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Optogenetic activation of intracellular adenosine A_{2A} receptor signaling in hippocampus is sufficient to trigger CREB phosphorylation and impair memory

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Abstract

Human and animal studies have converged to suggest that caffeine consumption prevents memory deficits in aging and Alzheimer's disease through the antagonism of adenosine A_{2A} receptors ($A_{2A}R$). To test if $A_{2A}R$ activation in hippocampus is actually sufficient to impair memory function and to begin elucidating the intracellular pathways operated by $A_{2A}R$, we have developed a chimeric rhodopsin-$A_{2A}R$ protein (*opto$A_{2A}R$*), which retains the extracellular and transmembrane domains of rhodopsin (conferring light responsiveness and eliminating adenosine binding pockets) fused to the intracellular loop of $A_{2A}R$ to confer specific $A_{2A}R$ signaling. The specificity of the *opto$A_{2A}R$* signaling was confirmed by light-induced selective enhancement of cAMP and phospho-MAPK (but not cGMP) levels in HEK293 cells, which was abolished by a point mutation at the C-terminal of $A_{2A}R$. Supporting its physiological relevance, *opto$A_{2A}R$* activation and the $A_{2A}R$ agonist CGS21680 produced similar activation of cAMP and phospho-MAPK signaling in HEK293 cells, of pMAPK in nucleus accumbens, of c-Fos/pCREB in hippocampus and similarly enhanced long-term potentiation in hippocampus. Remarkably, *opto$A_{2A}R$* activation triggered a preferential phospho-CREB signaling in hippocampus and impaired spatial memory performance while *opto$A_{2A}R$* activation in the nucleus accumbens

Conflict of Interest:

Authors declare no conflict of interest for the work presented in this manuscript.

triggered MAPK signaling and modulated locomotor activity. This shows that the recruitment of intracellular $A_{2A}R$ signaling in hippocampus is sufficient to trigger memory dysfunction. Furthermore, the demonstration that the biased $A_{2A}R$ signaling and functions depend on intracellular $A_{2A}R$ loops, prompts the possibility of targeting the intracellular $A_{2A}R$ interacting partners to selectively control different neuropsychiatric behaviors.

Keywords

adenosine A_{2A} receptor; hippocampus; memory; optogenetics; CREB; biased signalling; MAPK; intracellular domain of A_{2A} receptor; striatum

Introduction

Recently six longitudinal prospective studies have established an inverse relationship between caffeine consumption and the risk of developing cognitive impairments in aging and Alzheimer's disease (AD)[1-7]. This is in notable agreement with animal studies, which showed that caffeine prevents memory impairments in models of AD[8-10] and sporadic dementia[11] and in other conditions affecting memory performance[12, 13]; this seems to involve the antagonism of G-protein coupled adenosine A_{2A} receptors ($A_{2A}R$), since their selective pharmacological or genetic blockade mimic caffeine's effects[8, 12, 14, 15]. The convergence of human epidemiological and animal evidence led us to propose that $A_{2A}R$ represent a novel therapeutic target to improve cognitive impairments in neurodegenerative disorders. The validity of this target is supported by our finding that $A_{2A}R$ inactivation not only enhances working memory[16, 17], reversal learning[17], goal-directed behavior[18] and Pavlovian fear conditioning[19] in normal animals, but also reverse memory impairments in animal models of Parkinson's disease[20], aging[15] and AD[8, 9, 14]. Notably, pathological brain conditions associated with memory impairment (such as AD, stress or inflammation) are associated with increased extracellular levels of adenosine[21] and an up-regulation and aberrant signaling of $A_{2A}R$[22, 23]. This prompts the hypothesis that the "abnormal" activation of $A_{2A}R$ in particular brain region (such as the hippocampus) is sufficient to trigger memory impairment. This critical question has yet to be answered because of the inability to control forebrain $A_{2A}R$ signaling in freely behaving animals with a temporal resolution relevant to behavior.

Another major unsolved question is the mechanisms operated by brain $A_{2A}R$ to control memory function. In fact, $A_{2A}R$ signaling is different in different cellular elements with distinct functions under various physiological versus pathological conditions[22, 24, 25]. For example, striatal and extra-striatal $A_{2A}R$ exert opposite control of DARPP-32 phosphorylation[26], c-Fos expression[26], psychomotor activity[26, 27] and cognitive function[19]. Receptor-receptor heterodimerization has been postulated to contribute to the complexity of $A_{2A}R$ signaling[28]. Additionally, recent biochemical studies identified six G-protein interacting partners (GIP) linked to the intracellular C-terminal tail of $A_{2A}R$[29, 30], which raises the intriguing possibility that the interaction of $A_{2A}R$ intracellular domains with different GIPs may dictate the biased $A_{2A}R$ signaling in different cells. However, the inability to control intracellular GPCR signaling *in vivo* in a precise spatiotemporal manner,

has prevented translation of *in vitro* profiles of $A_{2A}R$ signaling into behavior in intact animals.

To determine if the abnormal activation of $A_{2A}R$ signaling in hippocampus is sufficient to impair memory function in freely behaving animals and to begin elucidating the nature of the biased $A_{2A}R$ signaling in different brain regions, we have developed a chimeric rhodopsin-$A_{2A}R$ protein (*opto$A_{2A}R$*): this merges the extracellular and transmembrane domains of rhodopsin conferring light responsiveness and the intracellular domains of $A_{2A}R$ conferring specific $A_{2A}R$ signaling, to investigate the biased $A_{2A}R$ signaling in defined cell populations of freely behaving animals in a temporally precise and reversible manner[31]. Furthermore, the selective retention of only the intracellular domains of $A_{2A}R$ in *opto$A_{2A}R$* chimaera permits a critical evaluation of its particular role controlling the biased $A_{2A}R$ signaling. After validating the specificity and physiological relevance of light-induced *opto$A_{2A}R$* recruitment of $A_{2A}R$ signaling in HEK293 cells, mouse brain slices and in freely behaving animals, we exploited its unique temporal and spatial resolution to provide direct evidence that the activation of intracellular $A_{2A}R$ signaling selectively in the hippocampus is sufficient to recruit cAMP and phosphorylated CREB, and alter synaptic plasticity and memory performance. Our findings also provide a direct demonstration that the intracellular control of the biased $A_{2A}R$ signaling in striatal and hippocampal neurons triggers distinct signaling and behavioral responses.

Materials and Methods

The detailed methods are described in the “Supplemental”.

Design and construction of the *opto$A_{2A}R$* vector

We constructed a fusion gene encoding a chimaera (*opto$A_{2A}R$*) where the intracellular loops 1, 2 and 3 and the C-terminal of rhodopsin were replaced with those of $A_{2A}R$ and the C-terminal sequence of bovine rhodopsin (TETSQVAPA) was added to the C-terminal of *opto$A_{2A}R$*. Lastly, codon optimized sequences of *opto$A_{2A}R$* were fused to the N-terminus of mCherry (with its start codon deleted) (Figure 1a).

