Rapid cAMP signaling regulates the postsynaptic modification underlying synaptic plasticity and memory

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Understanding how synaptic structure and function is modulated by synaptic activity is essential for elucidating the mechanisms of learning/memory. Here I propose to elucidate a novel role for postsynaptic cyclic AMP (cAMP), a ubiquitous second messenger, in synaptic plasticity and learning by establishing novel cAMP optogenetic techniques.

Strong synaptic activation such as LTP (long-term potentiation) or LTD (long-term depression), induce changes in postsynaptic structures (dendritic spines) in a process called structural potentiation. This process is crucial not only for activity-dependent synapse structural changes but also for functional plasticity such as LTP. We previously revealed that the major cytoskeletal protein actin interacts with the abundant postsynaptic enzyme CaMKIIβ (calcium/calmodulin-dependent protein kinase II β subunit), directly regulating dendritic spine structure during plasticity. Furthermore, we found that it also regulates LTP
through the activity-dependent dissociation of the CaMKIIβ/actin complex. However, the detailed signalling pathway for regulating this mechanism during plasticity remains elusive.

Pharmacological and genetic manipulation of the postsynaptic cAMP pathway revealed that cAMP signaling is essential for synaptic plasticity and memory. However the rapid spatial and temporal functions are not well known due to the lack of techniques to spatiotemporally control signaling activity.

To address how postsynaptic cAMP regulates synaptic plasticity and memory, I established optogenetic approaches to spatiotemporally manipulate cAMP amounts by light from the single synapse to the brain region of freely moving mice.

Utilizing this technique, combined with two-photon live imaging, electrophysiology, and mouse behavior assays, I demonstrated a novel role for postsynaptic cAMP modulating structural and functional synaptic plasticity and short-term learning.

This proposed research, in particular, the non-invasive light-dependent manipulation of cAMP levels *ex vivo*, could also serve as valuable tools for elucidating the role of cAMP signalling in a broad variety of research fields.
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Introduction

1.1 Synaptic plasticity

Neurons are information-carrying cells of the central nervous system. They communicate through several different mechanisms, depending on the location and neuron subtype. One way neurons send signals to other neurons is through a specialized structure called a chemical synapse (Krnjevic, 1974). The structure of the synapse is such that information flow is generally unidirectional (Figure 1). Presynaptic terminals produce neurotransmitter-containing vesicles that accumulate and dock at the plasma membrane. Upon receiving an action potential, the docked vesicles fuse to the membrane, thereby releasing their contents into the synaptic cleft. The neurotransmitters are detected by receptors on the postsynaptic structure, called a dendritic spine. If the detected signal is strong enough, a set of ion channels will open at the spine, allowing the influx of positively-charged atoms. The movement of these atoms creates a depolarization relay event that travels from the dendrites to the soma of the neuron and then to the axons to carry the signal forward.

Strong neural activity at a given synapse increases the efficiency of synaptic transmission of that synapse in a process called long-term potentiation or LTP (Bliss and Lomo, 1973). There has been considerable study into the molecular basis for LTP (Malenka and Bear, 2004). In addition to the influx of ions into the dendritic spine, LTP stimulation results in a change of dendrite morphology (Figure 2). Following LTP induction the size of the synapse increases, which in turn facilitates the insertion of neurotransmitter receptors, making the synapse more sensitive to further stimulation from the corresponding presynaptic site (Shi et al., 1999).
Figure 1. Model of synaptic communication

Presynaptic vesicles are transported to the presynaptic terminal by the action of motor proteins. Inside the vesicles is mainly the excitatory neurotransmitter glutamate. When an action potential is received by the presynaptic cell, the presynaptic vesicles fuse with the presynaptic terminal, thus releasing glutamate into the synaptic cleft. The free neurotransmitters cross the synaptic cleft and bind to receptors on the surface of the postsynaptic cell (the dendritic spine). This starts a reaction whereby glutamate-sensitive cation channels open, allowing entry of Na\(^+\) and Ca\(^{2+}\) ions into the cell, thus triggering another action potential and carrying the signal from the dendrite of the postsynaptic cell to its axon where the cycle begins anew thus propagating the signal.
Figure 2. Structural enlargement during synaptic plasticity.

