

Activation of $\alpha 7$ nicotinic acetylcholine receptors increases intracellular cAMP levels via activation of AC1 in hippocampal neurons

Qing Cheng, Jerrel L. Yakel*

Neurobiology Laboratory, NIEHS / NIH, 111 T.W. Alexander Dr., Durham, NC 27709, USA

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ABSTRACT

The activation of $\alpha 7$ nAChRs has been shown to improve hippocampal-dependent learning and memory. However, the molecular mechanism of $\alpha 7$ nAChRs' action remains elusive. We previously reported that activation of $\alpha 7$ nAChRs induced a prolonged enhancement of glutamatergic synaptic transmission in a PKA-dependent manner. Here, we investigated any connection between the activation of the $\alpha 7$ nAChR and cAMP signaling in hippocampal neurons. To address this question, we employed a FRET-based biosensor to measure the intracellular cAMP levels directly via live cell imaging. We found that application of the $\alpha 7$ nAChR-selective agonist choline, in the presence of the $\alpha 7$ nAChR positive allosteric modulator PNU-120596, induced a significant change in emission ratio of F535/F470, which indicated an increase in intracellular cAMP levels. This choline-induced increase was abolished by the $\alpha 7$ nAChR antagonist MLA and the calcium chelator BAPTA, suggesting that the cAMP increase depends on the $\alpha 7$ nAChR activation and subsequent intracellular calcium rise. The selective AC1 inhibitor CB-6673567 and siRNA-mediated deletion of AC1 both blocked the choline-induced cAMP increase, suggesting that calcium-dependent AC1 is required for choline's action. Furthermore, $\alpha 7$ nAChR activation stimulated the phosphorylation of synapsin, which serves as a downstream effector to regulate neurotransmitter release. Our findings provide the first direct evidence to link activation of $\alpha 7$ nAChRs to a cAMP rise via AC1, which defines a new signaling pathway employed by $\alpha 7$ nAChRs. Our study sheds light into potential molecular mechanisms of the positive cognitive actions of $\alpha 7$ nAChR agonists and development of therapeutic treatments for cognitive impairments.

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1. Introduction

The nicotinic ACh receptors (nAChRs) are in the superfamily of cys-loop cationic pentameric channels comprised of six α (2–7) and three β (2–4) nAChR subunits in the mammalian brain (Nashmi and Lester, 2006). They are activated by endogenous cholinergic inputs and exogenous compounds like nicotine. The $\alpha 7$ nAChR is one of most prevalent nAChR subtypes in the hippocampus (Jones et al., 1999; Albuquerque et al., 2009). Physiological studies have shown that $\alpha 7$ receptors have higher calcium permeability and lower affinity for acetylcholine than other nAChR subtypes (Albuquerque et al., 2009). Besides evoking brief current responses, nAChR

agonists also affect neuronal function in a long lasting fashion (Lena and Changeux, 1997; Zhong et al., 2008, 2013). Activation of $\alpha 7$ nAChRs via rapid stimulation of cholinergic inputs or acetylcholine application induced long-term potentiation (LTP) of synaptic transmission from CA3 to CA1 (Ji et al., 2001; Ge and Dani, 2005; Gu and Yakel, 2011). However how the $\alpha 7$ receptor is enhancing LTP, and furthermore the signaling mechanisms downstream of nAChR activation, remain elusive.

Recently, we found that activation of $\alpha 7$ nAChRs enhanced mossy fiber transmission in a PKA-dependent manner (Cheng and Yakel, 2014). In addition, inhibition of PKA in the dorsal hippocampus abolished nicotine's effect on learning (Gould et al., 2014). It is well accepted that activation of adenylyl cyclases (ACs) can increase glutamate release from hippocampal neurons (Chavez-Noriega and Stevens, 1994; Leenders and Sheng, 2005; Moulder et al., 2008). Moreover, $\alpha 7$ nAChRs were found to be physically associated with AC1 within lipid rafts of airway epithelium (Maouche et al., 2013). AC1 is one of the calcium- and calmodulin-

Abbreviations: ACh, acetylcholine; cAMP, cyclic adenosine monophosphate; nAChR, nicotinic acetylcholine receptor; AC, adenylyl cyclase.

* Corresponding author.

E-mail address: yakel@niehs.nih.gov (J.L. Yakel).

activated ACs and highly expressed in the dendritic arbors of the dentate gyrus and the mossy fiber projections (Nicol et al., 2005; Conti et al., 2007). Based on these facts, we hypothesized that $\alpha 7$ nAChRs exert their long-term actions in part through the mobilization of calcium and activation of calcium-dependent AC1. To test this hypothesis, we directly monitored intracellular cAMP levels through a FRET-based cAMP sensor $^{\text{T}}\text{Epac}^{\text{VV}}$ in real time in individual hippocampal neurons. We found that $\alpha 7$ nAChR activation led to a robust increase in cAMP level, which was blocked by $\alpha 7$ nAChR antagonists, the calcium chelator BAPTA, an AC1 inhibitor, and was reduced by siRNA against AC1. In addition, $\alpha 7$ nAChR activation resulted in the phosphorylation of synapsin. Our data suggest that the $\alpha 7$ nAChR employs the cAMP-PKA signaling pathway to phosphorylate synapsin, thereby mediating its modulation of synaptic transmission and positive actions on cognition. Delineating the downstream signaling pathway after $\alpha 7$ nAChR activation provides potential therapeutic targets, which could work in conjunction with nAChR agonists to treat cognitive disorders.

2. Materials and methods

2.1. Hippocampal neuronal culture

All animal procedures were conducted in accordance with National Institutes of Health animal welfare guidelines. Wild type C57BL/6J mice were purchased from Charles River. Postnatal day 0–2 pups of either sex were decapitated, and the hippocampi were removed, treated with papain (Worthington), and serially triturated. The dissociated neurons were plated onto poly-D-lysine coated glass coverslips. Cells were first cultured in FBS containing Neurobasal-A medium (Life technologies), and then switched to serum-free Neurobasal-A medium after 24 h.

2.2. Plasmids and siRNAs

We obtained a FRET-based cAMP sensor $^{\text{T}}\text{Epac}^{\text{VV}}$ from Dr. Jalink (University of Amsterdam, Netherlands) (Klarenbeek et al., 2011). The construct was confirmed by sequence analysis. The rat $\alpha 7$ nAChR plasmid was obtained from Dr. James Patrick at the Baylor College of Medicine (Houston, TX) (Seguela et al., 1993), and tested in $\alpha 7$ nAChR knockout mice (Gu et al., 2012). GCaMP3 was purchased from Addgene (plasmid no. 22692). Neurons were co-transfected with the cAMP sensor and $\alpha 7$ nAChR plasmids, or the GCaMP3 plasmid, after 3–4 days in culture via Lipofectamine 2000 (Life technologies). Imaging experiments were performed 4–7 days after transfection. Customized On-Target plus siRNA against mouse *Adcy1* were purchased from Dharmacon of GE Healthcare. Smart pool siRNA target sequences are: UGGCAAGUUCGAGAGUUA, GCUCAUGCUGCCGGA, GUACAACAUCUGCAACGA, GGACUUGACAUGAUCGAUA. Non-targeting pool siRNA sequences are: UGGUUUACA-UGCGACUAA, UGGUUUACA-UGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUCCUA. For siRNA experiments, siRNA and plasmids were co-transfected using DharmaFECT Duo Transfection reagent (Dharmacon of GE healthcare).

2.3. FRET imaging and data analysis

Hippocampal neurons less than 15 μm in diameter were imaged with a Zeiss LSM 510 META confocal microscope using a $63\times$, 1.1 NA water immersion objective. Images were collected with Zen2009 software (Carl Zeiss). The sensor was excited at 458 nm by an Argon ion laser, and emitted signals were detected simultaneously through 470–510 nm (F470) and 535–590 nm (F535) band-pass filters. Most FRET experiments were done at 30–60% power of the 458 nm Argon laser (output at 7.5 mW). The images were captured every 6s automatically. Images were analyzed with Zen2009 and OriginPro 8.5 (OriginLab Corporation). FRET ratio was expressed as ratio of F535 to F470 signals, and calculated for each region of interest (ROI). Mean FRET ratios were averaged from 8 to 12 ROIs selected randomly from peri-nuclear soma and primary process of each neuron. The mean baseline FRET ratio was obtained via averaging 1 min before the drug application. Choline-induced changes were calculated by averaging the FRET ratios during 9–10th min drug application. The effect of different chemicals was assessed by comparing the mean FRET ratios. Data are plotted as the mean $\pm$ SEM. Statistical tests were performed either with unpaired (for group comparison) or paired (for the same cell) Student's *t* tests.