Transfection and assessment of *opto$A_{2A}R$* signaling in HEK cells

48-hr after transfection, all-trans-retinal (25μM) was added to HEK293 cells that were then illuminated (500nm, 3mW/mm^2) for 60-sec. The cells were lysed 30-min after light illumination to analyze cAMP using cAMP-Glo™ assay (Promega), cGMP by HTRF-cGMP assay and IP1 by HTRF-IP1 assay kit (CisBio). For Western blot, cells were homogenized 10 min after light illumination using a PARIS Kit (Invitrogen).

Activation of *opto$A_{2A}R$* signaling in the brain

Recombinant AAV vectors were constructed with a CaMKIIα promoter by cloning the *opto$A_{2A}R$*-mCherry into pAAV-CaMKIIα-eNpHR 3.0-EYFP (Addgene). Viral particles were packaged and purified as serotype 5-positive at the University of North Carolina with titers of 1.5-2.0×10^{12} particles/mL. We injected 1.0μL of AAV5-CaMKIIα-*opto$A_{2A}R$*-mCherry virus or 0.75μL of AAV5-CaMKIIa-mCherry (“control”) virus into the right

nucleus accumbens or hippocampus. After 2 weeks, 473nm DPSS laser light was delivered via a patch cable with 50ms pulse width and ~3-5mW/mm^2 power density.

Behavioral tests

The Y-maze test for spatial recognition memory task was based on exploration of novelty, as previously described[12,32]. During the second trial (retrieval phase), the mice had access to the 3 arms for 5-min with light "ON". The time spent in each arm and total locomotor activity was measured by a video-tracking system.

Immunohistochemistry

The targeted expression of *optoA$_{2A}$R* and of its molecular signaling (c-Fos, pCREB, pMAPK) in NAc and hippocampus were determined by immunohistochemistry as described previously[17, 19,26]. Mice were killed 10 min after optical stimulation or 15-min after injecting 2.0μL of the A$_{2A}$R agonist CGS 21680 (0.5μg/μL) in NAc or hippocampus to activate endogenous A$_{2A}$R. We analyzed 3 fields/section, 3 sections/mouse, 3 mice/group.

Electrophysiological recordings in hippocampal slices

The recording of the evoked field excitatory postsynaptic potentials (fEPSP) in the CA1 *stratum radiatum* upon stimulation of Schaffer fibers every 20-sec, were as previously described[69] in hippocampal slices (400μm) prepared two weeks after transfection with AAV5-CaMKIIa-mCherry without or with *optoA$_{2A}$R* in hippocampus. A high frequency stimulation (HFS) train (100Hz, 1-sec) was used to induce long-term potentiation (LTP). Light stimuli, applied immediately before HFS, consisted of 3000 light pulses (465nm, 50ms pulse width, ~3-5mW/mm^2 power density) over 300-sec and the optic fiber was placed over the slice between the stimulation and recording electrodes. CGS21680 (30nM; Tocris) was added to the superfusion solution 20-min before HFS onwards.

Western blot and immunocytochemistry of total membranes and synaptosomes

Total membranes and synaptosomes from the hippocampus were prepared using sucrose/Percoll differential centrifugations and Western blot analysis was carried out as previously described[70] using an antibody against the third intracellular loop of A$_{2A}$R (1:500; Millipore). The immunocytochemical analysis to detect the presence of *optoA$_{2A}$R* in glutamatergic terminals, was done as previously[71], by detecting the co-localization of mCherry fluorescence with vGluT1 immunoreactivity (1:2500, Synaptic Systems).

Results

Light activation of *optoA$_{2A}$R* specifically recruits A$_{2A}$R signaling in HEK293 cells

We engineered a light activated chimeric protein able to recruit A$_{2A}$R signaling, *optoA$_{2A}$R*, by replacing the intracellular domains of rhodopsin with those of A$_{2A}$R (Figure 1a). 24-h after transfecting human embryonic kidney (HEK293) cells with *optoA$_{2A}$R*, we observed a single band with a 80 kD molecular weight, expected for *optoA$_{2A}$R* (Figure 1b), using an A$_{2A}$R antibody targeting the third *intracellular* loop of A$_{2A}$R. We also detected the red

fluorescence of mCherry, included in the *optoA$_{2A}$R* construct, largely restricted to the cell surface (Figure 1c), similar to that obtained using the $A_{2A}R$ antibody.

$A_{2A}R$ activate the G_S/G_{olf}-cAMP pathway as well as mitogen-activated protein kinases (MAPK) pathway in a G_S-independent manner[25]. Light stimulation of HEK293-*optoA$_{2A}$R* cells (for 60-sec) increased MAPK phosphorylation (p-MAPK), in contrast with the weak p-MAPK immunoreactivity in light stimulated cells transfected with the pcDNA3.1 vector (Figure 1d), which was confirmed by Western blot (Figure 1e). Light stimulation of HEK293-*optoA$_{2A}$R* cells also increased cAMP levels by 2-fold (immunoassay after 20-min), compared to non-stimulated HEK293-*optoA$_{2A}$R* cells and to light-stimulated cells transfected with pcDNA3.1 (Figure 1f; $p<0.001$, two way ANOVA). Thus, *optoA$_{2A}$R* specifically recruits the two parallel $A_{2A}R$ signaling pathways, namely G_S-cAMP and MAPK signaling in HEK293 cells.

Supporting the specificity of *optoA$_{2A}$R* signaling, light stimulation of HEK293-*optoA$_{2A}$R* cells induced cAMP and p-MAPK signaling (Figure 1e, 1f) but did not affect either cGMP (the rhodopsin transducing system, Figure 1g) or IP1 production (a degradation product of IP3, associated with Gq signaling, Figure 1h). Of note, light stimulation of *optoA$_{2A}$R* rapidly increased cAMP and pMAPK levels in HEK293 cells within 1-min, peaking at 15-30-min and declining to basal level at 60-90-min (Figure 1i; $p<0.05$, One way ANOVA). Further reinforcing the selectivity of *optoA$_{2A}$R* to trigger cAMP accumulation, a Ser400Ala point mutation in an $A_{2A}R$ phosphorylation site critical for $A_{2A}R$-D_2R receptor interaction[33], but not a Thr324Ala point mutation in an $A_{2A}R$ phosphorylation site critical for short-term desensitization[30], of the C-terminals of *optoA$_{2A}$R* abolished the light *optoA$_{2A}$R*-induced cAMP accumulation (Figure 1j; $^{**}p<0.01$, Student's *t* test). Thus, *optoA$_{2A}$R* signaling is specific and attributed to the unique amino acid composition of its C-terminus.

Light *optoA$_{2A}$R* activation triggers an $A_{2A}R$ signaling identical to the pharmacological activation of endogenous $A_{2A}R$ both in HEK293 cells and mouse brain

To demonstrate the physiological relevance of *optoA$_{2A}$R* signaling, we first compared in HEK293 cells cAMP levels and p-MAPK induced by either light activation of *optoA$_{2A}$R* or CGS21680 ($A_{2A}R$ agonist) activation of wild-type $A_{2A}R$. Light *optoA$_{2A}$R* activation increased cAMP (Figure 2b; $p<0.001$two-way ANOVA) to levels similar to these triggered by CGS21680 (200 nM) in cells transfected with $A_{2A}R$-mCherry (Figure 2a; $p<0.001$,two-way ANOVA). Moreover, in cells co-transfected with $A_{2A}R$-mCherry and *optoA$_{2A}$R*, co-stimulation with light and CGS21680 produced additive effects on both cAMP level (Figure 2c; light, $F(1,43)=7.243$, $p<0.01$; CGS21680, $F(3,43)=32.674$, $p<0.001$; light × CGS21680 interaction, $F(3,43)=0.336$, $p>0.05$, two-way ANOVA) and p-MAPK. Thus, *optoA$_{2A}$R* and CGS21680 produced similar $A_{2A}R$ signaling with additive effects in HEK293 cells.