At rest, CaMKII bundles F-actin filaments. When LTP is induced, CaMKII detaches from the actin filaments and phosphorylates various protein targets. Actin rapidly polymerizes, the volume of the spine increases, and additional AMPA receptors (AMPAR) are localized into the post-synaptic density. CaMKII then returns to the actin and helps to stabilize the enlarged spine structure. Adapted from Okamoto et al. (2009).
1.1.1 AMPA receptors

AMPA receptors (AMPAR) are one of two major types of glutamate-sensitive cation channels found in excitatory synapses. It consists of four subunits composed of four types (Keinanen et al., 1990). It is highly expressed at the post-synaptic density (PSD) and is usually the first step of the depolarization of the neuron (Takumi et al., 1999). Upon the binding of glutamate, AMPAR undergoes a conformational change resulting in the opening of a sodium-selective channel, which results in sodium ions flooding into the neuron and the neuron’s subsequent depolarization (Gill et al., 2008). AMPAR trafficking is shown to increase after LTP induction, leading to an increase in sensitivity of the dendritic spine to signals coming from the presynaptic terminals (Shi et al., 1999).

1.1.2 NMDA receptors

NMDA receptors (NMDAR) are the other major type of glutamate-sensitive cation channel found in excitatory synapses, but differ from AMPAR in that it is a channel for calcium. Upon the binding of glutamate, NMDAR is not calcium permeable since a magnesium ion occludes the pore opening (Ascher and Nowak, 1988). Only if the postsynaptic cell is depolarized first, will the magnesium ion be repelled from the pore opening and allow the channel to pass calcium ions through the pore in response to glutamate binding. In this way, it can be thought of as a coincidence detection mechanism. As with AMPAR trafficking, NMDAR trafficking is increased following LTP, thereby increasing the sensitivity of the dendritic spine to presynaptic signals (Grosshans et al., 2002).
1.1.3 Structural potentiation, calcium, and actin

In addition to trafficking receptors to the surface of the PSD, LTP also results in an increase in dendritic spine volume (Matsuzaki et al., 2004). The major protein responsible for this is CaMKII, which acts to reorganize the actin cytoskeleton that is present in the spine (Okamoto et al., 2007). CaMKII has two major isoforms, α/β, and is one of the most highly expressed proteins in the hippocampus. CaMKIIβ specifically has an actin-bundling domain that causes it to physically associate with the spine cytoskeleton. Upon calcium entry into the cell, CaMKII is activated by calcium-bound calmodulin and dissociates from actin. It undergoes a series of phosphorylation reactions, including with itself, that serves to reorganize and rapidly polymerize the existing pool of g-actin into f-actin. After about a minute of kinase activity, CaMKII is dephosphorylated and returns to the actin once more to stabilize spine structure.

1.2 Cyclic adenosine monophosphate

Cyclic adenosine monophosphate (cAMP) is a critical second messenger first described by Sutherland and Rall (1960). Its role is to transmit signals incoming from the surface of the cell to the cytoplasm where it can further affect signaling cascades (Beavo and Brunton, 2002). cAMP is formed from a catalysis of ATP into cAMP by adenylyl cyclase by way of a circularization of the phosphate group (Sutherland and Rall, 1958). As such, it has exactly the same number of atoms as its decomposed form, adenosine monophosphate (AMP).
Figure 3. The cAMP pathway in LTP.

The known molecular biology of structural synaptic plasticity. Shown proteins are: NMDAR (NMDA receptor), CaM (calmodulin), CaMKII (Ca\(^{2+}\)/calmodulin-dependent protein kinase II), AC (adenyl cyclase), HCN (hyperpolarization-activated cyclic nucleotide-gated ion channels), PKA (protein kinase A), I-1 (inhibitor 1), PP1 (protein phosphatase 1).
1.2.1 Adenylyl Cyclase

Adenylyl cyclases (AC) were first described by Sutherland et al. (1962) who also determined their role as the main protein which produces cAMP. AC is expressed in a large variety of cell types, including neurons (Krebs and Beavo, 1979). There are ten known isoforms of adenylyl cyclases, eight which are expressed in neurons, but only AC1 is neurospecific (Wong et al., 1999). In addition to AC activation by g-protein coupled receptors, AC1 is also activated by calcium-bound calmodulin (Wu et al., 1993).

