

# Optogenetic Activation of Adenosine A<sub>2A</sub> Receptor Signaling in the Dorsomedial Striatopallidal Neurons Suppresses Goal-Directed Behavior

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The striatum has an essential role in neural control of instrumental behaviors by reinforcement learning. Adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) are highly enriched in the striatopallidal neurons and are implicated in instrumental behavior control. However, the temporal importance of the A<sub>2A</sub>R signaling in relation to the reward and specific contributions of the striatopallidal A<sub>2A</sub>Rs in the dorsolateral striatum (DLS) and the dorsomedial striatum (DMS) to the control of instrumental learning are not defined. Here, we addressed temporal relationship and sufficiency of transient activation of optoA<sub>2A</sub>R signaling precisely at the time of the reward to the control of instrumental learning, using our newly developed *rhodopsin-A<sub>2A</sub>R chimeras* (optoA<sub>2A</sub>R). We demonstrated that transient light activation of optoA<sub>2A</sub>R signaling in the striatopallidal neurons in 'time-locked' manner with the reward delivery (but not random optoA<sub>2A</sub>R activation) was sufficient to change the animal's sensitivity to outcome devaluation without affecting the acquisition or extinction phases of instrumental learning. We further demonstrated that optogenetic activation of striatopallidal A<sub>2A</sub>R signaling in the DMS suppressed goal-directed behaviors, as focally genetic knockdown of striatopallidal A<sub>2A</sub>Rs in the DMS enhanced goal-directed behavior by the devaluation test. By contrast, optogenetic activation or focal AAV-Cre-mediated knockdown of striatopallidal A<sub>2A</sub>R in the DLS had relatively limited effects on instrumental learning. Thus, the striatopallidal A<sub>2A</sub>R signaling in the DMS exerts inhibitory and predominant control of goal-directed behavior by acting precisely at the time of reward, and may represent a therapeutic target to reverse abnormal habit formation that is associated with compulsive obsessive disorder and drug addiction.

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## INTRODUCTION

The striatum has an essential role in neuronal control of the balance between flexible, goal-directed actions and repetitive, habitual behaviors to achieve optimal performance of task (Brown Gould and Graybiel, 2010; Yin and Knowlton, 2006). The striatum is distinguished into the dorsomedial striatum (DMS), which mediates the acquisition and expression of goal-directed behavior through action-outcome learning, and the dorsolateral striatum (DLS), which mediates habit formation through stimulus-response learning (Brown Gould and Graybiel, 2010; Yin and Knowlton, 2006). The shift between goal-directed and habitual actions is associated

with changes in neural substrates from DMS to DLS (Yin and Knowlton, 2006) and critically involves the orbitofrontal and striatal circuits (Burguiere *et al*, 2013; Gremel and Costa, 2013). Dysfunction in normal shift between goal-directed and habit actions may contribute to obsessive compulsive disorder (Gillan *et al*, 2011), relapse of drug addiction (Ostlund and Balleine, 2008), habit learning deficit in Parkinson's patients (Knowlton *et al*, 1996), and preservative behaviors of Huntington's disease (Lawrence *et al*, 1998; Redgrave *et al*, 2010). Striatal control of instrumental learning involves critical functions of striatal dopamine and glutamate signaling (Lovinger, 2010; Yin *et al*, 2008): the nigrostriatal dopaminergic pathway provides a 'prediction error' signal for instrumental learning through reinforcement (Rossi *et al*, 2013; Steinberg *et al*, 2013); the activation of glutamatergic corticostriatal pathway is critical to the 'gain' control of cortical incoming information for action-outcome learning (Histed *et al*, 2009; Reynolds *et al*, 2001).

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The adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) are highly enriched in the postsynaptic striatopallidal neurons (Svenningsson *et al*, 1999) where A<sub>2A</sub>Rs interact with dopamine D<sub>2</sub> receptors (D<sub>2</sub>Rs) (Canals *et al*, 2003) and NMDA receptors (Higley and Sabatini, 2010), as well as metabotropic glutamate 5 receptors (Ferre *et al*, 2002). Thus, striatopallidal A<sub>2A</sub>Rs can integrate incoming information (glutamate) and neuronal sensitivity to this incoming information (dopamine) to control striatal synaptic plasticity and cognitions including goal-directed and habit behaviors (Chen, 2014). Indeed, genetic inactivation of striatal A<sub>2A</sub>Rs impairs habit formation (Yu *et al*, 2009) and pharmacological reduction of A<sub>2A</sub>R-mediated cAMP-pCREB signaling in the DMS enhances goal-directed ethanol drinking (Nam *et al*, 2013). However, the contributions of the striatopallidal A<sub>2A</sub>Rs in the DLS and DMS, two heterogeneous subregions underlying distinct DLS-related habitual or DMS-related goal-directed behavior, to the control of instrumental behavior are not defined.

Furthermore, the reward-based learning mechanism predicts that concurrent activation of the striatal neurons and reward-associated dopaminergic neuron activity is critical to reinforcement learning (Reynolds *et al*, 2001; Schultz *et al*, 1997). However, whether the transient activation of the striatopallidal A<sub>2A</sub>R signaling precisely at the time of reward is required or sufficient to modify instrumental learning is not known, largely because of the lack of methods to control A<sub>2A</sub>R signaling in intact animals with required spatiotemporal resolution. To overcome this limitation, we have developed chimeric rhodopsin-A<sub>2A</sub>R proteins (optoA<sub>2A</sub>R) by fusing the extracellular and transmembrane domains of rhodopsin with the intracellular loops of the A<sub>2A</sub>R (Li *et al*, 2015). We leveraged the spatiotemporal resolution of optoA<sub>2A</sub>R to activate striatopallidal A<sub>2A</sub>R signaling in a 'time-locked' manner precisely at the time of the reward. Coupling the optoA<sub>2A</sub>R approach with a satiety-based instrumental learning procedure (Derusso *et al*, 2010), we defined the contribution of striatopallidal A<sub>2A</sub>R signaling in the DMS and DLS, precisely at or randomly in relation to the time of the reward, to the control of goal-directed and habitual behaviors. We further validated the striatopallidal A<sub>2A</sub>R control of instrumental learning by focal knockdown of striatopallidal A<sub>2A</sub>Rs in the DMS and DLS using the AAV-Cre/flox strategy.

## MATERIALS AND METHODS

### Development of OptoA<sub>2A</sub>R Strategy

We have developed a optoA<sub>2A</sub>R, which retains the extracellular and transmembrane domains of rhodopsin (conferring light responsiveness), fused with the intracellular loops of A<sub>2A</sub>R (conferring specific A<sub>2A</sub>R signaling), as we described recently (Li *et al*, 2015). The specificity of the optoA<sub>2A</sub>R signaling was confirmed by light-induced selective enhancement of cAMP and phospho-MAPK levels, by the disappearance of light-induced optoA<sub>2A</sub>R signaling with a point mutation at the C-terminal region of A<sub>2A</sub>R, and by the demonstration that optoA<sub>2A</sub>R activation produced similar activation of signaling, synaptic plasticity, and behavioral responses in intact animals as the A<sub>2A</sub>R agonist CGS21680 (Li *et al*, 2015). We have constructed viral vectors for

optoA<sub>2A</sub>R (AAV5-EF1 $\alpha$ -DIO-mCherry-optoA<sub>2A</sub>R) and its control (AAV5-EF1 $\alpha$ -DIO-mCherry) using a double-floxed inverted (DIO) strategy to target mCherry-optoA<sub>2A</sub>R fusions in Cre-expressing striatopallidal neurons. The AAV5-EF1 $\alpha$ -DIO-mCherry-optoA<sub>2A</sub>R or AAV5-EF1 $\alpha$ -DIO-mCherry was injected to *adora2a*-cre mice (MMRRC: 031168-UCD) in which the expression of Cre recombinase under the control of A<sub>2A</sub>R gene regulatory elements was restricted to the striatopallidal neurons (but not cholinergic interneurons or the cortical-striatal projection neurons) (Durieux *et al*, 2009).