We further compared $A_{2A}R$ signaling (c-Fos and p-MAPK) in hippocampus and nucleus accumbens (NAc) induced by endogenous $A_{2A}R$ activation in wild-type mice and by *optoA$_{2A}$R* in transfected $A_{2A}R$ knockout mice. Intra-hippocampal injection of CGS21680 (0.93nmol/μL) significantly increased c-Fos expression within 15-min specifically in the cells surrounding the injection site (Figure 2d). By contrast, intra-accumbal injection of CGS21680 markedly induced p-MAPK (Figure 2e). Thus, endogenous $A_{2A}R$ activation

elicits a brain region-specific $A_{2A}R$ signaling in the forebrain (c-Fos in hippocampus and p-MAPK in NAc). Accordingly, light *opto$A_{2A}R$* stimulation in hippocampus significantly increased c-Fos expression specifically in the *opto$A_{2A}R$*-expressing cells underneath the cannula (Figure 2f) whereas *opto$A_{2A}R$* activation in NAc markedly induced p-MAPK (Figure 5d, 5e) but not c-Fos (Figure 2g). This demonstrates a similarly biased $A_{2A}R$ signaling triggered by *opto$A_{2A}R$* and CGS21680, *i.e*. c-Fos in hippocampus and p-MAPK in NAc, supporting the ability of *opto$A_{2A}R$* to mimic the endogenous $A_{2A}R$ signaling in the brain.

Targeted expression and light activation of *opto$A_{2A}R$* in glutamatergic terminals of the hippocampus controlling LTP in hippocampal slices

The $A_{2A}R$ antibody detected a single 45kD band (endogenous $A_{2A}R$) in striatal membranes, whereas $A_{2A}R$ levels were barely detectable in hippocampal samples, compatible with the 20-times lower density of $A_{2A}R$ in hippocampus *versus* striatum[70]. Importantly, this $A_{2A}R$ antibody designed against the third intracellular loop of $A_{2A}R$ recognized two bands of 80kD and 95kD selectively in hippocampus transfected with AAV-mCherry-*opto$A_{2A}R$*, but not with AAV-mCherry (Figure 3a), with a density in synaptosomes similar to that detected in total membranes (n=3, p>0.05, Student's *t* test). Furthermore, the immunocytochemistry analysis of purified synaptosomes (Figure 3b) allowed identifying mCherry fluorescence in glutamatergic nerve terminals (i.e. immunopositive for vesicular glutamate transporters type-1, vGluT1) only from hippocampus transfected with AAV-mCherry-*opto$A_{2A}R$* (n=3), but not with AAV-mCherry (not shown). This indicates that *opto$A_{2A}R$* is present in hippocampal glutamatergic nerve terminals, where endogenous $A_{2A}R$ have been identified[71].

Additionally, we observed (Figure 3c) that the amplitude of long-term potentiation (LTP) triggered by high-frequency light stimulation (HFS) was larger upon light stimulation (5-min before HFS) of hippocampal slices expressing *opto$A_{2A}R$* (197.3±2.9% over baseline) than without light stimulation (137.7±2.0% over baseline, n=3; p>0.05, Student's *t* test). By contrast, light stimulation did not modify LTP amplitude in hippocampal slices expressing only mCherry (138.9±2.7% *vs*. 142.5±1.5% over baseline with or without light stimulation, respectively, n=3; p>0.05, Student's *t* test) (data not shown). This enhancement upon light-induced *opto$A_{2A}R$* activation was similar to the impact of a pharmacological activation of endogenous $A_{2A}R$ with the $A_{2A}R$ agonist, CGS21680 (30nM) (Figure 3d) as well as in slices from mice transfected with AAV-mCherry (Figure 3e), as occurs in wild-type animals[72]. This shows that light activation of *opto$A_{2A}R$* can mimic an established physiological response operated by endogenous $A_{2A}R$ in hippocampus[44,69].

Optogenetic *opto$A_{2A}R$* activation in hippocampus recruits CREB phosphorylation and impairs memory performance

Two weeks after the focal injection of AAV5-(CaMKIIα promoter-driven)-*opto$A_{2A}R$*-mCherry (Figure 4a), light stimulation in the dorsal hippocampus significantly increased the levels of phosphorylated CREB (p-CREB) (Figure 4c,4d; p<0.001, Student's *t* test) specifically in the *opto$A_{2A}R$*-expressing neurons underneath the cannula, consistent with the $A_{2A}R$-G_S-cAMP pathway as the major $A_{2A}R$ signaling pathway in hippocampus[25]. Similar

to p-CREB recruitment, light stimulation significantly elevated c-Fos in the *optoA$_{2A}$R*-expressing neurons in hippocampus (Figure 2f; p<0.001, Student's *t* test), whereas it did not induce p-MAPK (Figure 4b,4d). Thus, in hippocampal neurons, light *optoA$_{2A}$R* activation preferentially stimulates the cAMP-PKA pathway, leading to p-CREB and c-Fos expression, without significant effect on the p-MAPK pathway.

To address the central question whether $A_{2A}R$ activation in hippocampus is sufficient to impair memory performance, we tested if triggering hippocampal *optoA$_{2A}$R* signaling affected spatial reference memory performance using a two-visit version of the Y-maze test. Light *optoA$_{2A}$R* activation in hippocampus during the 5-min testing period reduced about 2-fold the time spent in the novel arm compared with mice transfected with AAV-mCherry (control) only (Figure 4e; p<0.001, Student's *t* test). These short-term reference memory impairments were not due to changes in locomotion as gauged by the unaltered total distance travelled in the Y-maze (Figure 4f). Thus, *transient* activation of *optoA$_{2A}$R* in a set hippocampal neurons is sufficient to recruit p-CREB signaling and deteriorate memory performance.