Knockout experiments have implicated AC1 directly in learning and memory (Wu et al., 1993). By reducing the Ca\textsuperscript{2+}-stimulated activity of AC1 in the hippocampus to \(\sim 46\%\) of control mice, LTP was affected. The rate of field excitatory post-synaptic potentials (fEPSPs) increased following tetanus was less in AC1 knockouts and reached a magnitude of approximately 40\% of control mice. So too was the behaviour of these mice affected during the Morris water maze task. AC1 knockouts were able to learn where the hidden platform was located as measured by decreased latency to board the platform. However, when the platform was removed, AC1 knockouts spent less time searching for the platform in the correct quadrant, indicating a problem with memory formation.

The opposite approach has also given important insight into AC1 function (Wang et al., 2004). In an AC1 over-expression mouseline, AC1 is put under the control of the CaMKII promoter, restricting over-expression to the forebrain (cortex and hippocampus but not cerebellum). These mice had enhanced fEPSP slopes after tetanus, relative to controls. In control mice, the fEPSP slope gradually decreases after the initial increase following tetanus. However, there was no such decrease in the AC1 over-expressing mice. These mice also
showed increased performance on a novel object recognition task, which requires the hippocampus (Myhrer, 1988; Reed and Squire, 1997). In this test, mice are placed in an area containing two objects, and allowed to freely habituate for 5 min. The mouse is removed and one object is replaced with a new object. After 1 hour, the mice are returned to the arena. If the mice notice that the object has been changed, they are inclined to spend more time with it. AC1 over-expressing mice and control mice spent a comparable amount of time with the new object. However, when the test was repeated with 24 hours between habituating and returning to the arena, control mice did not spend significantly more time with either object. However, the AC1 over-expressing mice spent significantly longer with the novel object, suggesting that an increase in AC1 activity enhanced their ability to form a memory with respect to the arena objects.

1.2.2 Protein kinase A

Protein kinase A (PKA) expressed in the brain was first described by Miyamoto et al. (1969). Protein kinase A (PKA) is a four subunit (two regulatory, two catalytic) signaling kinase that is found in a variety of cell types, including neurons (McKnight et al., 1988). Its expression is normally limited to specific subcellular areas by the action of A-kinase-anchoring proteins (AKAPS). When PKA detects cAMP, the regulatory subunits detach from the catalytic subunits which go on to phosphorylate target proteins.

One of the first studies that specifically asked whether PKA signaling can affect neuronal output measured a neuron’s responsiveness to the excitatory neurotransmitter glutamate (Greengard et al., 1991). Pyramidal neurons from cultured P2-P7 rat hippocampus were recorded using a whole-cell patch-clamp technique. This is an electrophysiological technique
where a very thin recording pipette is presented to the surface of a neuronal cell body. Using suction, a small piece of the cell membrane (the patch) is taken into the pipette. The patch is then destroyed using stronger suction, and the contents of the recording pipette and the cell are intermixed. In this single-cell recording technique, neuronal responses to glutamate were enhanced when the neuron was first exposed to forskolin (a powerful activator of AC). Conversely, an inhibitor of PKA causes whole-cell glutamate responses to decrease, suggesting basal PKA activity.

Even as the previous study showed for the first time that PKA has an effect on neuronal output, it was not until much later that PKA was specifically shown to be elevated after LTP. In a series of experiments, the activity of PKA was estimated \textit{in vitro} exploiting the exclusive affinity of PKA to a small 7-amino acid peptide called Kemptide (Roberson and Sweatt, 1996). Using a liquid scintillation assay, the kinase activity of PKA was estimated from the amount of $^{32}$P-phosphorylated Kemptide that was able to be produced. LTP in the CA1 region of acute hippocampal slices from rats was induced with strong tetanus, which was able to produce LTP lasting $>$3h. The slices were immediately frozen and the CA1 region was microdissected for use in the liquid scintillation assay. PKA activity was found to be significantly heightened in LTP-induced slices. In addition, by providing a delay between LTP induction and freeing of the slices, they were able to observe the time dynamics of PKA. PKA is significantly produced within the first 2 min after induction, continues for 10 min, and decays by 45 min. Further, they found this activity is not due to an increased amount of PKA or an increased efficacy.

Genetic approaches investigating the function of PKA during LTP have been particularly insightful. A dominant negative PKA mutant dubbed R(AB) was generated such that the
mutant protein was expressed under the CaMKIIα promoter. The choice of this promoter restricted the R(AB) transgene to the hippocampus. After confirming otherwise normal physiology, LTP was induced in using either: one, two, or three trains of high frequency tetanus. The first two ways induced E-LTP lasting <2h and controls were comparable to R(AB) mice. However, when 3 trains were delivered, control hippocampal slices were put into a state of E-LTP which turned into L-LTP after the 3h mark. In stark contrast to the controls, R(AB) mice did not show any evidence of L-LTP after 3 trains of tetanus, as they rapidly decayed and reached baseline levels after 2h. This suggested that PKA was necessary for the establishment of L-LTP in the CA1 area of the hippocampus.