The experiments were conducted at room temperature in extracellular solution containing (mM): NaCl 140; KCl 3; MgCl_2 2; CaCl_2 2; HEPES 20; glucose 10; pH adjusted to 7.3 with NaOH. To isolate the responses from nAChRs, the imaging experiments were performed in the presence of CNQX and APV (to block glutamate channels), and TTX (to block voltage-gated sodium channels). To amplify the cAMP rise, 50 μM IBMX (PDE inhibitor) was added as well. For calcium-free condition, we removed CaCl_2 from extracellular solution, and pre-incubated the neurons with 20 μM BAPTA-AM (Life technology) in Ca-free condition for 30 min before imaging. TTX, CNQX, APV, and PNU-120596 were purchased from Tocris. AC1 selective

inhibitor CB-6673567 were obtained from ChemBridge. All other compounds were purchased from Sigma unless stated otherwise.

2.4. Immunocytochemistry

Cultured neurons were fixed in 4% paraformaldehyde in phosphate buffer for 15 min, permeabilized with 0.25% Triton X-100 for 15 min and blocked with 10% normal goat serum for 1 h. Then neurons were incubated with primary antibody, either rabbit anti-AC1 (1:500, Novus Biologicals, catalog # NBP1-19628) or rabbit anti-p-synapsin Ia/Ib (ser 9) antibody (1:500, Santa Cruz, catalog # sc-135710), at 4 $^{\circ}\text{C}$ for overnight, and followed by secondary antibody goat anti-rabbit with DyLight 633 (1:1000, Novus Biologicals, catalog # NBP1-76077) at room temperature for 1 h. After washing, coverslips were mounted in Vectashield anti-fade mounting medium with Dapi (Vector laboratories). Images were acquired via Zeiss LSM 710 confocal microscope with a $63\times$ /oil immersion objective, and analyzed and processed using MetaMorph (Molecular Devices) and Adobe Photoshop (Adobe system). For each condition, we analyzed three coverslips from different neuronal cultures and 8–12 field of views. The phospho-synapsin puncta were selected using integrated morphometric analysis after intensity thresholding. The morphometric parameter for the size of area is 0.1–3 μm^2 .

3. Results

3.1. The cAMP sensor $^{\text{T}}\text{Epac}^{\text{VV}}$ detects forskolin-induced rise of cAMP in cultured hippocampal neurons

First, we examined the functional properties of the FRET-based cAMP sensor $^{\text{T}}\text{Epac}^{\text{VV}}$ in cultured mouse hippocampal neurons. The sensor contains the cAMP-binding protein Epac1 (exchange protein activated by cyclic AMP) and mTurquoise as donor and a double acceptor consisting of a tandem of Venus and $^{\text{cp173}}$ Venus. The increase of cellular cAMP levels leads to a conformational change in Epac1 and a reduction of FRET between donor and acceptor fluorescent proteins. We observed uniform cytosolic distribution of $^{\text{T}}\text{Epac}^{\text{VV}}$ fluorescence in both the soma and processes of hippocampal neurons (Fig. 1A). The sensor-expressing neurons displayed normal morphology, even at high expression levels, without any apparent adverse effects in these neurons. Next, we tested if this sensor displayed a cAMP-dependent change of FRET ratio with forskolin, an activator of most forms of adenylyl cyclases (AC). To maximize the cAMP signal, IBMX (50 μM), a phosphodiesterase (PDE) inhibitor, was added to the bath solution to prevent the breakdown of cAMP and maintain a sustained cAMP elevation. The addition of forskolin (20 μM) increased the fluorescence intensity of mTurquoise, along with the concomitant decrease of Venus fluorescence intensity, in hippocampal neurons (Fig. 1B). Accordingly, the ratio of Venus to mTurquoise fluorescence (F535/F470) demonstrated a robust decrease (Fig. 1A and C) in all 6 neurons tested with forskolin (Fig. 1D). The averaged FRET ratio at the 10th minute of forskolin application showed a significant reduction of $19.6 \pm 3.6\%$ compared to control ($2.4 \pm 0.8\%$, $p = 0.0008$, $n = 6$; Fig. 1E). The forskolin-induced FRET ratio reduction was comparable in magnitude and latency to that seen for this sensor in stable cell lines (Klarenbeek et al., 2011; Polito et al., 2013). These results suggest that $^{\text{T}}\text{Epac}^{\text{VV}}$ maintains its function as a cAMP sensor in hippocampal neurons.

3.2. Activation of $\alpha 7$ nAChRs increases intracellular cAMP levels in hippocampal neurons

Next, we tested if activation of $\alpha 7$ nAChRs could alter the cAMP levels and thus change the FRET ratio of this sensor. To reduce the variability of endogenous $\alpha 7$ nAChR expression levels in our cultured hippocampal neurons (Zhang et al., 1998; Liu et al., 2001), we routinely co-transfected the $\alpha 7$ nAChR with $^{\text{T}}\text{Epac}^{\text{VV}}$ for our experiments. The expression of $\alpha 7$ nAChRs did not change the expression pattern of $^{\text{T}}\text{Epac}^{\text{VV}}$, and the basal FRET ratio was similar throughout the soma and neuronal processes (Fig. 2A). We bath applied choline ($\alpha 7$ nAChR-selective agonist, 2 mM) together with

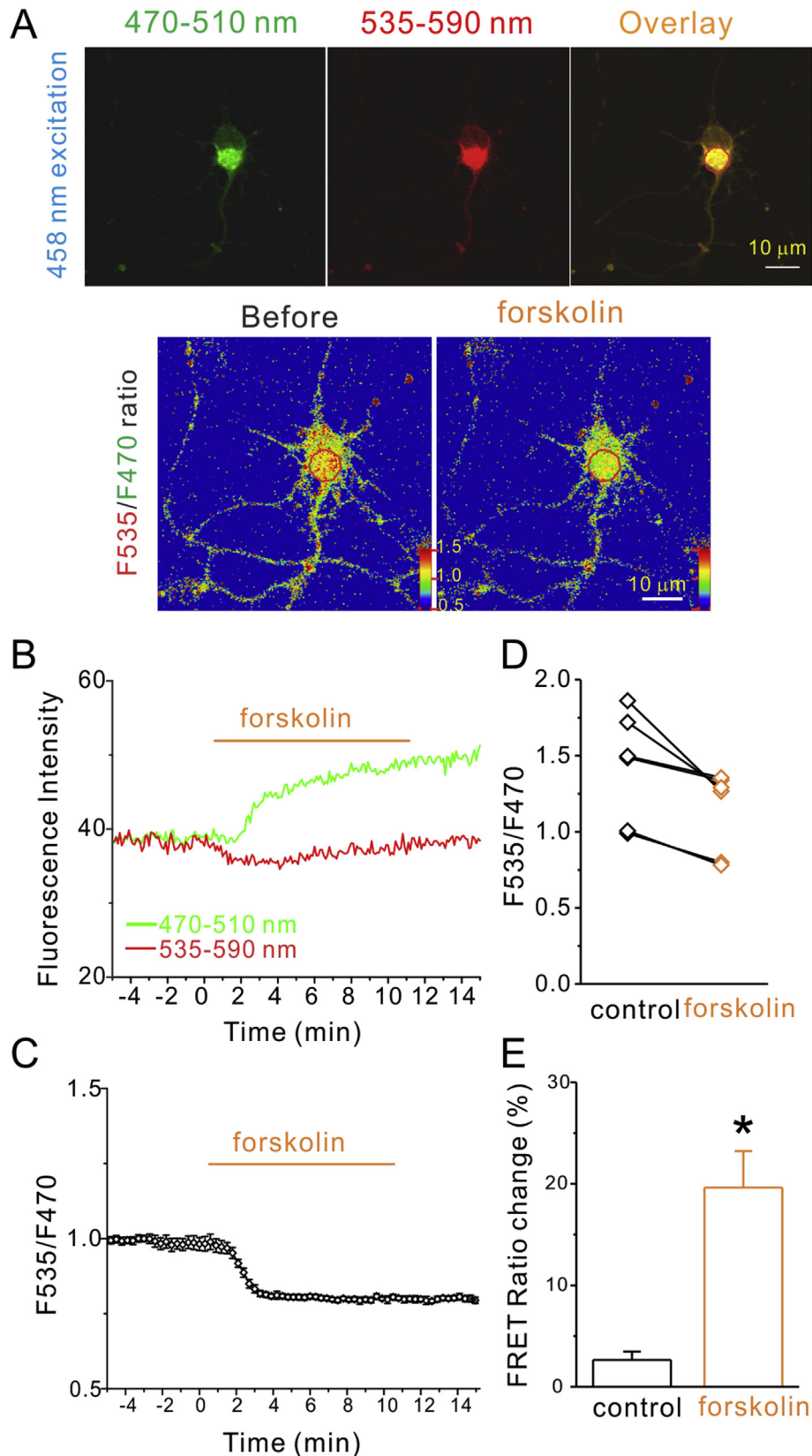


Fig. 1. cAMP sensor maintains its function in cultured hippocampal neurons. (A) Fluorescent images of a representative hippocampal neuron expressing the cAMP sensor $T_{\text{Epac}}^{\text{Vv}}$ excited with 458 nm laser and viewed with 470–510 nm emission spectrum (F470, left upper panel), or 535–590 nm emission spectrum (F535, middle upper panel), and overlay (right upper panel). The lower panel displayed F535/F470 ratio images before (left) and during forskolin application (right) at 10 min time point. (B) The fluorescence intensity of red circle outlined in the overlay panel of (A) in F470 (green) and F535 (red) spectrums were plotted against time of the bath application of forskolin (20 μM). (C) The FRET ratio (F535/F470 spectrum) was plotted against the time of bath application of forskolin (20 μM). (D) FRET ratios of 6 tested neurons were plotted against listed conditions. (E) The histogram shows forskolin-induced net decrease of normalized FRET ratio in listed conditions. Data shown are mean $\pm$ SEM; statistical significance was determined by Student's *t*-test (* denotes $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