Light *optoA$_{2A}$R* stimulation in nucleus accumbens recruits MAPK phosphorylation and selectively modulates motor activity

We next examined the impact of light *optoA$_{2A}$R* activation in NAc and hippocampus on the two $A_{2A}R$ signaling pathways (p-CREB and p-MAPK). Since GPCR can produce a biased signaling simply due to different receptor levels, we used the same CaMKIIα promoter to drive similar levels of *optoA$_{2A}$R* expression in hippocampal and striatal neurons[34, 35], to eliminate different *optoA$_{2A}$R* expression levels as a possible cause of a biased $A_{2A}R$ signaling in these two forebrain regions[36]. *OptoA$_{2A}$R* was selectively expressed in accumbal neurons (co-localized with Neu+ neurons, but not with GFAP+ astrocytes) in the core of NAc (Figure 5a). Using enkephalin (ENK) and substance-P (SP) immunostaining to identify the indirect and direct pathway neurons[37], we found that *optoA$_{2A}$R* was expressed in both ENK-containing (52%) and SP-containing (43%) neurons in NAc (Figure 5b). Importantly, light activation of *optoA$_{2A}$R* in NAc for 5-min markedly increased p-MAPK (Figure 5d,5e; p<0.001, Student's *t* test), with little induction of p-CREB and c-Fos (Figure 5c, 5e, 2g). Thus, the activation of $A_{2A}R$ signaling by *optoA$_{2A}$R* in NAc preferentially involved the MAPK pathway rather than the cAMP-PKA mediated c-Fos/CREB pathway.

In parallel with the preferential activation of p-MAPK signaling, light *optoA$_{2A}$R* activation in NAc for 5-min did not affect memory performance in the modified Y-maze test (Figure 5f; p=0.276, Student's *t* test), but robustly increased locomotor activity (83% increase of travelled distance in the Y-maze, Figure 5g, and in the open field test) (for AAV vector × behavioral phase, $F(1,45)=18.68$, p<0.001, two-way ANOVA). The observed motor stimulant effect resulting from *optoA$_{2A}$R* activation in NAc, instead of a motor depression observed upon accumbal administration of CGS21680[38, 39], was expected in view of the expression of *optoA$_{2A}$R* in both striatopallidal and striatonigral neurons using the CaMKIIα promoter[35, 36] (rather than the selective expression of endogenous $A_{2A}R$ in striatopallidal neurons prompted by the $A_{2A}R$ promoter[40]). This pitfall was however essential to circumvent the confounding effect of a 20-fold differential expression of $A_{2A}R$ in these two

brain regions as a possible cause for the biased $A_{2A}R$ signaling. Overall, the present findings show that $A_{2A}R$ trigger a biased $A_{2A}R$ signaling in different forebrain regions (cAMP in hippocampus and p-MAPK in NAc) in parallel with an impact on distinct behaviors (memory in hippocampus and locomotion in NAc).

Discussion

The development and validation of the *opto$A_{2A}R$* approach to mimic endogenous $A_{2A}R$ signaling allowed the novel conclusion that the recruitment of $A_{2A}R$ signaling in the dorsal hippocampus is sufficient to trigger a selective memory deficit. This is in accordance with the imbalance of the local extracellular adenosine levels[21] and up-regulation of $A_{2A}R$ in animal models of aging[41], sporadic dementia[11], AD[10], as well as in the human AD brain[42], namely in hippocampal nerve terminals[12, 13], a situation that was mimicked by hippocampal *opto$A_{2A}R$* expression under control of the CaMkIIα promoter. Indeed, *opto$A_{2A}R$* was detected in hippocampal synaptosomes, namely in glutamatergic synapses where endogenous $A_{2A}R$ are identified[71] and upregulated upon aging and neurodegeneration. This provides an anatomical basis for *opto$A_{2A}R$* control of p-CREB signaling, synaptic activity and memory performance. Indeed, the light activation of *opto$A_{2A}R$* in hippocampal slices mimicked a well-established physiological response operated by endogenous $A_{2A}R$, the control of hippocampal long-term potentiation (LTP)[44,69]. Furthermore, opto$A_{2A}R$ activation in hippocampus triggers CREB phosphorylation and impairs memory performance. These findings are consistent with the canonical cAMP/PKA pathway activated by hippocampal $A_{2A}R$[25] and with the established role of CREB phosphorylation controlling synaptic plasticity and long-term memory[43] through neuronal excitability and transcription, and with specific deficits of memory retrieval observed in mice expressing a time-controlled active CREB variant[44]. This ability of hippocampal *opto$A_{2A}R$* activation in glutamate synapses to control memory dysfunction and its purported neurophysiological correlate LTP, decisively strengthens the relation between $A_{2A}R$ and memory performance that had so far largely relied on the demonstration that $A_{2A}R$ blockade alleviated memory dysfunction[8, 9, 12, 14, 15]. Furthermore, this ability to place $A_{2A}R$ functioning as a sufficient factor to imbalance memory bolsters the rationale to probe the therapeutic effectiveness of $A_{2A}R$ antagonists to manage memory impairment[22, 23]. This notion is further warranted by the striking convergence of epidemiological[1-7] and animal[8-20] evidence supporting the therapeutic benefit of caffeine and $A_{2A}R$ antagonists to improve cognition. This aim should be facilitated by the safety profile of $A_{2A}R$ antagonists, tested in over 3000 parkinsonian patients[22].

The design of *opto$A_{2A}R$* also allowed identifying a critical role solely attributable to the intracellular domains of $A_{2A}R$ to dictate the biased $A_{2A}R$ signaling and function in neurons of different brain regions. Contrary to the widely accepted view that ligand-receptor interactions are the molecular basis directing the biased GPCR signaling, the distinct molecular and behavioral responses obtained upon *opto$A_{2A}R$* activation in different brain regions show that they are only dependent on an intracellular mechanism probably related with the differential association with different GIPs in different cell types. In fact, the cell-specific expression of intracellular GIPs provides a rich molecular resource[45] whereby $A_{2A}R$ signaling in the brain is specifically wired according to the needs of each cell type. In

particular, the long and flexible $A_{2A}R$ C-terminus[29] contains several consensus sites (e.g. $YXXG_{\phi}$)[30] required for MAPK activation[46], interaction with BDNF receptors (TrkB)[47], with FGF[48], with p53[49, 50], with a large set of downstream signaling effectors such as G proteins, GPCR kinases, arrestins and with at least six GIPs (actinin, calmodulin, Necab2, translin associated protein X, ARNO/cytohesin-2, ubiquitin-specific protease-4)[29]. Thus, targeting $A_{2A}R$ intracellular domains offers an additional layer of selectivity to manipulate $A_{2A}R$ signaling that is not attainable only by the ligand-receptor interaction. Thus, selectively targeting $A_{2A}R$ intracellular domains and their interacting GIPs in specific brain regions emerges as a novel strategy to obtain therapeutic effects with minimal side effects, as achieved with trans-membrane peptides to specifically disrupt the intracellular interaction between NMDA receptors and PSD95[51, 52] and between 5-HT2c-PENT[53]. If the critical interaction between intracellular domains of $A_{2A}R$ and GIPs are general features of GPCRs, the "*optoGPCR*" approach targeting intracellular domains of GPCRs may represent a novel drug discovery strategy for the largest protein superfamily in the human genome.