In addition to testing the nature of LTP in R(AB) mice, they were also tested on their ability to remember the location of the sunken platform in the Morris Water Maze. Transgenic mice did significantly worse than control mice. To rule out any defects in vision or motivation, the test was modified such that proximal cues were removed, and both the starting point and submerged platform were always the same relative to only one distal cue. In effect, the mice had to swim to the distal cue and would hit the platform on the way, thus not involving the memory system. Control and R(AB) mice performed equally as well when the test was modified like this, indicating that visual acuity, motivation, and neuromuscular coordination were intact in the R(AB) animal. Taken together, these results suggest that PKA is necessary in the hippocampus to form memories about the location of the hidden platform.

1.2.3 cAMP response element-binding protein

An important protein that PKA phosphorylates is the nuclear effector cAMP response element-binding protein (CREB), first described by Montminy and Bilezikjian (1987). CREB
is a leucine zipper transcription factor that, upon being phosphorylated by PKA, translocates to the nucleus where it affects the transcription of certain genes that are regulated by the cAMP response element (CRE) binding sequence. This sequence of events leads to the eventual translation of proteins, which is thought to be cAMP’s ultimate role in LTP.

1.2.4 Phosphodiesterases

The main class of enzymes that catalyzes cAMP to AMP is called the phosphodiesterases (PDEs). There are a number of different PDEs in the human genome, each having different selectivity to cyclic nucleotides (either cAMP or cGMP). The PDE4 family is cAMP-specific and contains four members (A-D). The expression patterns of the family members are diverse, suggesting that their roles are not functionally redundant. PDE4B is of particular interest since it is expressed in the hippocampus (Reyes-Irisarri et al., 2008). Non-selective inhibitors of PDE have previously been shown to enhance cognition and memory deficits in mouse models of ischemic stroke, Alzheimer’s disease, and age-related cognitive decline (de Lima et al., 2008; Gong et al., 2004; Li et al., 2011). Unfortunately these non-selective inhibitors are not well tolerated in humans. Inhibition of PDE4 in the brainstem and gut leads to severe nausea and vomiting at doses required for their cognitive effect (Menniti et al., 2006; Mori et al., 2010).

When looking at PDE4B in the context of LTP, there are a few intriguing pieces of evidence that hint at the mechanism of memory formation. A recent study explored what effect a PDE4B mutant protein (PDE4B<sup>Y358C</sup>; catalytically reduced) would have on LTP in acute hippocampal slices obtained from mice (McGirr et al., 2016). They applied high frequency (100 Hz) tetanus trains to wild-type slices while recording fEPSPs from the CA1 region,
which resulted in a dramatic increase in potentiation. When they performed the same experiment in the PDE4B<sup>Y358C</sup> mice, the slices were potentiated to a greater degree.

In that same study they also examined what effect PDE4B<sup>Y358C</sup> would have on behaviour trials meant to test learning in the mice, such as the Morris Water Maze. PDE4B<sup>Y358C</sup> mice are especially adept at finding the platform compared to wild-type mice. In a variation of this test, the platform is removed completely, and the mice are recorded on how much time they spend swimming in each quadrant of the pool. Wild-type mice will spend a lot of time swimming in the same quadrant as the former location of the platform. Here too do the PDE4B<sup>Y358C</sup> mice outperform the wild-type mice by spending a greater amount of time in the correct quadrant. This effect is also independent of the swimming speed or time spent swimming.

They also tested the cognitive abilities of mice in a displaced object recognition test. When previously given 10 min of habituation, both wild-type and PDE4B<sup>Y358C</sup> prefer to spend time with the displaced objects, suggesting that they are aware that the positions have changed. However, if only given 5 min of acquisition, PDE4B<sup>Y358C</sup> mice prefer to spend time with the displaced object and wild-type mice do not prefer either object. Taken together, this indicates that cognitive performance is increased when PDE4B is reduced (and thereby increasing cAMP) in the hippocampus in mice.
1.3 Hippocampal learning and memory

1.3.1 The hippocampus in human beings

Much of what we know about the physical location and formation of declarative memories comes from studies on humans in which their brains are damaged in some way. One such example is the case of Henry Molaison who is more famously known as Patient HM (Scoville and Milner, 1957). HM was diagnosed with intractable epilepsy in 1953 (at the age of 27) and underwent a bilateral medial temporal lobe resection including removal of the hippocampus in an attempt to cure this disease. Following the surgery, HM’s epilepsy was partially controlled but he also developed severe anterograde amnesia. He was not able to commit new events to his clear explicit memory, although his working memory and procedural memory were both intact. His long-term memory was also mostly intact, save for a span of 1-2 years immediately before the surgery.