### Stereotaxic AAV Injection, Optic Fiber Implantation, and Optogenetic Activation of OptoA<sub>2A</sub>R Signaling

For optoA<sub>2A</sub>R stimulation experiment, AAV5-EF1 $\alpha$ -DIO-mCherry-optoA<sub>2A</sub>R or AAV5-EF1 $\alpha$ -DIO-mCherry (200 nl per striatum) was injected to the DMS (AP, 0.98 mm; ML, 1.20 mm; DV, 2.50 mm) or DLS (AP, 0.98 mm; ML, 2.20 mm; DV, 2.60 mm) of *adora2a*-cre mice unilaterally. Optic fiber with 200  $\mu$ m diameter was implanted into relevant brain tissue 0.5 mm above the virus injection site. The mice were maintained for 3 weeks to achieve sufficient virus expression before behavioral training.

Optogenetic stimulation of optoA<sub>2A</sub>R signaling was achieved by turning on light (473 nm, 10 mW power at the tip) for 2 s per reward (within average 30 or 60 s interval per reward session). To achieve 'time-locked' activation of optoA<sub>2A</sub>R for 2 s precisely at the time of reward delivery, we programmed optical stimulation to be activated each time contingent on the mouse *active* lever pressing and delivery of sucrose reward (Figure 2b). 'Random' light stimulation was programmed to randomly deliver light in relation to the reward (ie anytime within the interval periods between every two rewards) with same light stimulation parameters as 'time-locked' stimulation (Figure 2b). Light stimulation manipulations were conducted only during random interval (RI) training sessions (Figures 2c, e and 3b).

### The Cre-Flox-Mediated Conditional A<sub>2A</sub>R-Knockdown Strategy

Conditional knockdown of the A<sub>2A</sub>R gene was achieved by injecting Cre recombinase-expressing AAV into distinct striatal subregions of the A<sub>2A</sub>R-floxed (A<sub>2A</sub>R<sup>flox/flox</sup>) mice with the exon 2 of the A<sub>2A</sub>R gene being flanked by insertion of flox sequences, as we described recently (Lazarus *et al*, 2011). Specifically, AAV8-Cre-zsGreen (200 nl per striatum) was injected into the DMS and DLS of wild-type (WT, A<sub>2A</sub>R<sup>+/+</sup>) and the floxed (A<sub>2A</sub>R<sup>flox/flox</sup>) mice bilaterally.

### Satiety-Based Instrumental Training

**Training session (CRF  $\rightarrow$  RI30  $\rightarrow$  RI60).** Mice were subjected to satiety-based instrumental learning paradigm as we described previously (Yu *et al*, 2009). In brief, mice underwent 3 or 4 days of continuous reinforcement (CRF) training, followed by RI schedule, which promoted habitual behavior: mice were trained 2 days on RI 30 s schedule, followed by 4 days on the RI 60 s schedule (with a 0.1 probability of reward availability every 3 s (RI30) or 6 s (RI60) contingent upon lever pressing).

**Devaluation test.** Following the training sessions, a 2-day devaluation test was conducted. A specific satiety procedure was applied to alter the current value of a specific reward. On each day, the mice were allowed to have free access to home chows (at least 0.5 g per mouse) or sucrose solution (at least 1 ml per mouse) for at least an hour to achieve sensory-specific satiety. Immediately after the unlimited prefeeding session, mice were given a 5-min extinction test during which the lever was inserted and pressing times was recorded without reward delivery. For each mouse, lever press rate during the devaluation test was normalized to the lever press rate during the last day of RI60 training session before the devaluation test.

### Immunofluorescence

Immunofluorescence was performed on free-floating sections (30  $\mu$ m) using the procedure as we described recently (Augusto *et al*, 2013; Shen *et al*, 2013). Primary antibodies were incubated following the manufacturer's protocols: A<sub>2A</sub>R (Santa Cruz; 1:100), p-MAPK (Cell Signal; 1:200), mCherry (Clontech; 1:500), enkephalin (Abcam; 1:500), and substance-P (Abcam; 1:500). Sections were then rinsed and incubated with Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen; 1:1000). Slices were washed and mounted and images were acquired and quantified as mean integrated optical density using Image Pro Plus.

### Statistical Analysis

Acquisition data were analyzed using two-way ANOVA for repeated measurements with training sessions as within-subjects effect and optoA<sub>2A</sub>R stimulation types or conditional knockdown genotypes as between-subjects effect. For the devaluation test, we performed two-way ANOVA for repeated-measures with optogenetic stimulation types or A<sub>2A</sub>R conditional knockdown genotypes as one factor and outcome devaluation as another factor. This was followed by simple main-effect analyses to determine the within-subject effect of devaluation test in each group. In addition, as per the experimental design, we also performed planned comparisons within each group between the devalued and valued conditions using a paired *t*-test.

## RESULTS

### Targeted Expression of OptoA<sub>2A</sub>R and MAPK Signaling by OptoA<sub>2A</sub>R Activation in the Striatopallidal Neurons

Two weeks after the injection of AAV5-EF1 $\alpha$ -DIO-mCherry-optoA<sub>2A</sub>R and its control vector into the striatum of the adora2a-Cre mice (Figure 1a), we verified the selective expression of optoA<sub>2A</sub>R in the striatopallidal neurons. Quantitative analysis of double immunofluorescence staining result indicated that 88% of mCherry (optoA<sub>2A</sub>R-mCherry)-positive cells were colocalized with enkephalin (a marker for the striatopallidal neurons), whereas only 17% mCherry-positive cells were colocalized with substance-P (a marker for the striatonigral neurons) in the striatum (Figure 1b). Representative double-immunofluorescence staining images illustrated the colocalization of optoA<sub>2A</sub>R-mCherry with