The significance of these novel insights is decisively strengthened by the demonstrated specificity and rapid induction of the *opto$A_{2A}R$* signaling. The specificity of *opto$A_{2A}R$* signaling is supported by the selective optogenetic induction of cAMP and MAPK signaling without affecting cGMP (rhodopsin) and IP3 (G_q) signaling and by the mutational analysis demonstrating that *opto$A_{2A}R$* signaling is specifically attributed to the unique amino acid composition of the $A_{2A}R$ C-terminus. Moreover, the comparable activation of $A_{2A}R$ signaling in HEK293 cells and the indistinguishable pattern of the biased $A_{2A}R$ signaling in NAc and hippocampus as well as the similar enhancement of hippocampal LTP triggered by *opto$A_{2A}R$* and CGS21680 supports that *opto$A_{2A}R$* signaling largely captures the physiological function of the native $A_{2A}R$. Different from opsin-based optogenetics, *opto-$A_{2A}R$* signals through GPCR signaling allows a control of intracellular $A_{2A}R$ signaling by light, which we now report to involve a rapid induction, consistent with similar rapid physiological response ($T_{on}1/2$= ~1-sec) of other GPCR light activated chimaera[58-61]. Thus, the temporal and spatial control of specific $A_{2A}R$ signaling afforded by *opto$A_{2A}R$* in freely behaving animals paves the way to probe the role of $A_{2A}R$ in defined forebrain circuits responsible for behaviors ranging from motor control, fear, addiction, mood or decision making[54, 55].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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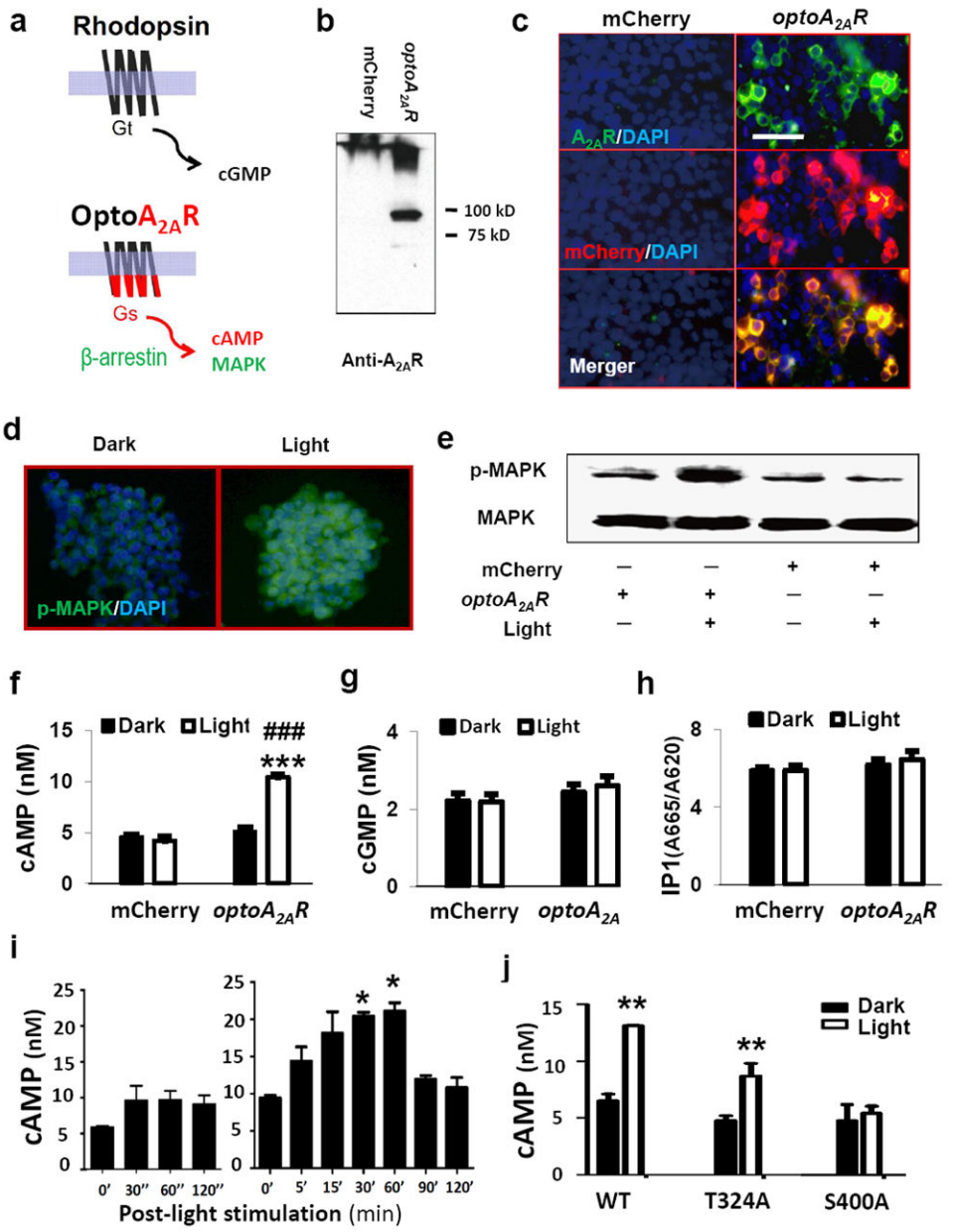

Figure 1. Characterization of *optoA$_{2A}$R* signaling in HEK293 cells

Design (**a**) of *optoA$_{2A}$R* and its expression (**b,c**) in HEK293 cells 24-hr after transfection. (**b**): Western blot analysis of *optoA$_{2A}$R* with a molecular weight of 75-100 kD. (**c**): co-localization of A_{2A}R-immunostaining (green) with mCherry-expressing (red) in *optoA$_{2A}$R*–positive cells. (**d,e**) Light induction of p-MAPK in *optoA$_{2A}$R*-expressing cells. **d**: p-MAPK-immunostaining of *optoA$_{2A}$R*-expressing cells before and after light stimulation. **e**: Western blot analysis of p-MAPK and MAPK expression in response to light. (**f,h**). Light-induced increase of cAMP (**f**; plasmid: $F(1,62)=126.7$, $p<0.001$; light stimulation, $F(1,62)=67.8$, $p<0.001$; plasmid × light, $F(1,62)=89.4$, $p<0.001$, two way ANOVA) but not of cGMP (**g**; light, $F(1,62)=0.110$, $p>0.05$, two way ANOVA) or IP_1 production (**h**; light, $F(1,62)=0.110$, $p>0.05$, two way ANOVA) in HEK293 cells transfected with *optoA$_{2A}$R*. ***=p<0.001,

comparing *optoA$_{2A}$R* with control; ###=p<0.001 compared to the dark, n=16, two-way ANOVA, Bonferroni *post-hoc* t-test. (**i**) Time course of *opto-A$_{2A}$R*-induced cAMP accumulation after light stimulation. One-way ANOVA, Bonferroni *post-hoc* t-test. (**j**) Effect of the mutations Ser400Ala and Thr324Ala of the C-terminal of *optoA$_{2A}$R* on light *optoA$_{2A}$R*-induced cAMP accumulation. **=p<0.01, Student's *t* test. Each experiment was done in duplicates or triplicates and repeated at least three times. Scale bar = 50μm