In another case, Patient R.B. developed severe anterograde amnesia following an ischemic stroke at 52 after surgical complications resulted in the loss of around 5 L of blood (the average human body contains 5.5 L). After surgery, Patient R.B.’s amnesia was immediately apparent upon casual observation. He was given a battery of tests with the goal of characterizing his cognitive abilities. Patient R.B. had normal scores for things like IQ and recall of past events, but was severely deficient in the ability to form new memories. Examination of his brain post-mortem revealed a bilateral lesion in the CA1 region of his hippocampus and extended down the entire structure. This was one of the first pieces of evidence that suggests that the hippocampus is an important brain subregion for the creation of new memories.
1.3.2 The hippocampus in non-human primates

Another major source of knowledge about the function of the hippocampus comes from the study of experimentally-induced brain lesions in nonhuman primates. These lesions were first produced to approximate in monkeys the brain lesion that produced such profound anterograde amnesia in patient H.M (Mishkin, 1978). The lesion included the hippocampus and several other major brain areas (parahippocampal cortex, amygdala, periamygdaloid cortex, entorhinal cortex, and perirhinal cortex). This lesion was dubbed the H+A+ lesion, where H is for hippocampus, A is for amygdala, and the plus signs are used to denote surrounding cortex to both regions. This was the first case of a model for amnesia in nonhuman primates.

The H+A+ model is quite large in terms of the affected areas, and there has been considerable interest in disentangling the specific effects of the brain subregions involved. A more selective insult, the H+ lesion, was therefore constructed. The cortex surrounding the hippocampus (posterior entorhinal cortex and parahippocampal gyrus) is necessarily damaged as the experimenters must move through these areas to damage the hippocampus. Animals with H+ lesions show selective deficiencies in memory formation tasks that resemble the deficiencies shown by amnesiac humans (Zolamorgan et al., 1989b). The test most sensitive to H+ lesions is called Delayed Nonmatching to Sample. In this test, the animal is briefly visually presented with an object, and then a delay period elapses. The animal then is presented with a choice of the already-presented object and a novel object. If the animal chooses the novel object, they are rewarded and said to have remembered the first object. New pairs of objects are used each time to prevent the effect of long-term memory
formation, which is independent of the hippocampus. Control animals (mock surgery) start to perform worse as the delay increases. The H+ lesion starts to cause memory impairment in this test at shorter delays than controls. As the delay increases, so too does the memory impairment, always to a greater degree than the controls. This is some of the first evidence that specifically implicates the hippocampus as the brain region that is responsible for working memory in nonhuman primates.

Even as the H+ lesion in monkeys is more specific than the H+ A+ lesion, the surgical insult done in this way still does not (in fact, cannot) be restricted to only the hippocampus. Another approach to studying the effect of hippocampal damage to working memory is to use a model of ischemic stroke. By using a technique of bilateral occlusion of the carotid artery (15 min), paired with hypotension induced pharmacologically, cell death can be mainly restricted to the CA1 region of the hippocampus and somatostatin-positive cells in the DG (Zolamorgan et al., 1992). In the delayed nonmatching to sample test, ischemic monkeys are impaired like the H+ lesion animals, but to lesser extent. This is one of the first examples that damage to mainly the hippocampus is sufficient to induce memory impairment.

An even more precise technique for damaging only the hippocampus and leaving surrounding tissue intact came after the development of magnetic resonance imaging (MRI; Alvarezroyo et al. (1991)). By mapping the brains of individual monkeys, researchers were able to precisely place radiofrequency electrodes into the hippocampus and then deliver current to raise the temperature of the tissue, thereby destroying it. This procedure generates H lesion animals, as the cortex is generally not damaged. In the delayed nonmatching to sample test, H lesion animals performed about the same as the ischemic animals, and significantly better than the H+ lesion animals(Alvarez et al., 1995). This was more evidence
that even incomplete damage to the hippocampus is enough to produce measurable memory deficits.

