) A diagram to illustrate the UAS×Gal4 binary system for the expression of EKO in DA neurons. DA-specific driver TH-Gal4 encodes a transcription factor protein (Gal4P), which binds to upstream activating sequence (UAS) and then leads to EKO expression in DA neurons. (*bottom*) A representative image of a third instar *Drosophila* larva. (B) Examples of the larval crawling path for WT and TH-EKO larvae (four examples per each strain). Each path was recorded for 30 sec. Scale bar=3 cm. (C) Locomotion speed (mm/min) for various fly lines: WT and TH-Gal4 for control, TH-A53T for a PD gene α-Syn (TH-A53T), and TH-EKO. Number (n) of larvae tested in parenthesis. Mean ± SEM. ***p<0.001 (one-way ANOVA and Dunnett's multiple comparison test).

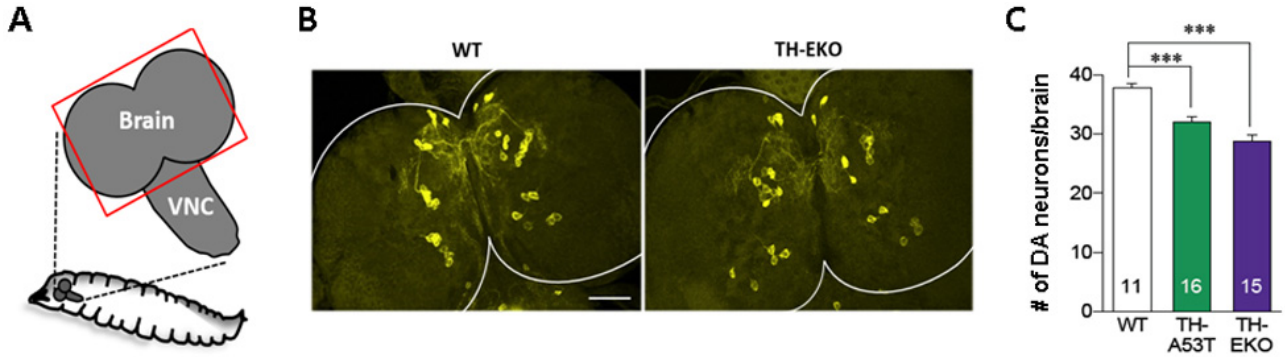

Fig. 2. Reduced number of DA neurons in *Drosophila* larval brain by a PD gene α-Syn and EKO. (A) A diagram showing *Drosophila* larval CNS including brain and ventral nerve cord (VNS). (B) Anti-TH(+) DA neurons in whole mount brains from WT & TH-EKO larvae. (C) Number of DA neurons in the whole brain from WT, TH-A53T and TH-EKO larvae. Mean±SEM. ***p<0.001 (one-way ANOVA and Dunnett's multiple comparison test).

showed that TH-EKO larvae displayed significantly reduced number of TH-positive DA neurons (Fig. 2). This reduction is slightly more severe in TH-EKO brains than TH-A53T. Along with locomotion deficits, these results clearly show that the reduced excitability in DA neurons causes PD symptoms mirroring effects of a mutant PD gene α-Syn (A53T).

Optogenetic rescue of locomotion defects in Drosophila larvae expressing a PD gene α-Syn