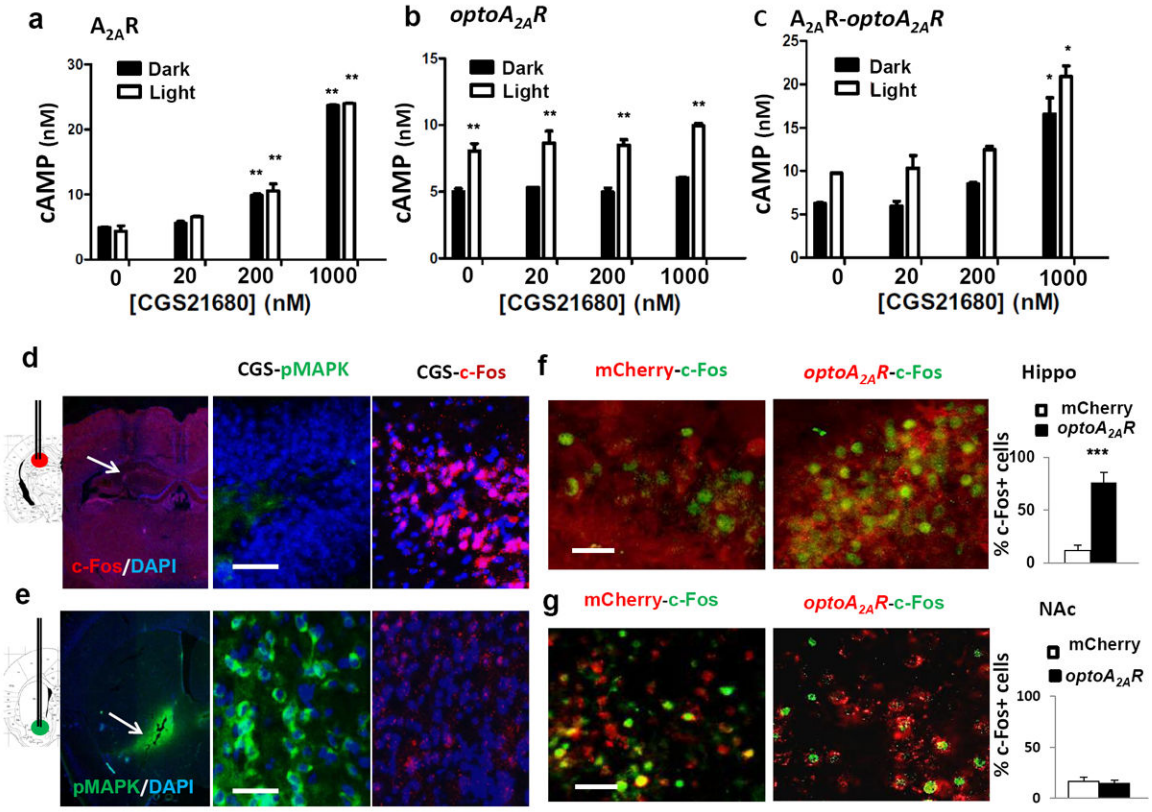

Figure 2. *OptoA$_{2A}$R* and CGS21680 produced additive induction of cAMP in HEK293 cells and indistinguishable biased A$_{2A}$R signaling in NAc and hippocampus

(**a,b,c**): HEK293 cells were transfected with wild-type A$_{2A}$R (*a*) or *optoA$_{2A}$R* (*b*) or co-transfected with wild-type A$_{2A}$R and *optoA$_{2A}$R* (*c*). (*a*) 24-hrs after transfection, cells transfected with wild-type A$_{2A}$R were treated with the A$_{2A}$R agonist CGS21680 (20, 100, 200 and 1000nM) and displayed a concentration-dependent increase of cAMP levels. Light activation of *optoA$_{2A}$R*-transfected HEK293 cells increased cAMP levels (*b*; for light, $F(1,31)=60.721$, $p<0.001$; for CGS21680, $F(3,31)=2.163$, $p>0.05$; light × CGS21680 interaction, $F(3,31)=0.155$, $p>0.05$, two-way ANOVA) similar to that induced by 200nM CGS21680 (**a**; for light, $F(1,46)=0$, $p>0.05$; for CGS21680, $F(3,46)=312.799$, $p<0.001$; light × CGS21680 interaction, $F(3,46)=0.547$, $p>0.05$, two-way ANOVA). Co-stimulation of light and CGS21680 in cells co-transfected with *optoA$_{2A}$R* and wild-type A$_{2A}$R produced an additive effect on cAMP levels (**c**; for light stimulation, $F(1,43)=7.243$, $p<0.01$; for CGS21680, $F(3,43)=32.674$, $p<0.001$; light × CGS21680 interaction, $F(3,45)=0.336$, $p>0.05$, two-way ANOVA). *=$p<0.05$, **=$p<0.01$, two-way ANOVA, Bonferroni *post-hoc* t-test. Following intra-accumbal injection, CGS21680 markedly induced p-MAPK expression (green) but not c-Fos expression around the injection site (**d**, lower panel). Following intra-hippocampal injection, CGS21680 induced c-Fos expression (right) but not p-MAPK expression (left) around the injection site (**e**, upper panel). (**f**): Two weeks after intra-hippocampal injection of AAV5-*optoA$_{2A}$R*, light stimulation for 5-min induced c-Fos expression in *optoA$_{2A}$*R-positive cells but not in cells transfected with AAV5-mCherry. By contrast, light stimulation of *optoA$_{2A}$R* in NAc did not affect c-Fos expression (**g**). Bar

graphs (**f, g**) are c-Fos cell counts (averaged from 3 fields of each section, 3 section per mouse, 3 mice per group). ***=p<0.001, Student's *t* test comparing the *optoA$_{2A}$R* with the mCherry. Scale bar=50 μm (**d**-**g**).