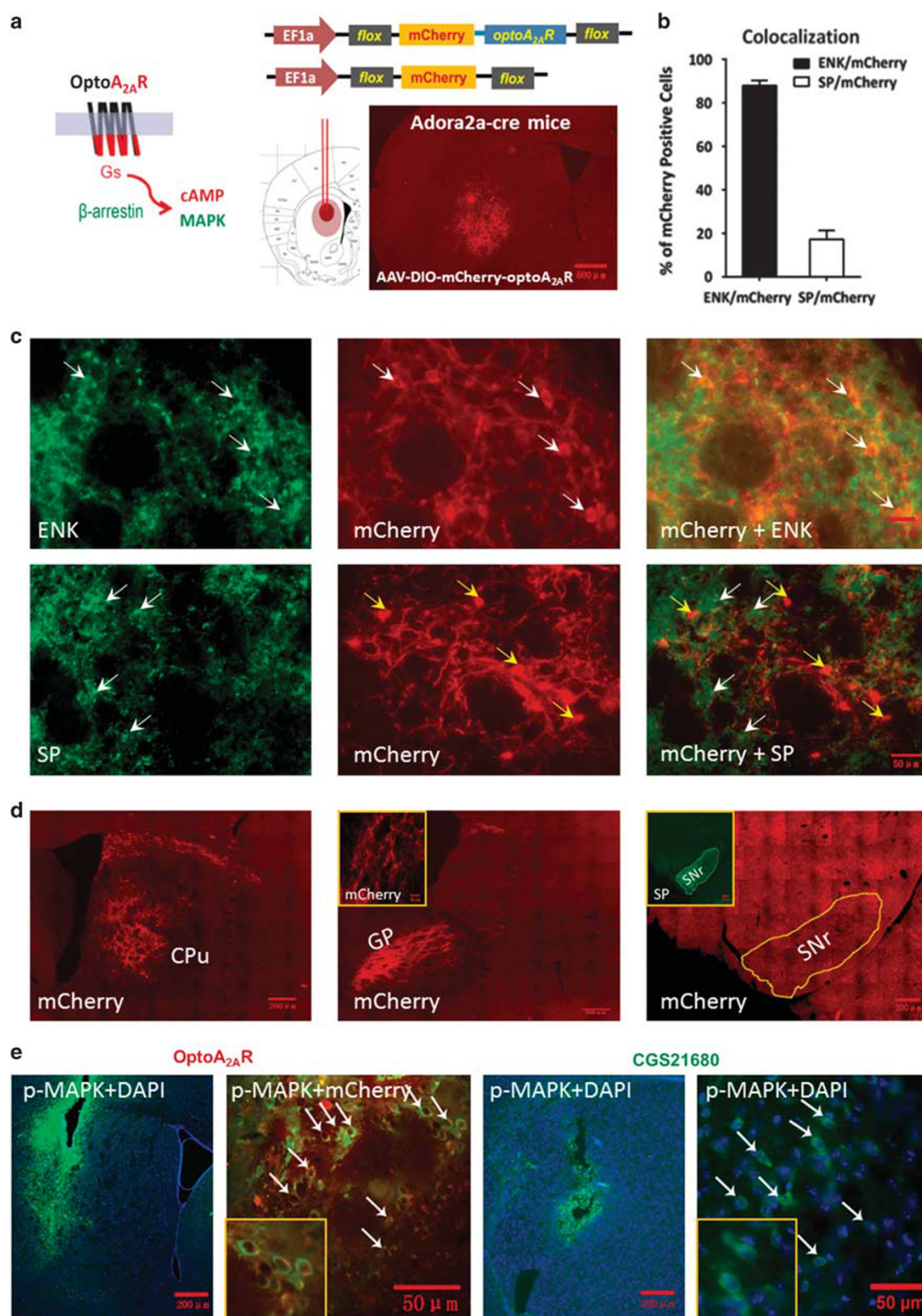
enkephalin but not substance-P (Figure 1c). Furthermore, the red (mCherry) fluorescence was specifically expressed in the terminals of the striatopallidal neurons in the globus pallidus, but was absent in the terminals of striatonigral neurons in the substantia nigra pars reticulata where substance P are highly expressed (Figure 1d). These results confirmed the selective expression of optoA<sub>2A</sub>R in the striatopallidal neurons. Moreover, optoA<sub>2A</sub>R stimulation in the striatum for 5 min induced p-MAPK in the mCherry-positive cells underneath the optic fiber (Figure 1e) in a similar pattern as the A<sub>2A</sub>R agonist CGS21680. Quantified analysis showed that light-induced p-MAPK activation was detected in 57% mCherry-optoA<sub>2A</sub>R-positive cells ( $n=1218$  from 4 mice). Thus, optoA<sub>2A</sub>R and CGS21680 produced indistinguishable p-MAPK signaling in the striatum.

### Optogenetic Activation of Striatopallidal A<sub>2A</sub>R Signaling in the DMS, Precisely at (but not Randomly in Relation to) the Time of the Reward, Suppressed Goal-Directed Behavior

To determine the effect of optoA<sub>2A</sub>R signaling in the DMS and DLS on goal-directed and habitual actions using a satiety-based instrumental learning paradigm, we first performed an devaluation time-course study to select specific RI training schedule that were most likely sensitive to bidirectional manipulation of the A<sub>2A</sub>R activity in the DMS and DLS. Devaluation test revealed that after the CFR  $\rightarrow$  RI30  $\rightarrow$  RI60 training, mice showed a clear goal-directed behavior on the 3rd day, developed habitual behavior on the 4th day, and became a stable habitual behavior on the 5th day after RI60 training (Supplementary Figure 1). Since the mice on the 4th day of RI60 schedule were at the transition period from goal-directed to habitual behavior and were most sensitive to bidirectional manipulation of A<sub>2A</sub>Rs in the DMS and DLS, we used the RI60 training for 4 days for the rest of the experiments.

We verified that the locations of the optical fiber implantation sites and expression of optoA<sub>2A</sub>R were restricted to the DMS by immunofluorescence (Figure 2a). At the RI sessions, we used the 'time-locked' method to deliver optoA<sub>2A</sub>R stimulation (for 2 s per reward) precisely at the time of reward delivery (Figure 2b). Mice with 'light off' serviced as controls. All mice gradually increased their lever pressing rates to obtain reward and reached the lever pressing plateau at the second day of RI training. There was no main effect of optoA<sub>2A</sub>R stimulation ( $F_{1,14}=0.371$ ,  $p>0.05$ ) nor optoA<sub>2A</sub>R stimulation  $\times$  RI training course interaction effect ( $F_{5,70}=0.098$ ,  $p>0.05$ ) by repeated-measures ANOVA. Thus, optogenetic activation of the striatopallidal A<sub>2A</sub>R signaling in the DMS did neither impair lever pressing performance nor affect acquisition of instrumental learning (Figure 2c).

The devaluation test (Figure 2d) revealed that there was no normalized devaluation  $\times$  optoA<sub>2A</sub>R interaction effect ( $F_{1,14}=0.429$ ,  $p=0.523$ ) by repeated-measures ANOVA. However, preplanned *t*-test showed that the optoA<sub>2A</sub>R mice with 'light off' displayed a goal-directed behavior with sensitivity to devalued reward ( $t_{1,7}=6.861$ ,  $***p<0.001$ ,  $n=8$ ). The goal-directed behavior in the



'light-off' group probably reflects unstable (transient) nature of instrumental behavior for the 4-day RI60 training schedule and might be partially attributed to the relatively low level of lever pressing in this group (and the total

rewards received) when the optical fiber implanted in the DMS compared with other experimental groups. Importantly the optoA<sub>2A</sub>R with 'time-locked' stimulation during the RI sessions failed to show sensitivity to outcome devaluation

(preplanned *t*-test,  $t_{1,7}=0.709$ ,  $p>0.05$ ,  $n=8$ ), indicating that their responding was habitual.

To better define the temporal importance of optoA<sub>2A</sub>R signaling precisely at the time of reward and to exclude the nonspecific effect caused by light, we have performed behavioral analyses with separate set of four experimental groups: mice expressing mCherry with 'time-locked' light stimulation ( $n=7$ ), mice expressing optoA<sub>2A</sub>R with 'light off' ( $n=9$ ), mice expressing optoA<sub>2A</sub>R with 'time-locked' light stimulation ( $n=8$ ), and mice expressing optoA<sub>2A</sub>R with 'random' ( $n=8$ ) light stimulation. The light stimulation scheme was illustrated in Figure 2b. Consistent with the result in Figure 2c, there was neither between-subject effect ( $F_{3,28}=1.481$ ,  $p=0.241$ ) nor RI training sessions  $\times$  manipulation groups interaction effect ( $F_{15,140}=1.284$ ,  $p=0.220$ ) in the acquisition phase by repeated-measures ANOVA (Figure 2e). However, analyses of the devaluation test (Figure 2f) revealed that there was a significant effect of optogenetic manipulation  $\times$  (normalized) devaluation interaction effect (repeated-measures ANOVA,  $F_{3,28}=3.258$ ,  $p=0.036$ ). The simple main-effect analyses of the devaluation test, respectively, in each group confirmed that only mice with optoA<sub>2A</sub>R expression in the DMS and time-locked light stimulation performed habitually ( $F_{1,8}=7.141$ ,  $*p<0.05$  for light off and  $F_{1,7}=6.074$ ,  $*p<0.05$  for random stimulation groups,  $F_{1,6}=16.050$ ,  $**p<0.01$  for mCherry group). Taken together, statistical analyses of both sets of the experiments (Figure 2d by the preplanned *t*-test and Figure 2f by the repeated-measures ANOVA) support that optogenetic activation of striatopallidal A<sub>2A</sub>R signaling in the DMS modulated the mode of instrumental behaviors by acting precisely at the time of the reward.