Our results suggest that both α-Syn and EKO impaired DA synaptic signaling and *Drosophila* larval locomotion by reducing DA release. In the following study, we wanted to test whether a reduced locomotion as well as a decreased TH expression level can be rescued by restoring DA neuronal excitability using an optogenetic approach. We prepared a crossed fly line expressing mutant human α-Syn (A53T) and channelrhodopsin (ChR2) in DA neurons (TH-ChR2-A53T or TH-Gal4; UAS-α-Syn(A53T)×UAS-ChR2). ChR2 is sensitive to blue light (BL, 470 nm) and thus DA neurons are expected to be excited when BL is on. Two different light patterns were used first: acute BL during the locomotion test and 1 hour BL before the test. The resulting locomotion score for the BL-stimulated TH-ChR2-A53T larvae was 74.2±5.6 mm/min, which was significantly greater than that without BL (~56 mm/min). Next, we wanted to determine whether longer BL exposure had any effect on the resulting larval locomotion. They were then exposed to BL for one hour prior to the test. TH-ChR2-A53T larvae with 1 hr BL showed a significant increase in locomotion, from 56.0±2.7 to 78.9±3.3 mm/min. When TH-ChR2-A53T larvae were exposed to 24 hr BL before the test, the locomotor activity (65.4±5.3 mm/min) was also rescued but 24 hr BL was not as effective as 1 hr BL, indicating certain aversive effects of longer stimulation of ChR2. In contrast, 12 hr BL exposure resulted in similar rescue of locomotion defects like 1 hr BL (data not shown). In these experiments, BL after 1 hr, 12 hr or 24 hr exposure was not given during test to eliminate a short-term effect of BL (Fig. 3B). The results clearly confirmed that ChR2 activation by BL is enough to rescue locomotion defects in PD. Further, longer activation (up to 12 hours) of ChR2 in DA neurons can rescue locomotion defects without possible detrimental effects.

Optogenetic activation of DA neurons restores motor function and TH expression in EKO larvae

In the following study, we wanted to test whether the rescue of EKO effects can restore a reduced locomotion as well as a decreased TH expression level. Larvae (TH-ChR2-EKO) were driven to express ChR2 (Pulver et al, 2011) specifically in DA neurons in addition to EKO. First, blue light (BL) was shed during the locomotion test in TH-ChR2-EKO. This acute BL led the rescue of

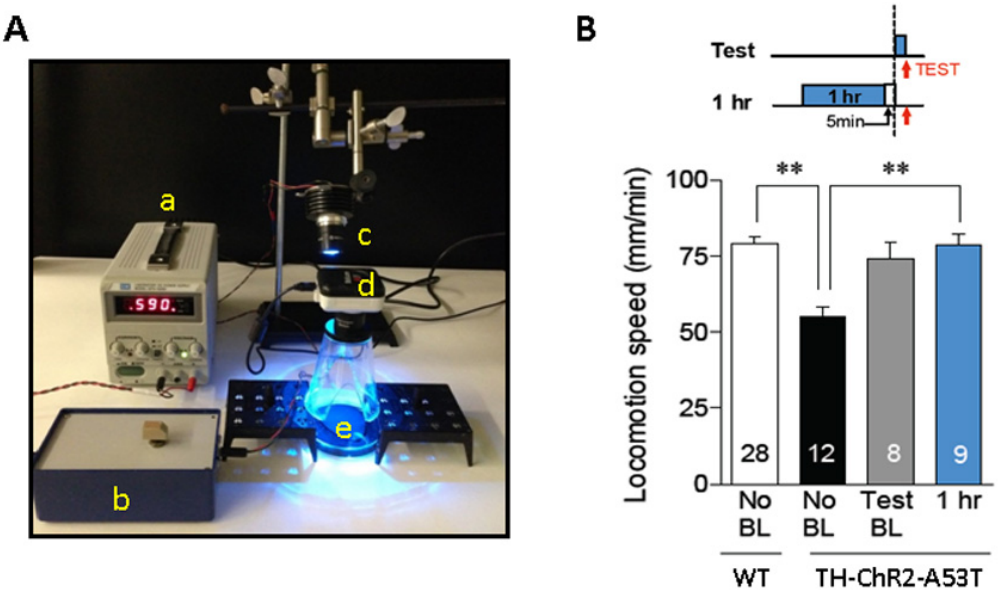

Fig. 3. Optogenetic rescue of locomotion defects by a PD gene α-Syn. (A) Optogenetic setup for the locomotion assay (also refer to Materials and Methods): (a) power supply, (b) multi-channel switch, (c) LED light, (d) moticam3 digital camera and (e) black agar plate for locomotion assay. (B) Locomotion speed of TH-ChR2-A53T larvae before and after optogenetic activation. (*upper panel*) Experimental protocol for optogenetic activation of DA neurons in TH-ChR2-A53T. Larvae were exposed to blue light (BL) during test for 1 min. Other group of larvae were exposed to BL prior to test, but not during test. (*bottom panel*) Locomotion speed in control (WT) and TH-ChR2-A53T with 3 different treatments (no BL, BL during test and 1 hour before test). Mean±SEM. **p<0.01 (one-way ANOVA and Dunnett's multiple comparison test).