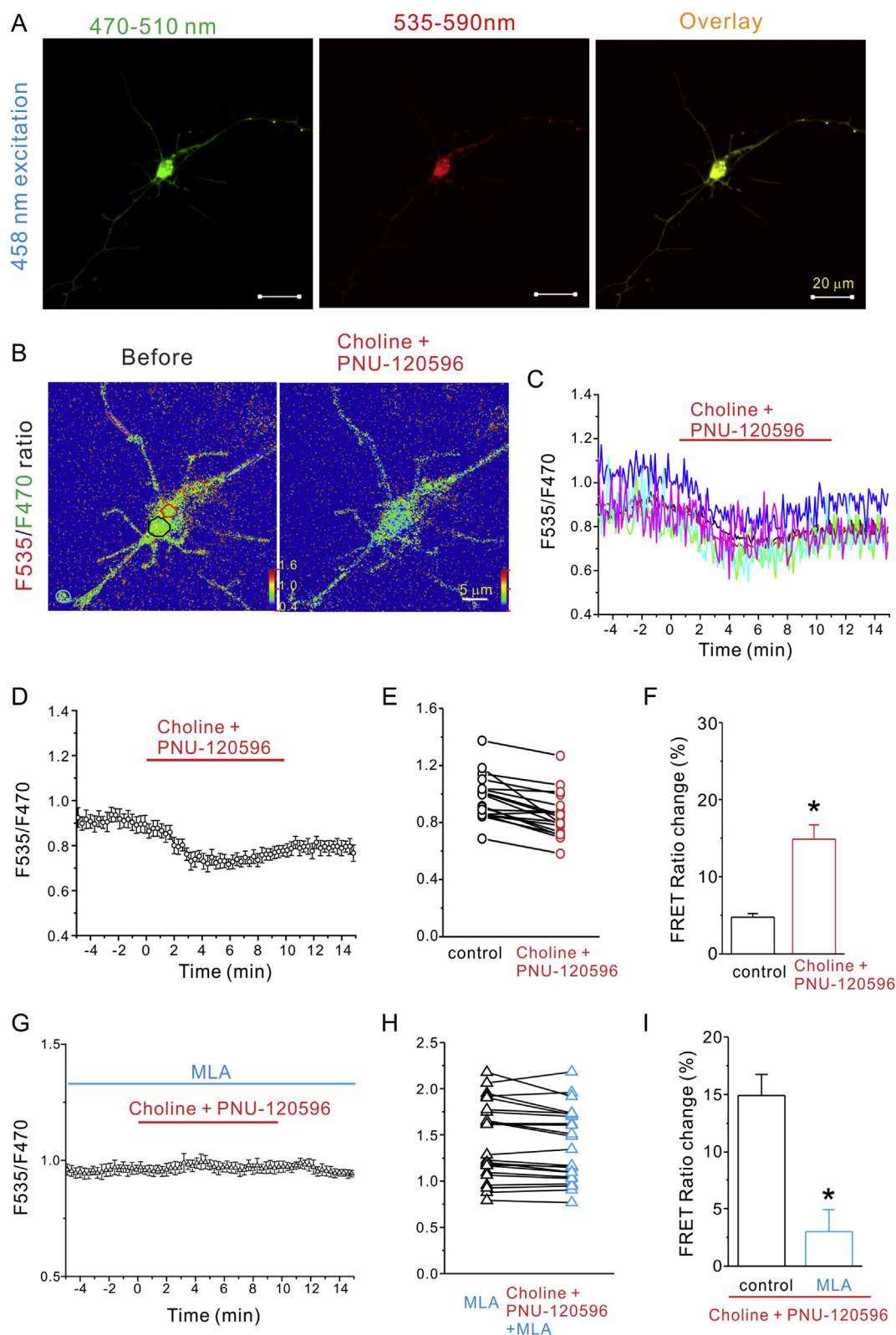


Fig. 2. Choline and PNU-120596 decreased FRET ratio in hippocampal neurons expressing cAMP sensor and $\alpha 7$ nAChRs is dependent on activation of $\alpha 7$ nAChRs. **A)** Fluorescent images of a representative hippocampal neuron expressing the cAMP sensor $T\text{Epa}^{\text{CV}}$ and $\alpha 7$ nAChRs excited with 458 nm laser and viewed with 470–510 nm emission spectrum (F470, left), or 535–590 nm emission spectrum (F535, middle), and overlay (right) (scale bar = 20 μ m). **(B)** F535/F470 ratio images before (left) and during choline and PNU-120596 (right) application at 10 min time point with color coded ROIs. FRET ratios of individual ROIs (**C**) and the averaged FRET ratio (**D**) were plotted against the time of bath application of choline (2 mM) and PNU-120596 (5 μ M) (from 0 to 10 min). The red line indicates the duration of choline and PNU-120596 perfusion. **(E)** and **(H)** FRET ratios of tested neurons were plotted against listed conditions. **(F)** and **(I)** The histogram shows choline and PNU-120596-induced net decrease of normalized FRET ratio in listed conditions. **(G)** The averaged FRET ratio of a representative neuron was plotted against the time before, during and after application of choline (2 mM) and PNU-120596 (5 μ M) in the presence of MLA (40 nM). Data shown are mean $\pm$ SEM; statistical significance was determined by Student's *t*-test (* denotes $p < 0.05$).

PNU-120596 (an $\alpha 7$ nAChR positive allosteric modulator [PAM]; 5 μ M) to induce substantial and prolonged activation of $\alpha 7$ nAChRs. After 3–5 min of choline and PNU-120596 application, we observed an initial reduction in the FRET ratio among all selected regions (soma and processes), which reached a maximum around 8–10 min after application (Fig. 2C, D). The slowness in response initiation was due to slow bath perfusion, which was necessary to prevent movement during imaging. The averaged FRET ratio at the 10th minute of forskolin application showed a significant reduction of $14.9 \pm 1.8\%$ compared to control ($4.8 \pm 0.5\%$, $p = 0.02$, $n = 20$; Fig. 2F). Therefore the choline and PNU-120596-induced FRET ratio reduction indicated a significant increase in intracellular cAMP levels. We further analyzed the FRET ratio data by separating FRET signals into two groups: soma and neurite (neuronal processes at least 10 μ m away from cell bodies). We found that the basal FRET ratio is very similar between the two groups (soma: 1.29 ± 0.03 , and neurites: 1.29 ± 0.16 , t -test, $p = 0.96$), however the neurite group has a larger standard error due to its lower fluorescence intensity (5.3 a.u. versus 32.5 a.u. for the soma). This data suggests that while

the cAMP level is similar throughout the neurons, the difference between different compartments could not be differentiated by the current cAMP sensor (with an EC_{50} value of about 14 μ M) due to the lower signal-to-noise ratio in the neurites. The treatment of choline and PNU-120596 resulted in a significant FRET ratio change of $13.9 \pm 1.5\%$ in the soma group (compared to the control of $2.9 \pm 0.7\%$), and $12.9 \pm 1.7\%$ in the neurite group (not statistically different from control of $13.9 \pm 1.2\%$). Though we noticed that the FRET ratio did show a comparable degree of reduction for both the soma and neurite groups, the measurement from neurites is undermined by the low fluorescence intensity and inherent higher noise level. Therefore we could not gain much information from the measurement derived from the neurites.