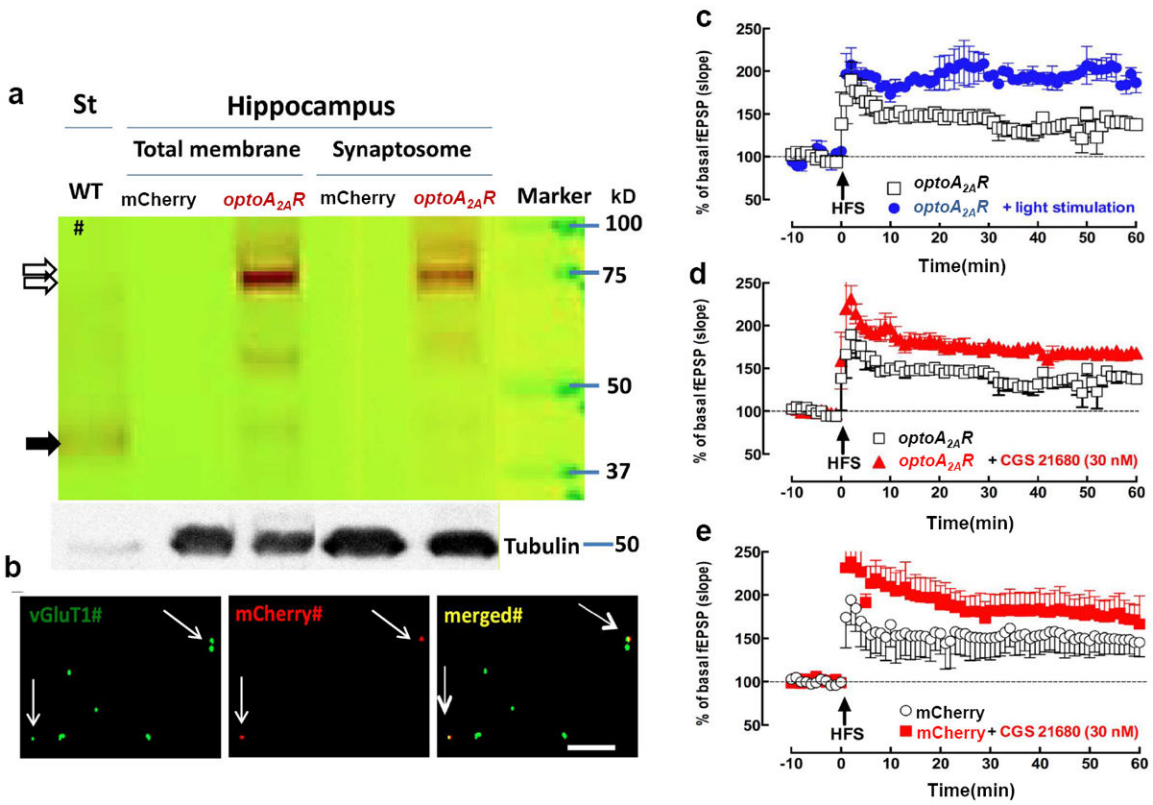

Figure 3. Targeted expression and light activation of *optoA$_{2A}$R* in glutamatergic terminals of hippocampus induced hippocampal long-term potentiation (LTP) in brain slices

(**a**) Representative Western blot analysis showing that an antibody against the third intracellular loop of $A_{2A}R$ recognized two bands at 80 and 95 kDa in hippocampal synaptosomes as well as in total membranes from mice transfected with AAV-mCherry-*optoA$_{2A}$R* (*optoA$_{2A}$R*) but not from mice transfected with AAV-mCherry, compatible with the localization of *optoA$_{2A}$R* in hippocampal synapses (n=3). (**b**) Representative single nerve terminal immunocytochemistry identifying that vesicular glutamate transporter type 1 (vGluT1, a marker of glutamatergic terminals; green) and mCherry immunoreactivity (red) were found to be co-localized (arrows identifying yellow in 'merged') in hippocampal synaptosomes from mice transfected with AAV-mCherry-*optoA$_{2A}$R* (*optoA$_{2A}$R*), whereas this was not observed for mice transfected with AAV-mCherry (not shown) (n=3). (**c**) Accordingly, light stimulation (3000 pulses of 50-ms duration each during 300-sec) of slices from mice transfected with AAV-mCherry-*optoA$_{2A}$R*, applied before a high-frequency train (100Hz for 1-sec), enhanced the amplitude of LTP compared to non-light stimulated slices, measured as an increased slope of field excitatory post-synaptic potentials (fEPSP) recorded in the *stratum radiatum* of the CA1 area upon stimulation of the afferent Schaffer fibers (**c**), whereas light stimulation failed to modify LTP amplitude in mice transfected with AAV-mCherry (not shown). This essentially mimics the effect of the pharmacological activation of endogenous $A_{2A}R$ with the selective $A_{2A}R$ agonist CGS21680 (30nM), in slices from mice transfected either with AAV-mCherry-*optoA$_{2A}$R* (**d**) or with AAV-mCherry (**e**). Representative images (**a,b**) and data (mean±SEM, **c-e**) are from n=3 independent mice. *=p<0.05, Student's *t* test.

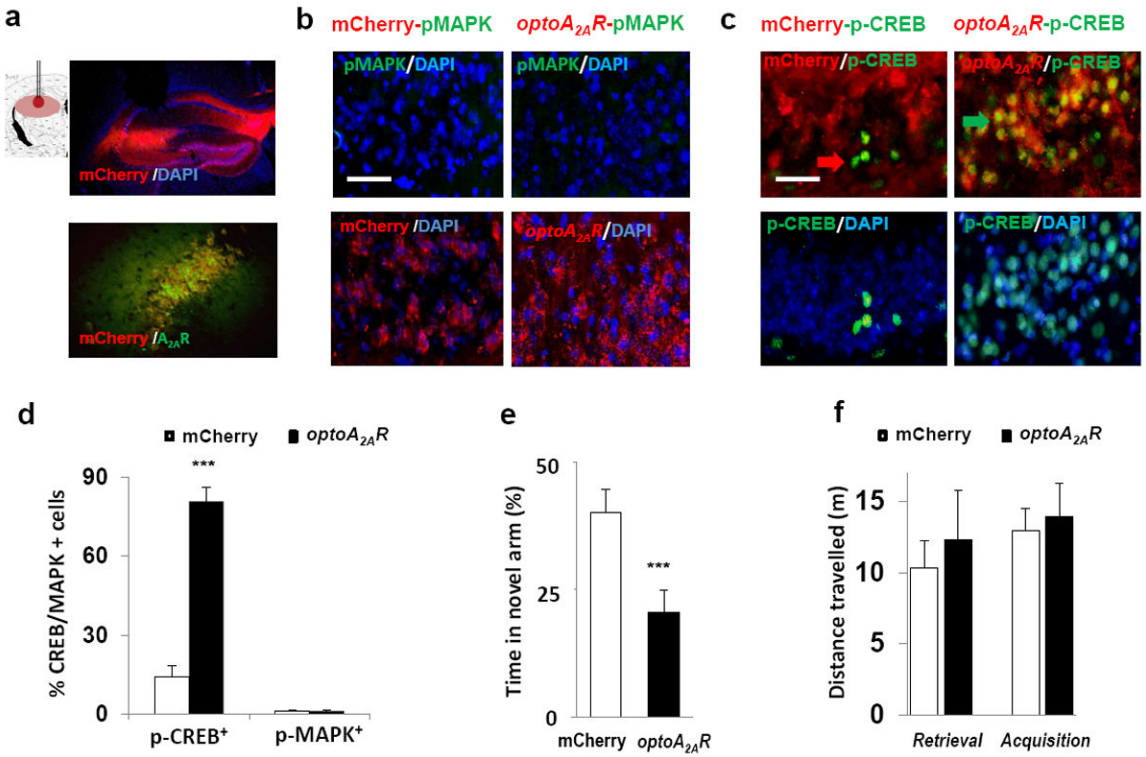

Figure 4. Light activation of hippocampal *optoA$_{2A}$R* triggers CREB phosphorylation and memory impairment

(**a**) Upper panel: Schematic illustration of transplanted cannula targeting hippocampus and expression of mCherry (*optoA$_{2A}$R*) under CaMKIIa promoter in hippocampus after focal injection of AAV5-*optoA$_{2A}$R*-mCherry (AP, -2.2 mm; ML, ±1.5mm; DV, +2.3 mm). Lower panel: co-localization of A$_{2A}$R-immunostaining (green) with mCherry-expressing (red) in *optoA$_{2A}$R*–expressing cells. (**b,c**) Light stimulation of *optoA$_{2A}$R* in hippocampus for 5-min induced phospho-CREB (**b,d**) but not phospho-MAPK (**c,d**, p=<0.001, Student's *t* test) in *optoA$_{2A}$R*-positive cells but not in cells transfected with AAV5-mCherry (3 fields/section, 3 sections/mouse, 3 mice/group). (**e**) Light stimulation of *optoA$_{2A}$R* signaling in the hippocampus during the retrieval phase impaired spatial recognition memory with decreased time in the novel arm (p=<0.001, Student's *t* test) of the Y-maze but had no effect on total distance travelled (**f**; AAV vector, F(1,41) = 0.45, p>0.05, two-way ANOVA) during on the 5-min "Light-On" period, compared with that of the control. Green arrow: co-localization of *optoA$_{2A}$R* with phospho-CREB; red arrow: phospho-CREB expression only. Scale bar=50μm.