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locomotion deficiency (Fig. 4), but it is likely due to enhanced DA synaptic release from existing DA presynaptic terminals, not by an increase of TH expression level. In the subsequent experiments, therefore, we looked for a long term effects of BL in order to examine TH expression. BL stimulation for 6 and 24 hours was given to TH-ChR2-EKO larvae. 6 hour stimulation was enough to rescue EKO effects but there was no rescue for 24 hour stimulation. We found that acute light stimulation during the locomotion test could restore locomotion partially (Fig. 4), whereas a long-term (6-hour) light stimulation before, but not during, locomotion test fully restored locomotion and significantly restored TH expression. These results are consistent with the idea that the reduced excitability of DA neurons causes impairment in both TH expression and motor behavior, and thus strongly reconfirm our hypothesis that reduced DA neuronal excitability can induce decrease in TH expression and locomotion defects.

DISCUSSION

Motor dysfunction in Parkinson's disease (PD) is primarily due to reduced synaptic DA release which is caused by neurodegeneration of DA neurons. A PD gene α-Syn is also known to decrease DA synaptic release [7, 8]. In this study, we demonstrated that reduced excitability of DA neurons by a genetically modified K^+ channel EKO [16] led to a decrease in TH signal and locomotion deficits, which mimics cellular and behavioral alterations in *Drosophila* larvae expressing a human PD gene α-Syn. Our findings strongly support that PD is a failure of DA synaptic transmission. It became possible to detect DA release from *Drosophila* larval CNS using carbon-fiber electrodes [22]. Therefore, it will be very interesting to examine actual DA synaptic release from EKO and α-Syn larvae.

Since its first use [23], optogenetic techniques have become a popular tool to precisely manipulate activities of certain neural circuits mediating normal or pathological behaviors by expressing light sensitive channels (e.g., ChR2). Indeed, it has been used to manipulate direct D1 or indirect D2 pathways in rodent basal ganglia [24]. They showed that changes in DA circuit activity can cause or rescue PD-like symptoms. Excitation of indirect D2 pathway induced a parkinsonian state while PD symptoms in 6-OHDA-treated mice were rescued by optogenetic activation of direct D1 pathway. Although *Drosophila* DA circuits mediating locomotion are anatomically not the same, acute and prolonged exposure to blue light (BL) was sufficient to activate ChR2, and thus rescue PD symptoms by α-Syn. We believe it is for the first time to confirm that locomotion defects by a genetic PD factor such as α-Syn can be rescued by increasing DA neuronal excitability with ChR2. Both acute (1 min during test) and long exposure to BL (1~12 hours) rescued locomotion defects. However, it seems there were adverse effects (e.g., Ca2+ overload, excitotoxicity) with 24 hour exposure to BL. It will be an interesting future experiment to examine various light patterns (e.g., 1~12 hours with for a 'on' for 30 min BL and subsequent 'off' for 30 min to minimize ChR2 desensitization), which would reveal a better recue effects of PD symptoms with an optimal light pattern and help to develop better optogenetic strategies for PD cure.

Drosophila larvae expressing EKO in DA neurons caused locomotion defects and reduced TH expression. The latter finding is