3.3. Choline-induced cAMP increase requires $\alpha 7$ nAChR activation and subsequent calcium rise

To determine whether the choline and PNU-120596-induced cAMP increase requires activation of $\alpha 7$ nAChRs, we measured

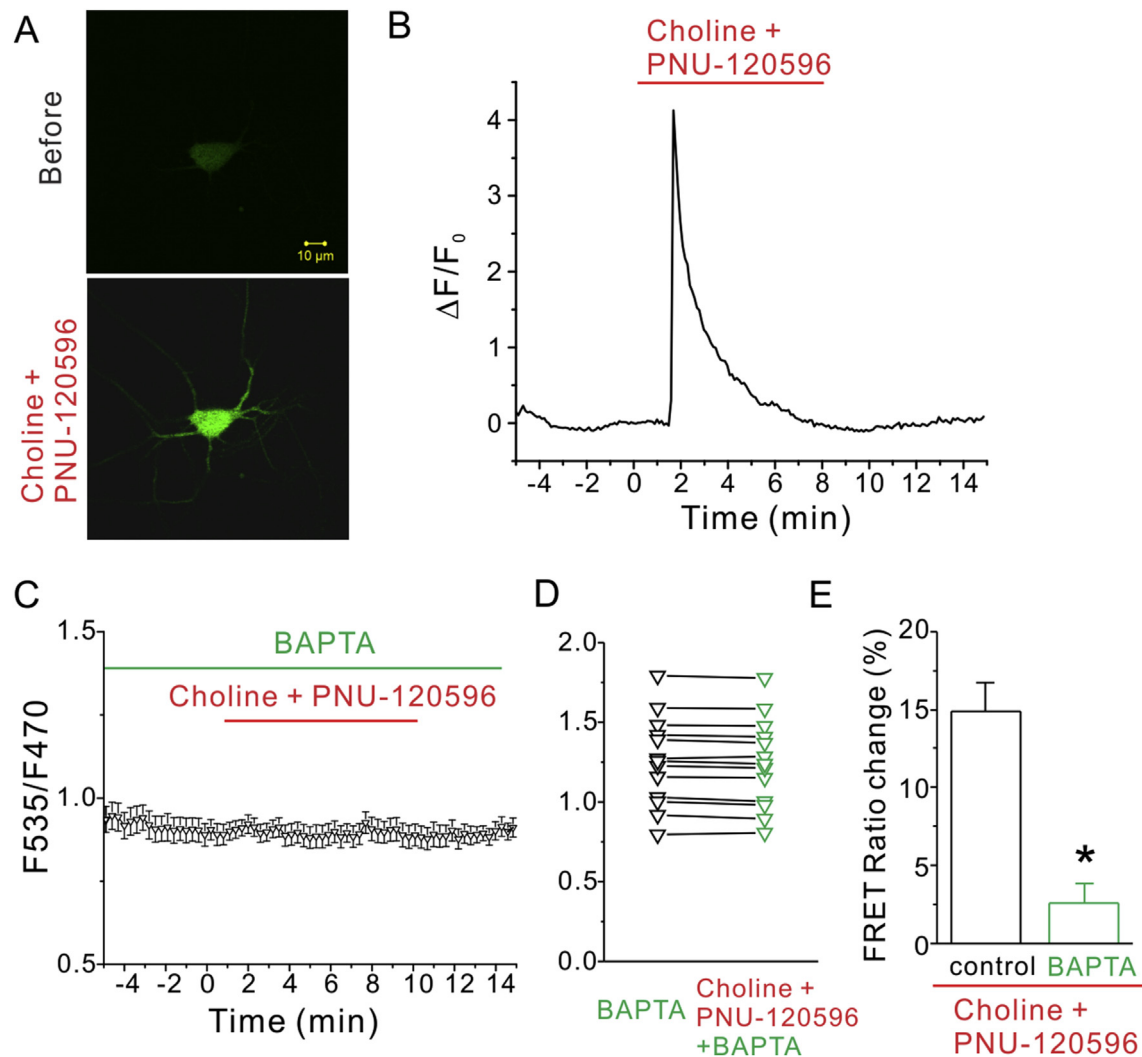


Fig. 3. Choline and PNU-120596 induced a robust intracellular calcium rise which was required for choline and PNU-120596-induced decrease of FRET ratio. (A) Fluorescent images of a representative hippocampal neuron expressing GCaMP3 were shown before (top) and during the application of choline and PNU-120596 (bottom). (B) Time course of fluorescence intensity of GCaMP3 revealed calcium rise evoked by application of choline and PNU-120596. (C) The averaged FRET ratio of a representative neuron transfected with the cAMP sensor $^{T}Epac^{VV}$ and $\alpha 7$ nAChRs was plotted against the time before, during and after application of choline (2 mM) and PNU-120596 (5 μ M) in calcium-free conditions. (D) FRET ratios of individual tested neurons were plotted against indicated conditions. (E) The histogram shows choline and PNU-120596-induced net decrease of normalized FRET ratio in listed conditions. * denotes $p < 0.05$.

the FRET ratio in the presence of MLA (a selective $\alpha 7$ nAChR antagonist). Application of MLA (40 nM) completely blocked the choline and PNU-120596-induced FRET ratio change ($2.6 \pm 1.9\%$, $n = 24$, $p = 0.0006$, Fig. 2G–I). Furthermore, choline and PNU-120596 also failed to induce any FRET ratio change in neurons transfected with the cAMP sensor alone (i.e. without the $\alpha 7$ nAChR) at the age of 6 DIV (data not shown), when the endogenous expression of $\alpha 7$ nAChRs could not be detected both electrophysiologically and biochemically (Colon-Saez and Yakel, 2011). These results suggested that the cAMP increase depends on $\alpha 7$ nAChR activation.

Since the $\alpha 7$ nAChR is highly permeable to calcium, we tested whether the choline and PNU-120596-induced FRET ratio change depended on calcium. First, we confirmed that there was an increase in cytoplasmic calcium levels induced by choline and PNU-120596 using the genetic calcium indicator, GCaMP3. As expected, the fluorescence of GCaMP3 increased sharply upon choline and PNU-120596 application, reaching a peak in 20–30 s, and decreased back to baseline within 3–5 min (Fig. 3A, B). Next, we removed calcium from the extracellular solution and incubated the neurons with BAPTA-AM (20 μ M) for 30 min before imaging. In this calcium-free condition, choline and PNU-120596 failed to induce any FRET ratio change ($3.2 \pm 1.3\%$, $n = 16$, $p = 0.0002$, Fig. 3C–E). This data indicated that the choline and PNU-120596-induced cAMP increase depends on the $\alpha 7$ nAChR-evoked intracellular calcium rise.

3.4. Choline-induced cAMP increase requires AC1 activation

Based on our observation that activation of the $\alpha 7$ nAChR induced increases in cytoplasmic levels of calcium and cAMP, and that the latter depended on calcium, we hypothesized that the $\alpha 7$ nAChR-induced calcium rise may be activating a calcium-dependent adenylyl cyclase (AC), which in turn led to the production of cAMP. There are two known types of calcium-activated ACs in hippocampal neurons, AC1 and AC8 (Hanoune and Defer, 2001; Conti et al., 2007; Wang and Zhang, 2012). Our previous study showed that the PKA-dependent enhancement of glutamate release was mediated by activation of $\alpha 7$ nAChRs in the hippocampal mossy fiber terminals, which originated from dentate granule cells. AC1 is highly expressed in both dentate granule cell dendrites and mossy fiber axons (Conti et al., 2007). Therefore, we asked if the choline and PNU-120596-induced cAMP rise required the activity of AC1. To test this, we measured the FRET ratio change in the presence of a selective AC1 inhibitor CB-6673567 (50 μ M) (Brand et al., 2013). The addition of CB-6673567 alone did not change the basal FRET ratio, suggesting that the basal AC1 activity is low. In the presence of CB-6673567, the choline and PNU-120596-induced FRET ratio change was greatly reduced ($3.1 \pm 2.5\%$, $n = 14$, $p = 0.002$, Figs. 4A–C), suggesting that calcium-dependent AC1 is required for $\alpha 7$ nAChR's action.

Next we asked if the knockdown of AC1 would block choline's action. To test this, we compared the effect of small interfering RNAs (siRNAs) targeted against AC1 and a scrambled siRNA in transfected neurons. To evaluate the efficiency of these siRNAs, we measured the AC1 immunoreactivity from neurons 72 h after siRNA and EPAC transfection. The EPAC expression was comparable among control neurons, and neurons treated with either scrambled siRNA, or siRNA against AC1 (47.9 ± 5.7 AU, 43.3 ± 4.4 AU and 45.6 ± 2.9 AU respectively, Fig. 4F). On the contrary, the immunoreactivity to AC1 of EPAC-expressing neurons was greatly reduced after treatment of siRNA against AC1 (45.4 ± 3.3 AU, $n = 87$) compared to that of scrambled siRNA (75.2 ± 5.2 AU, $n = 73$, $p < 0.01$) (Fig. 4D, E). This data suggested that siRNA against AC1 successfully reduced the level of AC1. Then, we examined the

choline and PNU-120596-induced FRET ratio change in siRNA-treated neurons. Choline and PNU-120596 failed to reduce the FRET ratio in neurons treated with siRNA targeted against AC1 (Fig. 4H), while they reduced the FRET ratio in non-target scrambled siRNA treated neurons (Fig. 4G). The choline-induced FRET ratio change in non-target scrambled siRNA treated neurons was not significantly different from that of non-treated control neurons ($13.9 \pm 2.6\%$, $n = 17$, $p = 0.80$, Fig. 4I), but in neurons treated with siRNA targeted against AC1, the choline-induced FRET ratio change was significantly smaller than that of control neurons ($4.2 \pm 1.5\%$, $n = 21$, $p = 0.017$, Fig. 4I). These results suggested that AC1 is required for the choline-induced cAMP rise in hippocampal neurons.