The H+A+ monkeys had the strongest amnesia of all the tested subtypes. As such, it became important to distinguish if damage to only the amygdala was responsible for the enhanced memory impairment. The same radiofrequency ablation approach used to destroy the hippocampus in H lesion animals was also able to selectively destroy the amygdala, to generate A lesion animals (Zolamorgan et al., 1989a). A lesion animals showed no memory deficits in the delayed nonmatching to sample test, where H+ and H+A+ did. To determine if damaging the amygdala would produce memory impairment only if the hippocampus was already destroyed, H+A animals were generated. These H+A animals did indeed have memory impairments, but they were no greater than the H+ animals.

These results did not however explain the increased memory deficits in H+A+ animals. To recapitulate those defects while leaving the amygdala intact, monkeys were prepared with lesions which damaged the hippocampus, anterior entorhinal cortex, and a large part of the perirhinal cortex, producing a H++ animal (Squire and Zolamorgan, 1991). In the delayed nonmatching to sample test, the H++ animal nearly performed as poorly as the H+ A+ animal, and significantly worse than either the H+ or H+ A lesion animals. Taken together, this is evidence for the hippocampus and surrounding cortex (and not the amygdala) as being the subregion which is exclusively responsible for memory formation.
1.3.3 The hippocampus in rodents

1.3.3.1 The odour discrimination task

Lesion studies have had similar results in rats. In one task, rats with lesions in the fornix (the main output tract of the hippocampus) were tested for how well they were able to discriminate between two odours (Eichenbaum et al., 1988). Two odours were presented simultaneously and the rats were rewarded if they put their nose into the hole from which the correct odour originated. The “correct” odour was arbitrarily chosen for each rat and dispensed out of either the right or left hole in a random manner. Rats that had the fornix lesions took much longer to learn this discrimination task than mock surgery rats, indicating that they had trouble learning.

1.3.3.2 Timing-related deficiencies

In addition to problems discriminating odours, rats with hippocampal lesions also have problems remembering certain timing-related tasks (Meck et al., 1984). In one study, fornix-lesioned rats were trained to press a reward lever after 20s of white noise. During testing, the white noise played for 50s (well over twice the length of training noise) and the time until the rats pressed the reward lever (now not dispensing a reward) was recorded. Overall, the lesioned rats performed comparable to the control rats. However, if a period of 5s of silence occurred before 20s of white noise had elapsed, control rats would pause their internal “timer” and resume when the white noise started playing again. In other words, these rats would press the lever after a total of 20s of white noise, even if 25s had elapsed since the start of the experiment. In contrast, fornix-lesioned rats would begin their internal timers
anew after the pause. In other words, the fornix-lesioned animals would press the lever after 20s had elapsed from the end of the 5s pause, ignoring the duration of white noise that came earlier. This is an interesting result as the hippocampus had previously been thought to mainly encode location-based memory (Okeefe and Nadel, 1979), and there is no location discrimination in these timing trials.

1.3.3.3 Configural discrimination studies

Rats with lesions in the hippocampus proper have deficiencies in configural discrimination, that is, pattern recognition (Rudy and Sutherland, 1989). Rats were trained to press a lever when they either heard a tone, saw a light, but not when both occurred together. Control rats are able to learn within a week to avoid pressing the lever when both stimuli were presented. By contrast, rats with hippocampal lesions could not recall or be retrained even after two weeks to avoid pressing the lever when both stimuli were presented. It is interesting to note that the hippocampal lesioned rats pressed the lever at a statistically higher rate than control rats. One explanation is that, since the lever presses were rewarded, the lesioned rats had decreased impulse control. To control for this, the rats were trained to avoid pressing the lever when they heard the tone. In this scenario, the lesioned rats pressed the lever as often as the control rats, indicating that their impulse control was unaffected by their lesion.