### Optogenetic Activation of Striatopallidal A<sub>2A</sub>R Signaling in the DLS had Relatively Limited Effects on Habitual Formation

Next, we examined the effect of optoA<sub>2A</sub>R signaling in the DLS on instrumental behaviors. Similarly, we confirmed the optical fiber implantation sites and expression of optoA<sub>2A</sub>R to be restricted to DLS by immunofluorescence (Figure 3a). Following the RI training sessions, optoA<sub>2A</sub>R mice with 'light off' ( $n=10$ ) or with 'time-locked' stimulation ( $n=13$ ) gradually increased lever presses. There was no main effect of optoA<sub>2A</sub>R stimulation ( $F_{1,21}=0.156$ ,  $p>0.05$ ) and no interaction effect of training session  $\times$  optoA<sub>2A</sub>R stimulation

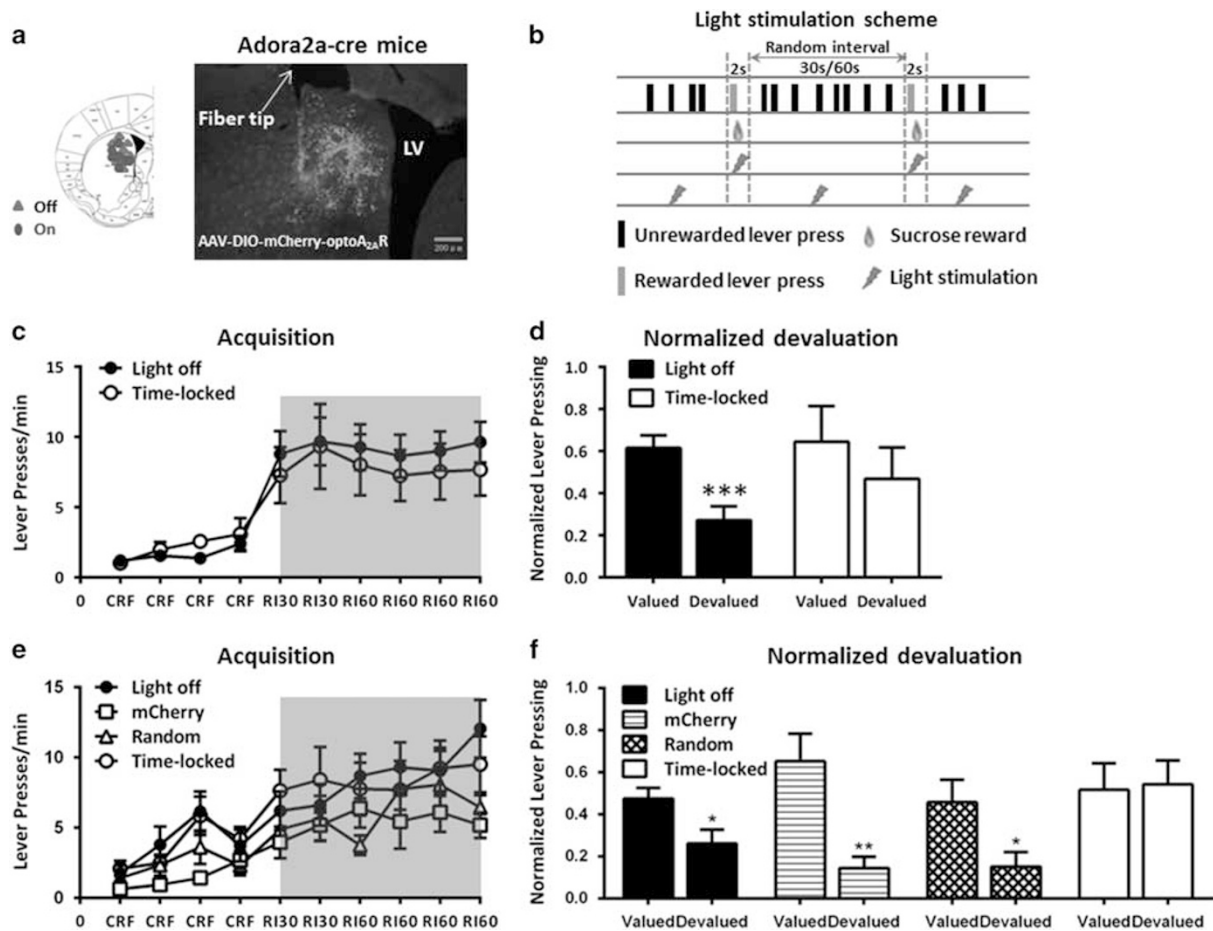
in the RI sessions ( $F_{5,105}=0.916$ ,  $p>0.05$ ) by repeated-measures ANOVA (Figure 3b). After the 4th day of RI60 training, repeated-measures ANOVA analyses of the devaluation test revealed that there was no optogenetic manipulations  $\times$  normalized devaluation interaction effect ( $F_{1,21}=0.022$ ,  $p=0.884$ ). However, the preplanned *t*-test showed that optoA<sub>2A</sub>R mice with 'time-locked' stimulation tended to perform goal-directed behavior (normalized devaluation test,  $t_{1,12}=3.725$ ,  $**p<0.01$  (Figure 3c); devaluation test,  $t_{1,12}=2.030$ ,  $p>0.05$  (Supplementary Figure 2c)). Conversely, optoA<sub>2A</sub>R mice with 'light off' displayed habitual behavior (normalized devaluation test,  $t_{1,9}=1.270$ ,  $p>0.05$  (Figure 3c); devaluation test,  $t_{1,9}=1.868$ ,  $p>0.05$  (Supplementary Figure 2c)). Thus, optogenetic activation of striatopallidal A<sub>2A</sub>R signaling in the DLS tended to promote goal-directed behavior, but its effect was relatively limited.

### Knockdown of A<sub>2A</sub>Rs in the DMS Enhanced Goal-Directed Behavior, Whereas Knockdown of the A<sub>2A</sub>Rs in the DLS had a Limited Effect on Habitual Behavior

We further evaluated the effects of focal knockdown of the A<sub>2A</sub>Rs in the DMS and DLS on instrumental learning. Figures 4a and 5a provided representative outline of the AAV transfection and A<sub>2A</sub>R focal knockdown areas of the DMS and DLS. Fluorescent images showed that A<sub>2A</sub>Rs expression (the red fluorescence) was reduced selectively in the Cre-expressing regions (indicated by green fluorescence). Quantitative analysis of the A<sub>2A</sub>R immunoreactivity (Figures 4b and 5b) confirmed selective knockdown of A<sub>2A</sub>Rs in the DMS (by 91%) and DLS (by 94%) after transfection with AAV-Cre-zsGreen only in A<sub>2A</sub>R<sup>lox/lox</sup> mice but not in WT mice (A<sub>2A</sub>R<sup>+/+</sup>).