3.5. $\alpha 7$ nAChR activation leads to the phosphorylation of synapsin

Lastly to identify possible downstream targets of the $\alpha 7$ nAChR-AC1-PKA cascade, we examined the phosphorylation status of one of the prominent PKA substrates at presynaptic terminals, synapsin (Huttner and Greengard, 1979). To determine if the phosphorylation of synapsin occurred upon activation of $\alpha 7$ nAChRs, we stained the hippocampal neurons with a phospho-specific antibody directed against the PKA phosphorylation site (serine 9) of synapsin I (Hilfiker et al., 2005). At the basal level, synapsin phosphorylation at serine 9 was very sparse and only observed in a small subset of terminals (Fig. 5A). Incubation with 20 μ M forskolin resulted in the widespread phosphorylation of synapsin as expected. Treatment of choline and PNU-120596 for 4 min produced a similar effect to that of forskolin. Quantitative measurements showed a significant increase in the number of puncta containing phosphorylated synapsin in either forskolin or choline and PNU-120596 treated neurons (Fig. 5B). The size and fluorescence intensity of the puncta also displayed significant increases for either forskolin or choline and PNU-120596 treated neurons (Fig. 5C and D). These data suggested that activation of $\alpha 7$ nAChRs leads to the phosphorylation of synapsin at the PKA site.

4. Discussion

cAMP has long been thought of as a gating molecule to control the expression of short-term plasticity and its conversion to long-term plasticity (Kandel, 2012; Kandel et al., 2014). In this study, we investigated its involvement in $\alpha 7$ nAChR-mediated signaling mechanisms with a cAMP biosensor in live cultured mouse hippocampal neurons. We found that activation of $\alpha 7$ nAChRs induced an elevation in intracellular cAMP levels, which depended on the rise of cytoplasmic calcium and activation of the calcium-activated adenylyl cyclase, AC1.

In contrast to biochemical measurements, fluorescence sensors allow us to study the dynamic change in cAMP levels in individual live neurons. We employed the ¹Epac^{vv} sensor (Klarenbeek et al., 2011) for its brightness, reduced pKa value (less sensitive to pH change), and large dynamic range. Even in the presence of IBMX, we observed low basal cAMP levels in our cultured neurons, indicating little basal AC activity, similar to the report from cortical neurons (Yamashita et al., 1997). In addition, the intracellular cAMP level in neurons can vary depending on cell types, preparations, and developmental stages (Nikolaev et al., 2004; Willoughby and Cooper, 2008; Castro et al., 2010). In contrast to the consistent forskolin-induced cAMP increase, there was more variability in the $\alpha 7$ nAChR-induced increase in cAMP levels; this could stem from differential density of $\alpha 7$ nAChRs, calcium buffering capacity, and distribution of calcium-dependent AC1 in cultured hippocampal neurons.

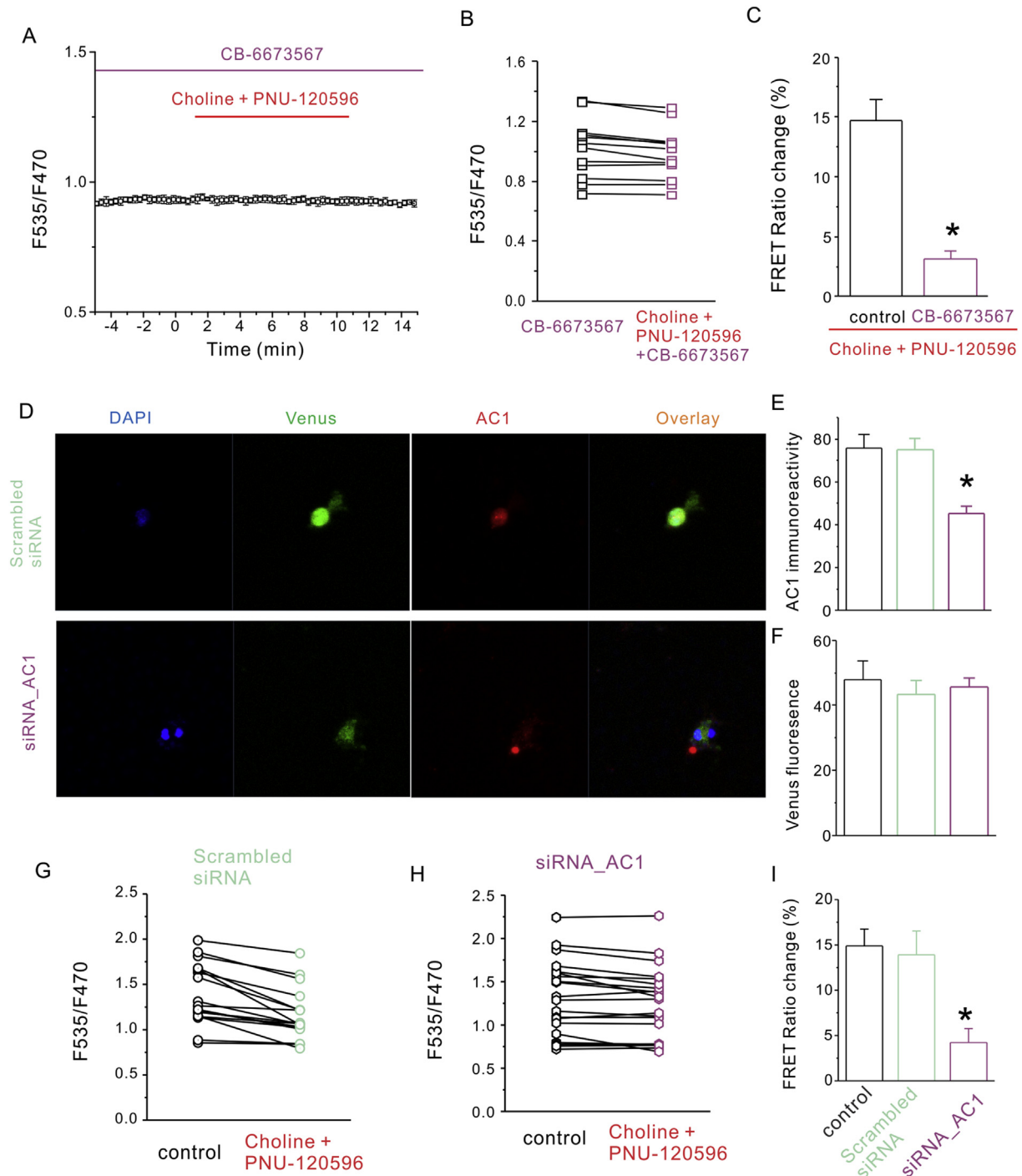


Fig. 4. Either AC1 inhibitor or knockdown of AC1 with siRNA reduced choline and PNU-120596-induced decrease of FRET ratio. (A) The averaged FRET ratio of a representative neuron was plotted against the time before, during and after application of choline (2 mM) and PNU-120596 (5 μ M) in the presence of CB-6673567 (50 μ M). (B) FRET ratios of individual tested neurons were plotted against indicated conditions. (C) The histogram shows choline and PNU-120596-induced net decrease of normalized FRET ratio in listed conditions. (D) Images of neurons transfected with EPAC and $\alpha 7$ nAChRs treated with either scrambled siRNA (upper panel) or siRNA against AC1 (lower panel) were shown with DAPI staining (blue), EPAC fluorescence (green) and immunostaining against AC1 (red). Immunoreactivity against AC1 (E) but not fluorescence intensity of Venus (F) was decreased for neurons treated with siRNA_AC1. The FRET ratios of individual tested neurons were plotted for scrambled siRNA (G) and siRNA against AC1 (H). (I) The histogram shows choline and PNU-120596-induced net decrease of normalized FRET ratio in listed conditions. Data shown are mean $\pm$ SEM; statistical significance was determined by Student's *t*-test (* denotes $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Here we have provided several lines of evidence suggesting that the choline and PNU-120596-induced cAMP increase depended on activation of the $\alpha 7$ nAChRs: 1) the cAMP increase was induced by choline, the $\alpha 7$ nAChR-selective agonist, and PNU-120596, the $\alpha 7$ nAChR-selective PAM; 2) the cAMP increase was blocked by a low

concentration of MLA (40 nM), an $\alpha 7$ nAChR-selective antagonist; and 3) the cAMP increase was present only in neurons co-transfected with the cAMP sensor and the $\alpha 7$ nAChR subunit. The $\alpha 7$ nAChRs have a high calcium permeability, comparable to that of NMDA receptors (Uteshev, 2012), which confers an ability to