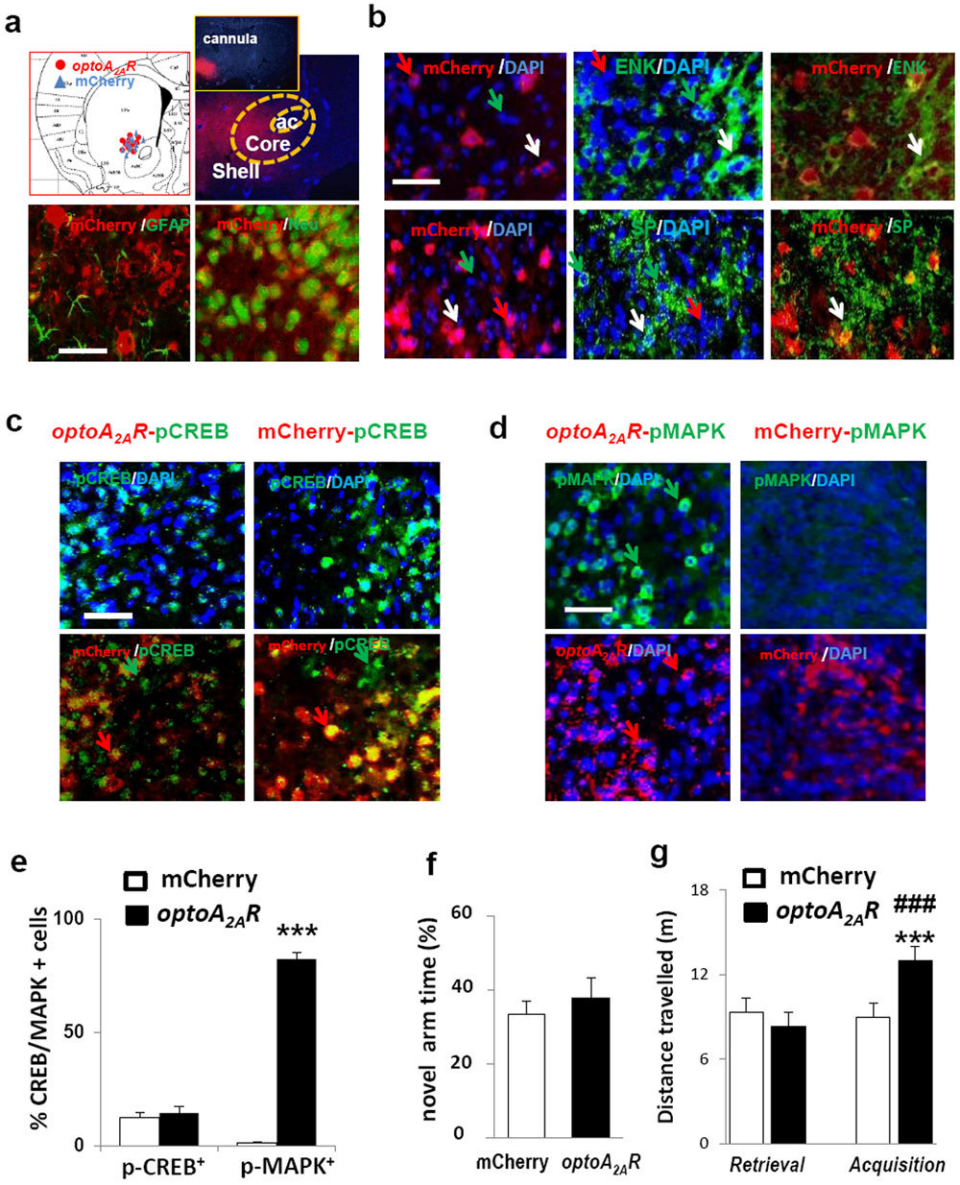

Figure 5. Light activation of accumbal *optoA$_{2A}$R* triggered p-MAPK phosphorylation and locomotor response

(**a**) Top left: Schematic illustration and mCherry (*optoA$_{2A}$R*) under CaMKIIa promoter in NAc after focal injection of AAV5-*optoA$_{2A}$R*-mCherry int NAc (AP, +1.1 mm; ML, ±1.4mm; DV, +4.5 mm). Top left: Schematic illustration and location of mCherry in the core and shell of NAc. Top right: A$_{2A}$R-immunostaining (green) localized in mCherry-expressing (red) *optoA$_{2A}$R*–positive cells. Bottom left: *optoA$_{2A}$R*-mCherry were co-localized in NeuN-positive (green) but not GFAP-positive cells. (**b**) Expression of *optoA$_{2A}$R* in enkephalin (ENK)-positive and substance-P (SP)-positive medium-sized spiny neurons. (**c,d**) Light induction of p-CREB (**c**) and p-MAPK (**d**) (green) in NAc at two weeks after intra-NAc injection of AAV5-*optoA$_{2A}$R* (right) or AAV5-mCherry (left). Light stimulation produced scattered expression of p-CREB but triggered a robust induction of p-MAPK with mCherry-expressing in *optoA$_{2A}$R*–positive cells (right panels). (**e**) Quantitative analysis of

light induction of phospho-CREB and phospho-MAPK in *optoA$_{2A}$R*-positive cells but not in cells transfected with AAV5-mCherry. pCREB/pMAPK-positive cell accounts were obtained from 3 fields per section, 3 sections per mouse, 3 mice per group. ***=p<0.001, Student's *t* test comparing the optoA$_{2A}$R with the mCherry. (**f, g**) Light stimulation of *optoA$_{2A}$R* in NAc increased the total distance travelled at the acquisition phase (**g**; for AAV vector, F(1, 44)=10.11,p<0.003; for behavioral phase, F(1,44)=25.08, p<0.001; AAV vector × behavioral phase, F(1,44)=18.68, p <0.001, two-way ANOVA; ***p<0.001, $^{###}$p<0.001, Bonferroni *post-hoc* test, comparing *optoA$_{2A}$R* with mCherry) but had no effect time spent in the novel arm during the 5-min "Light-On" period, compared with that of the mCherry (**f**; p = 0.276, Student's *t* test). Scale bar = 50 μm. Green arrow: co-localization of *optoA$_{2A}$R* with phospho-CREB; red arrow: phospho-CREB expression only; white arrow: co-localization of *optoA$_{2A}$R* with ENK, SP, phospho-CREB or phospho-MAPK; green arrow: ENK, SP, phospho-CREB or phospho-MAPK only; red arrow: mCherry expression only.