Consistent with the optoA<sub>2A</sub>R results, focal knockdown of A<sub>2A</sub>Rs in the DMS (Figure 4c) and DLS (Figure 5c) did not affect the acquisition of instrumental learning as the A<sub>2A</sub>R<sup>lox/lox</sup> and WT mice transfected with AAV-Cre-zsGreen showed identical instrumental learning course at RI training session (DMS: genotype main effect,  $F_{1,13}<0.001$ ,  $p>0.05$ , RI period  $\times$  genotype interaction effect:  $F_{5,65}=0.859$ ,  $p>0.05$ ; DLS: genotype main effect,  $F_{1,11}=0.534$ ,  $p>0.05$ , RI period  $\times$  genotype interaction effect:  $F_{5,55}=1.234$ ,  $p>0.05$ ; by repeated-measures ANOVA). For the devaluation test, repeated-measures ANOVA analyses revealed that there was genotypes  $\times$  devaluation interaction effect in the DMS experiment (Figure 4d, normalized devaluation,  $F_{1,13}=9.161$ ,  $p=0.01$ , simple

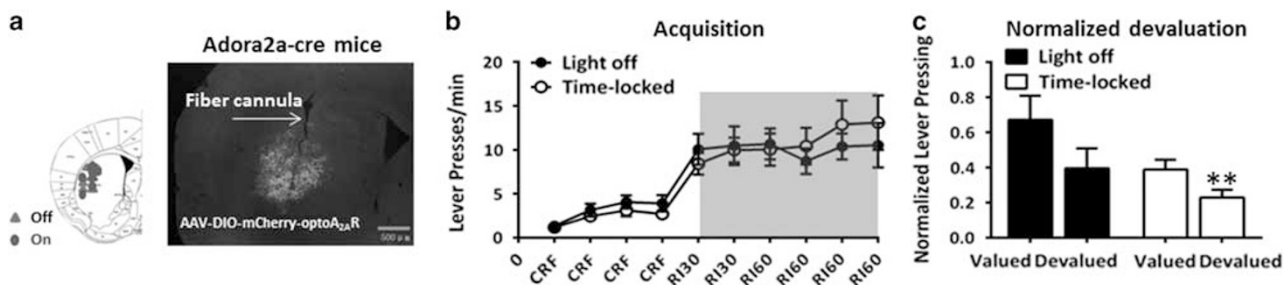
**Figure 1** Targeted expression and phospho-MAPK (p-MAPK) signaling of optoA<sub>2A</sub>R in striatopallidal neurons. (a) Schematic illustration of the optoA<sub>2A</sub>R chimera construction by replacing the intracellular loops 1, 2, and 3 and C terminal of the bovine rhodopsin with that of the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) to achieve control of A<sub>2A</sub>R signaling by 473 nm light (left panel). Representative fluorescent image shows the expression of mCherry-optoA<sub>2A</sub>R in the striatum after injection of AAV5-DIO-mCherry-optoA<sub>2A</sub>R to adora2a-cre mice for 2 weeks (right panel). (b) The quantitative data shows that 88% mCherry-positive cells ( $n=114$ , from four mice) were colocalized with enkephalin (ENK), whereas only 17% mCherry-positive cells ( $n=106$ , from four mice) were colocalized with substance P (SP). (c) Double immunostaining with the mCherry and the specific antibodies (ENK or SP) showed that optoA<sub>2A</sub>Rs were specifically expressed in ENK-positive striatopallidal neurons (white arrows, upper panels) but not SP-positive striatonigral neurons (yellow arrows, lower panels). (d) Following injection of AAV-DIO-mCherry-optoA<sub>2A</sub>R virus in the dorsomedial striatum (DMS) of adora2a-cre mice, the mCherry fluorescence of striatopallidal projection terminals was specifically expressed in the global pallidum (GP) but not in the substantia nigra pars reticulata (SNr). The green fluorescence of striatonigral projection terminals containing endogenous SP was specifically expressed in the SNr. (e) The expression of p-MAPK was induced by optoA<sub>2A</sub>R stimulation (white arrows, left panels) or CGS21680 injection (white arrows, right panels). Quantified analysis showed that light-induced p-MAPK activation was detected in 57% mCherry-optoA<sub>2A</sub>R-positive cells ( $n=1218$  from four mice).



**Figure 2** 'Time-locked' but not random optogenetic activation of striatopallidal adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) signaling in the dorsomedial striatum (DMS) suppresses goal-directed behavior. (a) Left panel: Schematic illustration of the locations of the fiber tips for each animal in the 'light-off' group (the red triangles) and 'time-locked' activation group (the blue circles). Right panel: Typical coronal section of mCherry-optoA<sub>2A</sub>R expression in the DMS of adora2a-cre(+) mice. The white arrow indicates the optical fiber tip. (b) Schematic illustration of timing of lever pressing, sucrose reward delivery, and optical stimulation. Light stimulation (the blue flash) was delivered to the DMS during a 2-s period in 'time-locked' manner with (the flashes between the two red dotted vertical lines) or in 'random' manner with (the flashes in the random interval periods) reward delivery (the liquid drops). (c) Two groups of mice expressing optoA<sub>2A</sub>R in the DMS were subjected to either 'time-locked' light stimulation or 'light off' ( $n = 8$  per group) during the random interval (RI) training session (as indicated by the blue bar). The two groups performed indistinguishably in the acquisition phase of instrumental learning by repeated-measures analysis of variance (ANOVA)—RI period  $\times$  optoA<sub>2A</sub>R stimulation interaction effect:  $F_{5,70} = 0.098$ ,  $p > 0.05$ ; optoA<sub>2A</sub>R stimulation main effect:  $F_{1,14} = 0.371$ ,  $p > 0.05$ . (d) Following the RI training sessions, a 2-day devaluation test without any experimental (optoA<sub>2A</sub>R activation) manipulation was conducted as described in the Materials and Methods section. Mice without optoA<sub>2A</sub>R activation during the RI training sessions significantly reduced their lever presses in devalued condition compared with valued condition (normalized devaluation:  $t_{1,7} = 6.861$ , \*\*\* $p < 0.001$ , preplanned  $t$ -test). By contrast, mice with optoA<sub>2A</sub>R 'time-locked' stimulation showed no significant devaluation effect (normalized devaluation:  $t_{1,7} = 0.709$ ,  $p > 0.05$ , preplanned  $t$ -test). However, there was no normalized devaluation  $\times$  optoA<sub>2A</sub>R interaction effect by repeated-measures ANOVA analysis ( $F_{1,14} = 0.429$ ,  $P = 0.523$ ). (e) We further performed instrumental behavioral analyses of a separate set of four experimental groups: mice expressing mCherry with 'time-locked' light stimulation ( $n = 7$ ), mice expressing optoA<sub>2A</sub>R with 'light off' ( $n = 9$ ), mice expressing optoA<sub>2A</sub>R with 'time-locked' light stimulation ( $n = 8$ ), and mice expressing optoA<sub>2A</sub>R with random light stimulation ( $n = 8$ ). Consistent with the result in (c) repeated-measures ANOVA analysis indicated that there was neither between-subject effect ( $F_{3,28} = 1.481$ ,  $p = 0.241$ ) nor RI training sessions  $\times$  manipulation groups interaction effect ( $F_{15,140} = 1.284$ ,  $p = 0.220$ ) in the acquisition phase. (f) Repeated-measures ANOVA analyses of the devaluation test revealed that there was significant effect of optogenetic manipulation  $\times$  (normalized) devaluation interaction effect:  $F_{3,28} = 3.258$ ,  $p = 0.036$ . Similarly, the simple main-effect analyses of the devaluation test in four groups indicated that only mice with optoA<sub>2A</sub>R expression in the DMS and time-locked light stimulation performed habitually, whereas other groups displayed goal-directed behavior (simple effect analyses:  $F_{1,8} = 7.141$ , \* $p < 0.05$  for 'light off' and  $F_{1,7} = 6.074$ , \* $p < 0.05$  for 'random' stimulation groups, and  $F_{1,6} = 16.050$ , \*\*\* $p < 0.01$  for mCherry group). Data are presented as the mean  $\pm$  SEM. The color reproduction of this figure is available on the *Neuropsychopharmacology* journal online.