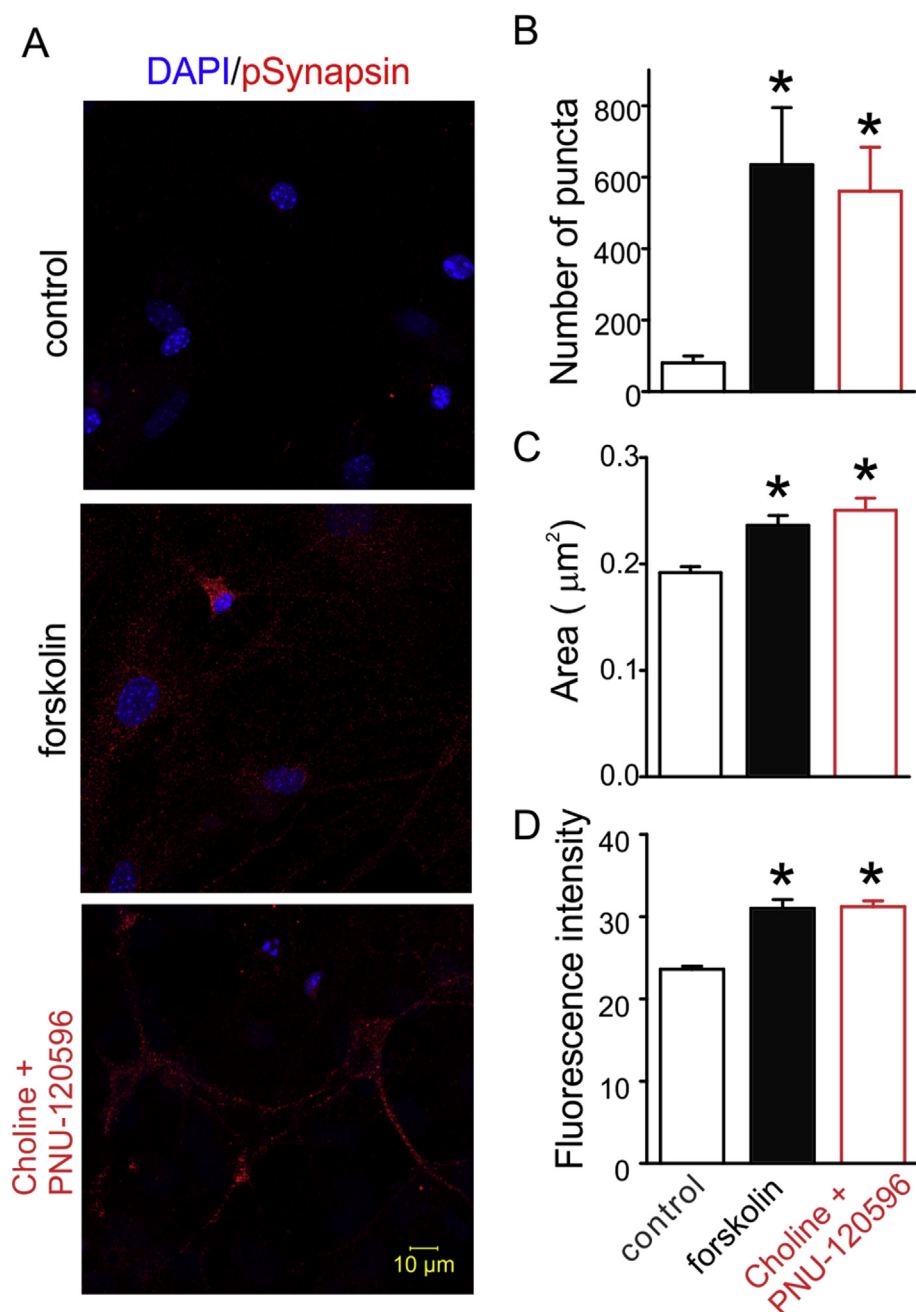


Fig. 5. Activation of $\alpha 7$ nAChR induces the phosphorylation of synapsin at PKA site. (A) Hippocampal neurons were stained against phospho-synapsin I (red) and DAPI (blue). Stimulation with either forskolin (center) or choline and PNU-120596 (right) caused a big increase of immunofluorescence compared to control (left). (B)–(D) Histograms show the changes of the size, fluorescence intensity, and number of phospho-synapsin puncta. Data shown are mean $\pm$ SEM; statistical significance was determined by Student's *t*-test (* denotes $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transduce a brief ionic current to a long-lasting activation of signaling molecules (Fagen et al., 2003; McKay et al., 2007). Indeed, we observed a prominent calcium rise upon activation of $\alpha 7$ nAChRs via fluorescent imaging using the genetically-encoded calcium sensor GCaMP3. The calcium rise could come from calcium influx through opened $\alpha 7$ nAChRs, as well as the secondary activation of voltage-gated calcium channels, or calcium-induced calcium release from intracellular calcium stores (Sharma and Vijayaraghavan, 2003; Dajas-Bailador and Wonnacott, 2004). When we removed external calcium and chelated internal calcium with BAPTA, activation of $\alpha 7$ nAChRs now failed to increase cAMP levels, suggesting that a subsequent calcium rise following $\alpha 7$

nAChR activation is required for the cAMP increase. This also suggests that there might not be a direct interaction between the $\alpha 7$ nAChR and ACs, but rather that calcium is the intermediate signaling molecule to couple the activation of $\alpha 7$ nAChRs to the cAMP pathway.

Traditionally, the calcium signaling cascade induced by $\alpha 7$ nAChRs is linked to the CaMKII pathway (Sharma et al., 2008), although several studies have linked the activation of $\alpha 7$ nAChRs to cAMP-dependent responses. In the perforant pathway to dentate granule cells, the acute nAChR enhancement of LTP required activation of PKA (Welsby et al., 2009). In cultured hippocampal neurons, nicotine increased the activity of ERK1/2 via $\alpha 7$ nAChR

activation in a calcium- and PKA-dependent manner (Dajas-Bailador et al., 2002). In addition, we recently reported that the action of $\alpha 7$ nAChRs at mossy fiber terminals also depended on PKA activity (Cheng and Yakel, 2014). AC1 and AC8 are the only two calcium-dependent ACs in rodent hippocampus. During the early development of the rodent brain, mRNA of AC1 was very high in the cerebral cortex, striatum, thalamus and brainstem, and decreased considerably after third the postnatal week (Matsuoka et al., 1997; Nicol et al., 2005). Conversely, AC8 has very low and limited expression during development and begins to express strongly in mature neurons (Nicol et al., 2005). AC1 plays a crucial role in the refinement of axonal arbors in the somatosensory and visual system in early development stages (Nicol et al., 2006, 2007; Dhande et al., 2012). AC1 is concentrated in the mossy fiber tract in the mature hippocampus (Conti et al., 2007), and deletion of AC1 led to deficiencies in mossy fiber LTP (Villacres et al., 1998) and impaired spatial memory (Wu et al., 1995). Moreover, calcium-stimulated AC-dependent PKA activation in presynaptic terminals was shown to be primarily driven by AC1, but not AC8, in hippocampus (Conti et al., 2009). Lastly, the $\alpha 7$ nAChR and AC1 were reported to associate physically and functionally in epithelium (Maouche et al., 2013). Here we have shown that either the selective AC1 inhibitor, or knockdown of AC1 with siRNA, blocked the $\alpha 7$ nAChR-mediated cAMP increase, suggesting that the calcium-dependent AC1 is the key signaling molecule for translating the short calcium rise to cAMP signaling when the $\alpha 7$ nAChRs are activated. If activation of $\alpha 7$ nAChRs, which increases the concentration of cAMP at presynaptic terminals, is accompanied by another depolarization and calcium influx, the strengthening of synaptic transmission would be further enhanced (Gu and Yakel, 2011).