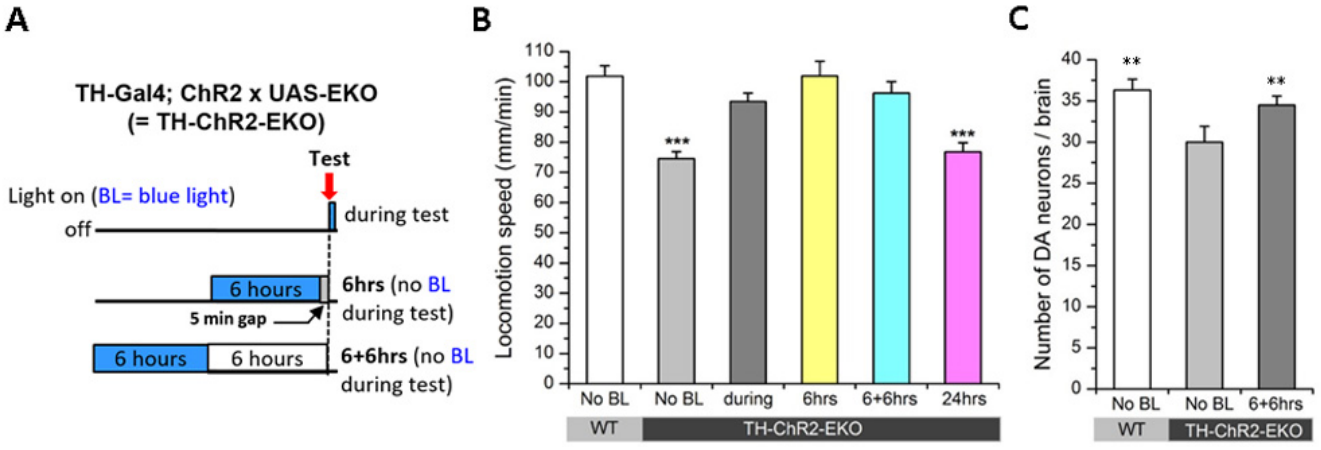

Fig. 4. Optogenetic rescue of locomotion defects and DA neurodegeneration by EKO. (A) Experimental protocol of optogenetic activation of TH-positive neurons of TH-ChR2-EKO. (B) Locomotion speed of TH-ChR2-EKO *Drosophila* larvae before and after optogenetic activation. Mean±SEM. ***p<0.001 (one-way ANOVA and Dunnett's multiple comparison test). (C) TH-positive cell number of TH-ChR2-EKO *Drosophila* larvae before and after optogenetic activation. Mean±SEM. **p<0.01 (one-way ANOVA and Dunnett's multiple comparison test).

consistent to other studies showing that number of TH-positive DA neurons is decreased, causing neurodegeneration when their excitability is reduced [32, 33]. Our results also showed that increased DA neuronal excitability by ChR2 rescues these EKO-induced symptoms, which confirms the notion that depolarizing stimuli applied to DA neuron is neuroprotective [33]. The results strongly support that DA excitability and synaptic release are key functional processes underlying health of DA neurons and larval locomotion. Further, we observed that number of TH-positive neurons was increased after 6 hour exposure to BL, indicating enhanced TH expression. Our result is consistent with previous results showing that TH expression is increased with higher neuronal excitability [25]. Fossom et al [26] showed that TH mRNA can be increased 2~5 fold in 20 min after cAMP stimulation. Therefore, it seems 6 hour BL is enough to increase TH enzyme expression and thus an increase of DA synaptic release.

Traditionally, TH-negative antibody signal is interpreted as death of DA neurons in PD models. However, some parkinsonian symptoms are known to be reversible [27, 28], suggesting that reduced DA synaptic release, but not DA neuronal loss. Our TH-EKO-ChR2 results also suggest that at least a subpopulation of DA neurons are TH-negative but still alive because DA neuronal number was increased just after 6 hour BL exposure in larvae expressing both EKO and ChR2 genes. It will be interesting to examine whether there is a reduction in other genes specifically or preferentially expressed in DA neurons such as DA transporter (DAT), vesicular monoamine transporter 2 (VMAT2) or DOPA decarboxylase (DDC) gene. DAT expression altered by α-Syn is currently controversial [7, 29, 30]. In addition, VMAT2 function can be interfered by α-Syn [31]. Therefore, DDC will be a useful marker to examine whether DDC(+)/TH(-) DA neurons are found in α-Syn or EKO larval brain, and thus these neurons can increase TH expression by ChR2 activation. By identifying DDC(+)/TH(-) neurons will provide new targets of PD interventions to restore DA synaptic release.

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