main-effect analyses,  $F_{1,6} = 35.683$ , \*\* $p < 0.01$  for A<sub>2A</sub>R focal knockdown mice; Supplementary Figure 2d, devaluation,  $F_{1,13} = 10.231$ ,  $p = 0.007$ , simple main-effect analyses,  $F_{1,6} = 40.197$ , \*\* $p < 0.01$  for A<sub>2A</sub>R focal knockdown mice). This indicated that the control mice displayed a clear habitual action without sensitivity to devaluation condition, whereas focal A<sub>2A</sub>R knockdown in the DMS altered

sensitivity to devaluation by markedly reducing lever presses in the devalued condition. In contrast to the DMS A<sub>2A</sub>R-knockdown effect, focal knockdown of A<sub>2A</sub>R in the DLS did not affect instrumental behavior and showed no sensitivity to devaluation condition (Figure 5d: genotypes  $\times$  normalized devaluation interaction effect,  $F_{1,11} = 1.993$ ,  $p = 0.186$  by repeated-measures ANOVA, and  $t_{1,6} = 0.646$ ,



**Figure 3** Optogenetic activation of striatopallidal adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) signaling in the dorsolateral striatum (DLS) exerts relatively limited and possibly opposite control over habitual action compared with the optoA<sub>2A</sub>R in the dorsomedial striatum (DMS). (a) Left: Schematic illustration of the sites of optical fibers implantation. Right: A representative image of mCherry-optoA<sub>2A</sub>R expression and fiber implantation. (b) Mice were under continuous reinforcement (CRF) training followed by RI30 and then RI60 training with or without optoA<sub>2A</sub>R stimulation as described in the Materials and Methods section. The performances of optoA<sub>2A</sub>R mice with 'time-locked' stimulation ( $n = 13$ ) or with 'light off' ( $n = 10$ ) during the acquisition phase were indistinguishable (repeated-measures analysis of variance (ANOVA), random interval (RI) training course  $\times$  optogenetic stimulation interaction:  $F_{5,105} = 0.916$ ,  $p > 0.05$ ; optoA<sub>2A</sub>R stimulation main effect:  $F_{1,21} = 0.156$ ,  $p > 0.05$ ). (c) OptoA<sub>2A</sub>R mice with 'time-locked' stimulation or 'light off' during the RI training sessions were subjected to devaluation test as described in the Materials and Methods section. Repeated-measures ANOVA analyses revealed that there was no normalized devaluation  $\times$  optogenetic stimulation interaction effect ( $F_{1,21} = 0.022$ ,  $p = 0.884$ ). However, preplanned  $t$ -test analysis revealed that optoA<sub>2A</sub>R mice receiving 'time-locked' stimulation tended to perform goal-directed behavior (only for the normalized devaluation test:  $t_{1,12} = 3.725$ ,  $**p < 0.01$ ; but not for devaluation test:  $t_{1,12} = 2.030$ ,  $p > 0.05$ ; Supplementary Figure 2c). Whereas optoA<sub>2A</sub>R mice with 'light off' displayed habitual behavior (normalized devaluation test:  $t_{1,9} = 1.270$ ,  $p > 0.05$ ; devaluation test:  $t_{1,9} = 1.868$ ,  $p > 0.05$ ; Supplementary Figure 2c). Data are presented as the mean  $\pm$  SEM. The color reproduction of this figure is available on the *Neuropsychopharmacology* journal online.

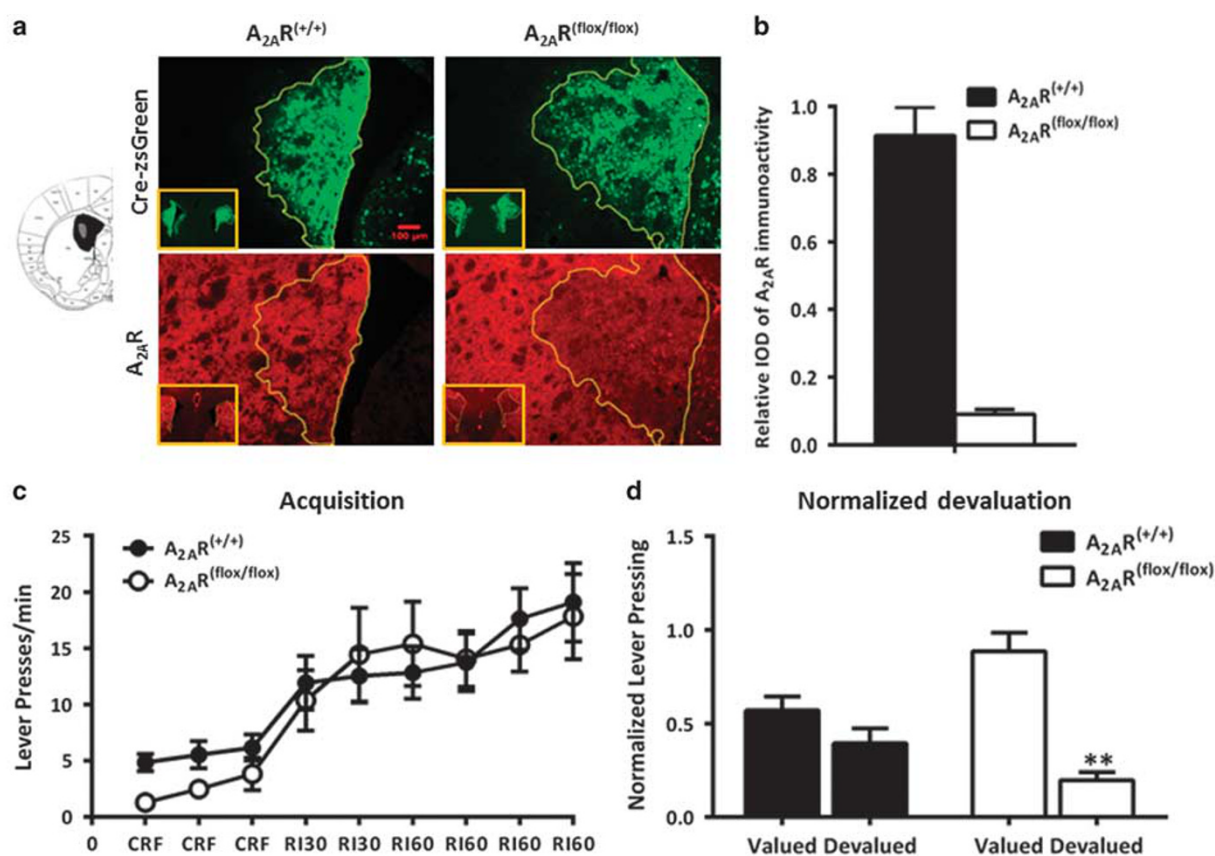
$p > 0.05$  for DLS A<sub>2A</sub>R-knockdown mice,  $t_{1,5} = 2.017$ ,  $p > 0.05$  for WT mice by preplanned  $t$ -test; the devaluation test showed a similar result; Supplementary Figure 2e). Thus, consistent with the results of the optoA<sub>2A</sub>R, these findings validate that focal knockdown of striatopallidal A<sub>2A</sub>Rs in the DMS selectively enhanced goal-directed behavior, whereas focal knockdown of striatopallidal A<sub>2A</sub>Rs in the DLS had little effect on habitual behavior.