Recently, we found that nAChR activation also converged with the cAMP-PKA signaling pathway to enhance neurotransmission at mossy fiber terminals (Cheng and Yakel, 2014). The cAMP-PKA pathway has long been shown to play a major role in synaptic plasticity at mossy fiber terminals (Huang et al., 1994; Weisskopf et al., 1994; Huang et al., 1995). However, the effectors downstream of this signaling pathway remain elusive. Evidence has shown the PKA-dependent enhancement of synaptic efficacy through the recruitment of synaptic vesicles (SVs) from the reserve pool to the readily releasable pool of vesicles (Trudeau et al., 1996; Kuromi and Kidokoro, 2000; Park et al., 2014). Synapsins, a prominent PKA substrate (Huttner and Greengard, 1979), can regulate neurotransmitter release via mobilization of SVs in the reserve pool (Hilfiker et al., 1999) and alteration of the kinetics of SV fusion (Hilfiker et al., 1998; Humeau et al., 2001; Samigullin et al., 2004; Hilfiker et al., 2005). Additionally, PKA-mediated synapsin I phosphorylation served as a key modulator for calcium-dependent synaptic plasticity (Menegon et al., 2006; Fiumara et al., 2007; Cousin and Evans, 2011). This makes synapsin a suitable candidate to transduce the $\alpha 7$ nAChR-induced calcium and subsequent cAMP rise into the enhancement of glutamate release. Indeed, we found that the activation of $\alpha 7$ nAChRs leads to the widespread increase of phosphorylated synapsin I (via its PKA site), similar to the extent of direct AC activation by forskolin. The enhancement of neurotransmitter release is most likely to involve other PKA substrates besides synapsin. Among them, RIM1 α is known to be essential for PKA-dependent LTP in the cerebellum (Lonart et al., 2003).

5. Conclusion

Activation of $\alpha 7$ nAChRs regulates the release of neurotransmitter and synaptic plasticity (Ji et al., 2001; Ge and Dani, 2005; Gu and Yakel, 2011; Cheng and Yakel, 2014), and promotes the maturation of adult-born neurons (Campbell et al., 2010) and glutamate

synapse formation (Lozada et al., 2012); these various actions are the result of triggering various signaling cascades. Our results identify a cAMP-dependent pathway as one of the molecular mechanisms employed by the $\alpha 7$ nAChR. Recent studies have shown that targeting $\alpha 7$ nAChRs offers symptomatic improvement of cognitive deficits in Alzheimer's disease, schizophrenia, and Down syndrome (Levin et al., 2006; Thomsen et al., 2010; Deutsch et al., 2013, 2014). Our finding provides insight for the development of therapeutic treatments for these and possibly other neuronal disorders. Future studies will determine which presynaptic proteins are the substrates of the $\alpha 7$ nAChR-activated PKA, and how they enhance the release of glutamate.

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References

- Albuquerque, E.X., Pereira, E.F., Alkondon, M., Rogers, S.W., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol. Rev.* 89, 73–120.
- Brand, C.S., Hocker, H.J., Gorfe, A.A., Cavaletto, C.N., Dessauer, C.W., 2013. Isoform selectivity of adenylyl cyclase inhibitors: characterization of known and novel compounds. *J. Pharmacol. Exp. Ther.* 347, 265–275.
- Campbell, N.R., Fernandes, C.C., Half, A.W., Berg, D.K., 2010. Endogenous signaling through $\alpha 7$ -containing nicotinic receptors promotes maturation and integration of adult-born neurons in the hippocampus. *J. Neurosci.* 30, 8734–8744.
- Castro, L.R., Gervasi, N., Guiot, E., Cavellini, L., Nikolaev, V.O., Paupardin-Tritsch, D., Vincent, P., 2010. Type 4 phosphodiesterase plays different integrating roles in different cellular domains in pyramidal cortical neurons. *J. Neurosci.* 30, 6143–6151.
- Chavez-Noriega, L.E., Stevens, C.F., 1994. Increased transmitter release at excitatory synapses produced by direct activation of adenylyl cyclase in rat hippocampal slices. *J. Neurosci.* 14, 310–317.
- Cheng, Q., Yakel, J.L., 2014. Presynaptic $\alpha 7$ nicotinic acetylcholine receptors enhance hippocampal mossy fiber glutamatergic transmission via PKA activation. *J. Neurosci.* 34, 124–133.
- Colon-Saez, J.O., Yakel, J.L., 2011. The $\alpha 7$ nicotinic acetylcholine receptor function in hippocampal neurons is regulated by the lipid composition of the plasma membrane. *J. Physiol.* 589, 3163–3174.
- Conti, A.C., Maas Jr., J.W., Moulder, K.L., Jiang, X., Dave, B.A., Mennerick, S., Muglia, L.J., 2009. Adenylyl cyclases 1 and 8 initiate a presynaptic homeostatic response to ethanol treatment. *PLoS One* 4, e5697.
- Conti, A.C., Maas Jr., J.W., Muglia, L.M., Dave, B.A., Vogt, S.K., Tran, T.T., Rayhel, E.J., Muglia, L.J., 2007. Distinct regional and subcellular localization of adenylyl cyclases type 1 and 8 in mouse brain. *Neuroscience* 146, 713–729.
- Cousin, M.A., Evans, G.J., 2011. Activation of silent and weak synapses by cAMP-dependent protein kinase in cultured cerebellar granule neurons. *J. Physiol.* 589, 1943–1955.
- Dajas-Bailador, F., Wonnacott, S., 2004. Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol. Sci.* 25, 317–324.
- Dajas-Bailador, F.A., Soliakov, L., Wonnacott, S., 2002. Nicotine activates the extracellular signal-regulated kinase 1/2 via the $\alpha 7$ nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. *J. Neurochem.* 80, 520–530.
- Deutsch, S.I., Burket, J.A., Benson, A.D., 2014. Targeting the $\alpha 7$ nicotinic acetylcholine receptor to prevent progressive dementia and improve cognition in adults with Down's syndrome. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 54C, 131–139.
- Deutsch, S.I., Schwartz, B.L., Schooler, N.R., Brown, C.H., Rosse, R.B., Rosse, S.M., 2013. Targeting $\alpha 7$ nicotinic neurotransmission in schizophrenia: a novel agonist strategy. *Schizophr. Res.* 148, 138–144.
- Dhande, O.S., Bhatt, S., Anishchenko, A., Elstrott, J., Iwasato, T., Swindell, E.C., Xu, H.P., Jamrich, M., Itoharu, S., Feller, M.B., Crair, M.C., 2012. Role of adenylyl cyclase 1 in retinofugal map development. *J. Comp. Neurol.* 520, 1562–1583.
- Fagen, Z.M., Mansvelder, H.D., Keath, J.R., McGehee, D.S., 2003. Short- and long-term modulation of synaptic inputs to brain reward areas by nicotine. *Ann. N. Y. Acad. Sci.* 1003, 185–195.
- Fiumara, F., Milanese, C., Corradi, A., Giovedi, S., Leitinger, G., Menegon, A., Montarolo, P.G., Benfenati, F., Ghirardi, M., 2007. Phosphorylation of synapsin domain A is required for post-tetanic potentiation. *J. Cell. Sci.* 120, 3228–3237.
- Ge, S., Dani, J.A., 2005. Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. *J. Neurosci.* 25, 6084–6091.

- Gould, T.J., Wilkinson, D.S., Yildirim, E., Poole, R.L., Leach, P.T., Simmons, S.J., 2014. Nicotine shifts the temporal activation of hippocampal protein kinase A and extracellular signal-regulated kinase 1/2 to enhance long-term, but not short-term, hippocampus-dependent memory. *Neurobiol. Learn Mem.* 109, 151–159.
- Gu, Z., Yakel, J.L., 2011. Timing-dependent septal cholinergic induction of dynamic hippocampal synaptic plasticity. *Neuron* 71, 155–165.
- Gu, Z., Lamb, P.W., Yakel, J.L., 2012. Cholinergic coordination of presynaptic and postsynaptic activity induces timing-dependent hippocampal synaptic plasticity. *J. Neurosci.* 32, 12337–12348.
- Hanoune, J., Defer, N., 2001. Regulation and role of adenylyl cyclase isoforms. *Annu. Rev. Pharmacol. Toxicol.* 41, 145–174.
- Hilfiker, S., Schweizer, F.E., Kao, H.T., Czernik, A.J., Greengard, P., Augustine, G.J., 1998. Two sites of action for synapsin domain E in regulating neurotransmitter release. *Nat. Neurosci.* 1, 29–35.
- Hilfiker, S., Pieribone, V.A., Czernik, A.J., Kao, H.T., Augustine, G.J., Greengard, P., 1999. Synapsins as regulators of neurotransmitter release. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354, 269–279.
- Hilfiker, S., Benfenati, F., Doussau, F., Nairn, A.C., Czernik, A.J., Augustine, G.J., Greengard, P., 2005. Structural domains involved in the regulation of transmitter release by synapsins. *J. Neurosci.* 25, 2658–2669.
- Huang, Y.Y., Li, X.C., Kandel, E.R., 1994. cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late phase. *Cell* 79, 69–79.
- Huang, Y.Y., Kandel, E.R., Varshavsky, L., Brandon, E.P., Qi, M., Idzerda, R.L., McKnight, G.S., Bourtschouladze, R., 1995. A genetic test of the effects of mutations in PKA on mossy fiber LTP and its relation to spatial and contextual learning. *Cell* 83, 1211–1222.
- Humeau, Y., Doussau, F., Vitiello, F., Greengard, P., Benfenati, F., Poulain, B., 2001. Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in Aplysia. *J. Neurosci.* 21, 4195–4206.
- Huttner, W.B., Greengard, P., 1979. Multiple phosphorylation sites in protein I and their differential regulation by cyclic AMP and calcium. *Proc. Natl. Acad. Sci. U. S. A.* 76, 5402–5406.
- Ji, D., Lape, R., Dani, J.A., 2001. Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. *Neuron* 31, 131–141.
- Jones, S., Sudweeks, S., Yakel, J.L., 1999. Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci.* 22, 555–561.
- Kandel, E.R., 2012. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol. Brain* 5, 14.
- Kandel, E.R., Dudai, Y., Mayford, M.R., 2014. The molecular and systems biology of memory. *Cell* 157, 163–186.
- Klarenbeek, J.B., Goedhart, J., Hink, M.A., Gadella, T.W., Jalink, K., 2011. A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PLoS One* 6, e19170.
- Kuromi, H., Kidokoro, Y., 2000. Tetanic stimulation recruits vesicles from reserve pool via a cAMP-mediated process in *Drosophila* synapses. *Neuron* 27, 133–143.
- Leenders, A.G., Sheng, Z.H., 2005. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol. Ther.* 105, 69–84.
- Lena, C., Changeux, J.P., 1997. Role of Ca²⁺ ions in nicotinic facilitation of GABA release in mouse thalamus. *J. Neurosci.* 17, 576–585.
- Levin, E.D., McClernon, F.J., Rezvani, A.H., 2006. Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)* 184, 523–539.
- Liu, Y., Ford, B., Mann, M.A., Fischbach, G.D., 2001. Neuregulins increase alpha7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus. *J. Neurosci.* 21, 5660–5669.
- Lonart, G., Schoch, S., Kaeser, P.S., Larkin, C.J., Sudhof, T.C., Linden, D.J., 2003. Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. *Cell* 115, 49–60.
- Lozada, A.F., Wang, X., Gounko, N.V., Massey, K.A., Duan, J., Liu, Z., Berg, D.K., 2012. Glutamatergic synapse formation is promoted by alpha7-containing nicotinic acetylcholine receptors. *J. Neurosci.* 32, 7651–7661.
- Maouche, K., Medjber, K., Zahm, J.M., Delavoie, F., Terryn, C., Coraux, C., Pons, S., Cloez-Tayarani, I., Maskos, U., Birembaut, P., Tournier, J.M., 2013. Contribution of alpha7 nicotinic receptor to airway epithelium dysfunction under nicotine exposure. *Proc. Natl. Acad. Sci. U. S. A.* 110, 4099–4104.
- Matsuoka, I., Suzuki, Y., Defer, N., Nakanishi, H., Hanoune, J., 1997. Differential expression of type I, II, and V adenylyl cyclase gene in the postnatal developing rat brain. *J. Neurochem.* 68, 498–506.
- McKay, B.E., Placzek, A.N., Dani, J.A., 2007. Regulation of synaptic transmission and plasticity by neuronal nicotinic acetylcholine receptors. *Biochem. Pharmacol.* 74, 1120–1133.
- Menegon, A., Bonanomi, D., Albertinazzi, C., Lotti, F., Ferrari, G., Kao, H.T., Benfenati, F., Baldelli, P., Valtorta, F., 2006. Protein kinase A-mediated synapsin I phosphorylation is a central modulator of Ca²⁺-dependent synaptic activity. *J. Neurosci.* 26, 11670–11681.
- Moulder, K.L., Jiang, X., Chang, C., Taylor, A.A., Benz, A.M., Conti, A.C., Muglia, L.J., Mennerick, S., 2008. A specific role for Ca²⁺-dependent adenylyl cyclases in recovery from adaptive presynaptic silencing. *J. Neurosci.* 28, 5159–5168.
- Nashmi, R., Lester, H.A., 2006. CNS localization of neuronal nicotinic receptors. *J. Mol. Neurosci.* 30, 181–184.
- Nicol, X., Muzerelle, A., Bachy, I., Ravary, A., Gaspar, P., 2005. Spatiotemporal localization of the calcium-stimulated adenylyl cyclases, AC1 and AC8, during mouse brain development. *J. Comp. Neurol.* 486, 281–294.
- Nicol, X., Muzerelle, A., Rio, J.P., Metin, C., Gaspar, P., 2006. Requirement of adenylyl cyclase 1 for the ephrin-A5-dependent retraction of exuberant retinal axons. *J. Neurosci.* 26, 862–872.
- Nicol, X., Voyatzis, S., Muzerelle, A., Narboux-Neme, N., Sudhof, T.C., Miles, R., Gaspar, P., 2007. cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map. *Nat. Neurosci.* 10, 340–347.
- Nikolaev, V.O., Bunemann, M., Hein, L., Hannawacker, A., Lohse, M.J., 2004. Novel single chain cAMP sensors for receptor-induced signal propagation. *J. Biol. Chem.* 279, 37215–37218.
- Park, A.J., Havekes, R., Choi, J.H., Luczak, V., Nie, T., Huang, T., Abel, T., 2014. A presynaptic role for PKA in synaptic tagging and memory. *Neurobiol. Learn Mem.* 114, 101–112.
- Polito, M., Klarenbeek, J., Jalink, K., Paupardin-Tritsch, D., Vincent, P., Castro, L.R., 2013. The NO/cGMP pathway inhibits transient cAMP signals through the activation of PDE2 in striatal neurons. *Front. Cell. Neurosci.* 7, 211.
- Samigullin, D., Bill, C.A., Coleman, W.L., Bykhovskaia, M., 2004. Regulation of transmitter release by synapsin II in mouse motor terminals. *J. Physiol.* 561, 149–158.
- Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J.A., Patrick, J.W., 1993. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* 13, 596–604.
- Sharma, G., Vijayaraghavan, S., 2003. Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. *Neuron* 38, 929–939.
- Sharma, G., Grybko, M., Vijayaraghavan, S., 2008. Action potential-independent and nicotinic receptor-mediated concerted release of multiple quanta at hippocampal CA3-mossy fiber synapses. *J. Neurosci.* 28, 2563–2575.
- Thomsen, M.S., Hansen, H.H., Timmerman, D.B., Mikkelsen, J.D., 2010. Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology. *Curr. Pharm. Des.* 16, 323–343.
- Trudeau, L.E., Emery, D.G., Haydon, P.G., 1996. Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. *Neuron* 17, 789–797.
- Uteshev, V.V., 2012. alpha7 nicotinic ACh receptors as a ligand-gated source of Ca(2+) ions: the search for a Ca(2+) optimum. *Adv. Exp. Med. Biol.* 740, 603–638.
- Villacres, E.C., Wong, S.T., Chavkin, C., Storm, D.R., 1998. Type I adenylyl cyclase mutant mice have impaired mossy fiber long-term potentiation. *J. Neurosci.* 18, 3186–3194.
- Wang, H., Zhang, M., 2012. The role of Ca(2+)-stimulated adenylyl cyclases in bidirectional synaptic plasticity and brain function. *Rev. Neurosci.* 23, 67–78.
- Weisskopf, M.G., Castillo, P.E., Zalutsky, R.A., Nicoll, R.A., 1994. Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. *Science* 265, 1878–1882.
- Welsby, P.J., Rowan, M.J., Anwyl, R., 2009. Intracellular mechanisms underlying the nicotinic enhancement of LTP in the rat dentate gyrus. *Eur. J. Neurosci.* 29, 65–75.
- Willoughby, D., Cooper, D.M., 2008. Live-cell imaging of cAMP dynamics. *Nat. Methods* 5, 29–36.
- Wu, Z.L., Thomas, S.A., Villacres, E.C., Xia, Z., Simmons, M.L., Chavkin, C., Palmiter, R.D., Storm, D.R., 1995. Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. *Proc. Natl. Acad. Sci. U. S. A.* 92, 220–224.
- Yamashita, N., Yamauchi, M., Baba, J., Sawa, A., 1997. Phosphodiesterase type 4 that regulates cAMP level in cortical neurons shows high sensitivity to rolipram. *Eur. J. Pharmacol.* 337, 95–102.
- Zhang, X., Liu, C., Miao, H., Gong, Z.H., Nordberg, A., 1998. Postnatal changes of nicotinic acetylcholine receptor alpha 2, alpha 3, alpha 4, alpha 7 and beta 2 subunits genes expression in rat brain. *Int. J. Dev. Neurosci.* 16, 507–518.
- Zhong, C., Talmage, D.A., Role, L.W., 2013. Nicotine elicits prolonged calcium signaling along ventral hippocampal axons. *PLoS One* 8, e82719.
- Zhong, C., Du, C., Hancock, M., Mertz, M., Talmage, D.A., Role, L.W., 2008. Presynaptic type III neuregulin 1 is required for sustained enhancement of hippocampal transmission by nicotine and for axonal targeting of alpha7 nicotinic acetylcholine receptors. *J. Neurosci.* 28, 9111–9116.