## DISCUSSION

### Transient and 'Time-Locked' Activation of optoA<sub>2A</sub>R Signaling Precisely at the Time of Reward is Required and Sufficient to Modulate Goal-Directed Behavior

The contemporary theory of striatum-dependent learning postulates that the concurrent activation of presynaptic nigral-striatal dopamine (reinforcement) signaling and corticostriatal glutamate (sensorimotor) signaling and post-synaptic striatopallidal neuronal activity (modulated by neuromodulator such as adenosine) is critical to striatal synaptic plasticity and instrumental learning (Yagishita *et al*, 2014; Reynolds *et al*, 2001; Schultz *et al*, 1997). Indeed, modification of instrumental learning by optogenetic manipulation of striatal neurons was only effective in a narrow temporal window (ie before or concurrent with the onset of cue (Tai *et al*, 2012), or in the time segment (1.5 s) between action selection and outcome (Aquili *et al*, 2014)), supporting the temporal importance of dopamine, glutamate, and neuromodulator signaling in striatum-dependent instrumental learning. Different from rapid neurotransmitter release such as dopamine and glutamate, extracellular adenosine is generated by conversion of ATP to adenosine through a set of ectonucleotidases and by bidirectional nucleotide transporters (Chen *et al*, 2013). Striatopallidal A<sub>2A</sub>R activity may modulate instrumental learning by acting precisely at the time of the reward to integrate dopamine or glutamate signaling for coding the action-outcome contingency.

Alternatively, striatopallidal A<sub>2A</sub>Rs control instrumental learning by modulating the vigor of actions (Desmurget and Turner, 2010), by providing permissive role in learning association (Brainard and Doupe, 2000), or by modulating the 'off-line' processing of incoming signaling (glutamate) (Pomata *et al*, 2008). In these alternative schemes, the temporal relationship between striatopallidal activity (ie A<sub>2A</sub>R activity) and the reward is not essential. Thus, a critical question is whether the transient activation of A<sub>2A</sub>R precisely at the time of reward delivery was required and sufficient to modulate instrumental learning. This question has not been addressed owing to the lack of methods to control A<sub>2A</sub>R signaling in behaving animals with required temporal resolution. Our development of the optoA<sub>2A</sub>R (Li *et al*, 2015) offers the opportunity to optogenetically control the A<sub>2A</sub>R signaling with sufficient temporal resolution. We showed that transient (2 s per reward) and 'time-locked' light activation of the optoA<sub>2A</sub>R signaling in the striatopallidal neurons precisely at the time of the reward (but not random light stimulation) was required and sufficient to modify the sensitivity to outcome devaluation without affecting the acquisition. The requirement and sufficiency of 'time-locked' and transient activation of optoA<sub>2A</sub>R signaling at the time of the reward to modify instrumental learning demonstrated a temporally specific relationship between adenosine A<sub>2A</sub>R signaling and nigrostriatal dopamine signaling in association with the reward delivery and possibly corticostriatal glutamate signaling that converged on the striatopallidal neurons. Considering the extensive interaction between A<sub>2A</sub>Rs, D<sub>2</sub>Rs, and NMDA receptors in the striatopallidal neurons (Lovinger, 2010), we speculate that concurrent activation of A<sub>2A</sub>Rs, D<sub>2</sub>Rs, and NMDA receptors in the striatopallidal neurons allows the integration of adenosine, dopamine, and glutamate signaling, and coding of the mode of instrumental learning behavior (Abeliovich *et al*, 1992; Tai *et al*, 2012).

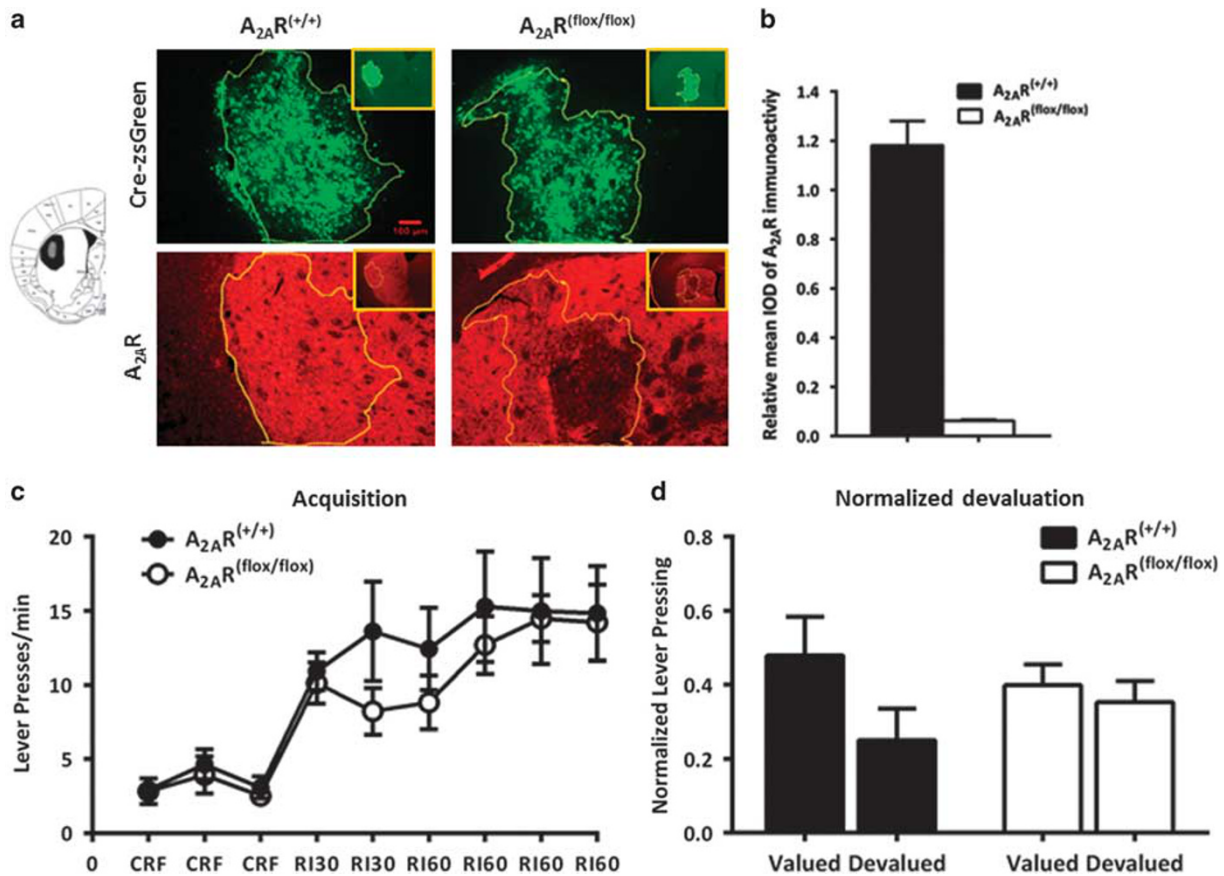


**Figure 4** Focal knockdown of adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) in the dorsomedial striatum (DMS) enhances goal-directed behavior. (a) Left: Schematic illustration of the maximal (black) and minimal (gray) A<sub>2A</sub>R knockdown areas in the DMS. Right: Representative immunofluorescent photomicrographs show focal knockdown expression of A<sub>2A</sub>Rs in the DMS after injection of AAV-Cre-zsGreen into the A<sub>2A</sub>R<sup>(flox/flox)</sup> (right panels) and A<sub>2A</sub>R<sup>(+/+)</sup> mice (left panels). Intensity of A<sub>2A</sub>Rs (red) were significantly decreased in the overlapping area with zsGreen expression (the yellow circle) in A<sub>2A</sub>R<sup>(flox/flox)</sup> mice but not in A<sub>2A</sub>R<sup>(+/+)</sup> mice. (b) Quantitative analysis showed that A<sub>2A</sub>R expression were markedly reduced in the virus-transfected regions of A<sub>2A</sub>R<sup>(flox/flox)</sup> mice compared with A<sub>2A</sub>R<sup>(+/+)</sup> mice. (c) Two–three weeks after bilateral injection of AAV-Cre-zsGreen into the DMS, A<sub>2A</sub>R<sup>(flox/flox)</sup> mice and A<sub>2A</sub>R<sup>(+/+)</sup> mice ( $n = 8$  per group) were under CRF–RI30–RI60 training paradigm as described in the Materials and Methods section. Both groups similarly increased their lever pressing rate during the acquisition phases (repeated-measures analysis of variance (ANOVA) revealed no random interval (RI) period  $\times$  genotype interaction effect:  $F_{5,65} = 0.859$ ,  $p > 0.05$ ; and no genotype main effect:  $F_{1,13} < 0.001$ ,  $p > 0.05$ ). (d) Mice with DMS A<sub>2A</sub>R knockdown significantly reduced their lever pressing in the devalued condition compared with that of the valued condition, but the A<sub>2A</sub>R<sup>(+/+)</sup> mice responded insensitively to the selective satiety devaluation treatment (normalized devaluation  $\times$  genotype interaction effect:  $F_{1,13} = 9.161$ ,  $p = 0.01$ ; simple effect analysis:  $F_{1,6} = 35.683$ ,  $**p < 0.01$  for A<sub>2A</sub>R focal knockdown mice by repeated-measures ANOVA). Data are presented as the mean  $\pm$  SEM. CRF, continuous reinforcement. The color reproduction of this figure is available on the *Neuropsychopharmacology* journal online.

### The Striatopallidal A<sub>2A</sub>R Signaling in the DMS Provides a ‘Break’ Mechanism to Constrain Instrumental Learning

As the DMS and DLS are distinctly involved in goal-directed and habitual behaviors, respectively (Balleine *et al*, 2009; Brown Gould and Graybiel, 2010; Yin and Knowlton, 2006), another important question is whether striatopallidal A<sub>2A</sub>Rs exert DMS- and DLS-specific control over instrumental learning. Our bidirectional manipulation of the striatopallidal A<sub>2A</sub>Rs by optogenetic activation of A<sub>2A</sub>R signaling and Cre-mediated knockdown of A<sub>2A</sub>Rs in the DMS and DLS demonstrated that A<sub>2A</sub>Rs in the DMS exerted an inhibitory and predominant control of goal-directed, whereas striatopallidal A<sub>2A</sub>Rs in the DLS had relatively limited but possibly opposite effects on habit formation. This is consistent with the associative corticostriatal–DMS loop being ‘default’ model of striatal function (Thorn *et al*, 2010) and with

previous finding that deletion of the indirect pathway in the DMS (but not DLS) produces pronounced psychomotor and cognitive effects (Durieux *et al*, 2012). This view is also supported by recent pharmacological study that reduction of A<sub>2A</sub>R-mediated PKA–pCREB signaling in the DMS enhanced acquisition of goal-directed ethanol drinking behaviors in mice (Nam *et al*, 2013). Given the prominent role of the DMS in control of goal-directed behavior, our finding that focal knockdown of striatopallidal A<sub>2A</sub>Rs in the DMS captures the goal-directed characteristics of striatum-specific A<sub>2A</sub>R knockout (KO) mice argue that striatum–A<sub>2A</sub>R KO mice displayed enhanced goal-directed behavior, but manifested as impaired habit formation (Yu *et al*, 2009). Although our analysis is designed to isolate the striatopallidal A<sub>2A</sub>R action from other action sites, this does not preclude the contribution of the A<sub>2A</sub>Rs in extrastriatal or cholinergic neurons to the control of instrumental learning, which needed to be further defined.



**Figure 5** Focal knockdown of the adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) in the dorsolateral striatum (DLS) exerts relatively limited effects on habitual behaviors. (a) Representative image shows that A<sub>2A</sub>Rs were knocked down selectively in the area with the AAV-Cre-zsGreen expression in the DLS of A<sub>2A</sub>R<sup>(flox/flox)</sup> mice but not in A<sub>2A</sub>R<sup>(+/+)</sup> mice. The yellow circle depicted the boundary of the AAV-Cre-zsGreen expression and A<sub>2A</sub>R knockdown area. (b) Quantitative analysis shows that A<sub>2A</sub>R expression was markedly reduced in the AAV-Cre-zsGreen transfected regions of A<sub>2A</sub>R<sup>(flox/flox)</sup> mice compared with A<sub>2A</sub>R<sup>(+/+)</sup> mice. (c) Focal A<sub>2A</sub>R knockdown in the DLS ( $n=7$ ) did not affect lever pressing during the acquisition phase compared with their A<sub>2A</sub>R<sup>(+/+)</sup> controls ( $n=6$ ) (repeated-measures analysis of variance (ANOVA) revealed no random interval (RI) period  $\times$  genotype interaction effect:  $F_{5,55}=1.234$ ,  $p>0.05$ ; and no genotype main effect:  $F_{1,11}=0.534$ ,  $p>0.05$ ). (d) There was no genotype  $\times$  devaluation interaction effect ( $F_{1,11}=1.993$ ,  $p=0.186$ , repeated-measures ANOVA) for the normalized devaluation test. Both groups similarly showed insensitivity to outcome devaluation (DLS A<sub>2A</sub>R knockdown mice: normalized devaluation;  $t_{1,6}=0.646$ ,  $p>0.05$ ; wild-type (WT) mice: normalized devaluation;  $t_{1,5}=2.017$ ,  $p>0.05$ ). Data are presented as the mean  $\pm$  SEM. CRF, continuous reinforcement.

It is worth noting that similar to striatal A<sub>2A</sub>R KO (Yu *et al*, 2009), either optoA<sub>2A</sub>R activation or focal A<sub>2A</sub>R knockdown of striatopallidal A<sub>2A</sub>R activity did not affect the acquisition (Figures 2c, 3b, 4c and 5c) or omission/extinction (Supplementary Figure 3) phase of instrumental learning, but specifically affect sensitivity to outcome devaluation. The lack of the optoA<sub>2A</sub>R effect during the acquisition and extinction/omission phases indicates that striatopallidal A<sub>2A</sub>Rs unlikely affect general arousal status or attention to influence instrumental learning, but instead it may modify the motivation control of action selection. This notion is consistent with the critical role of striatopallidal A<sub>2A</sub>Rs in the modulation of effort expenditure and motivation (Mingote *et al*, 2008; Nunes *et al*, 2013).

Lastly, bidirectional manipulation of the striatopallidal A<sub>2A</sub>Rs by optoA<sub>2A</sub>R and Cre-mediated A<sub>2A</sub>R knockdown demonstrates a critical role of the postsynaptic striatopallidal A<sub>2A</sub>Rs and the striatopallidal pathway in the DMS in control of instrumental learning. This collaborates with the recent

finding that transient optogenetic stimulation of striatopallidal neurons introduces opposing biases during decision making in mice (Tai *et al*, 2012), and that loss of striatal long-term depression largely restricted to striatopallidal neurons is associated with a shift in behavioral control from goal-directed action to habitual responding (Nazzaro *et al*, 2012). Taken together with increasing evidences from diverse learning paradigms that striatopallidal A<sub>2A</sub>Rs assume an inhibitory control over working memory (Wei *et al*, 2011; Zhou *et al*, 2009), fear condition (Singer *et al*, 2013; Wei *et al*, 2014), reversal learning (Wei *et al*, 2011), and instrumental learning (Yu *et al*, 2009), we postulate that postsynaptic striatopallidal A<sub>2A</sub>R function may provide a 'break' mechanism to constrain some cognitions including instrumental learning (Chen, 2014). If the postulated 'break' mechanism of the striatopallidal A<sub>2A</sub>R is validated by future experiments, this provides a framework for a pharmacological strategy by blocking striatopallidal A<sub>2A</sub>R activity to reverse abnormal

habit formation that is associated with compulsive obsessive disorder and relapse of drug addiction.

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