1.4 Long-term potentiation

The precise physiological mechanism for memory was first hypothesized by Santiago Ramón y Cajal, the father of modern neuroscience. He observed that neurons were post-mitotic, and that new information may be coded in the nature of the connections between the cells (Cajal,
1894). It was not until 1973, nearly 80 years later, that we had proof that this might be the case. In an elegant experiment performed by Tim Bliss, we saw the first signs of a physiological cellular mechanism for memory (Bliss and Lomo, 1973). In anaesthetized rabbits, electrodes were placed into their hippocampus. One electrode was used for stimulating the axon bundles of the perforant path (the stimulation electrode), and another was used for recording the responses from dendrites of the dentate gyrus (the recording electrode). For a given amount of stimulus in the axonal layer (triggering firing), there was a characteristic response in the dendrite layer. After obtaining a stable baseline, strong input stimulus was given from the stimulation electrode (a tetanus). When the stimulation electrode returned to pre-tetanus strength, the dendrite response at the recording electrode was enhanced compared to the previously stable baseline responses to the stimulation electrode. This is the phenomenon that they called long-term potentiation (LTP) as this enhanced dendrite response was stable over the course of many hours after the initial tetanus. Over the years LTP has been broken down into two major phases, representing distinct stages of the effect. There is early LTP (E-LTP) which is the stage <60 minutes post-induction and is protein synthesis independent, and late LTP (L-LTP) which occurs >60 minutes after induction and requires protein synthesis (Malenka and Bear, 2004).

### 1.4.1 LTP in memory

One of the first studies that investigated if there was a direct relationship between LTP and memory used an approach consisting of surgically implanted recording and stimulating electrodes in the rat hippocampus (McNaughton et al., 1986). They reasoned that if LTP and memory share the same pathway, then activation of LTP at all synapses should be able to
impair memory formation. To test memory, they exploited the rat’s preference for small dark holes in the presence of a bright light. Rats were placed on a table that was surrounded by 18 holes, one of which had a dark box underneath. A bright light shone on the table surface and rats were trained to run into the dark box in the correct hole which was always positioned relative to some identifiable markers in the room. After the last day of training, rats were connected to a recording rig via cables which attached to the protruding commutator connected to the implanted recording and stimulation electrodes. By stimulating the rats with HFS and then recording the responses, LTP was confirmed to be induced in the rats. The circular escape table was then turned 135° from the training position, and rats were placed back on the table. This allowed the assessment of if the rats remembered the location of the dark box. The first time they were tested, rats made the same number of errors as controls. This indicated that the original memory of the escape tunnel was unaffected by HFS. However, on subsequent trials, rats that were given HFS made significantly more errors in finding the new location of the escape tunnel than did controls. This raised the intriguing possibility that HFS inhibited the formation of a memory that the escape tunnel had been moved.

In another study, LTP was recorded from the dentate granule cell layer and induced to varying levels of saturation by tetanizing an area afferent to the hippocampus using an implanted multi-electrode array (Moser et al., 1998). This allows for much more widespread LTP than with a single electrode. Rats were tested in their ability to find the submerged platform in the Morris water maze. All animals were able to find the platform when it was visible, as measured by their decreased latency to reach it. However, the ability to search the
correct arena quadrant for the removed platform was negatively correlated with the degree of LTP saturation induced in the hippocampus of these rats.

1.5 Lack of available tools

Further study into the specific role of the cAMP cascade during LTP has been impeded by the absence of tools to precisely observe and manipulate cAMP subcellularly in networked neurons. Purely genetic approaches (like PKA knockouts) have high spatial resolution but poor temporal resolution. They are also often plagued by compensatory changes in the cells (Brandon et al., 1997). When combined with pharmacological approaches, these techniques have better temporal resolution but affect all cells, although this can be mitigated somewhat when combined with genetic techniques, like conditional knockouts. Electrophysiological approaches often have high temporal resolution, but stimulate many endogenous synapses and pathways.

1.6 Optogenetics

The emerging field of optogenetics is being rapidly adopted by the neuroscience community. Originally coined as a way to control neural activity using light, the term has spread to include any molecular biology approach that uses light to manipulate the state of the cell (Miesenboeck, 2009). cAMP in particular has been a target of optogenetic approaches, with several methods in use.

Caged cAMP is a method by which cAMP is chemically coupled to a large chemical group which renders it inert to the cell. Upon exposure to light, the link is broken and free cAMP is
released in the cell (Engels and Schlaeger, 1977). This approach suffers from the requirement of getting large amounts of the caged compound into the cell in the first place.

Light-activated G-protein coupled receptors are another approach (Karunarathne et al., 2013). When these proteins are illuminated, they undergo a conformational change whereby they are able to activate g-proteins, which stimulate AC. However, this approach has the downside of not being able to activate the cAMP pathway in isolation, as there are many other protein cascades that are activated by G-proteins.

The third way is to use photoactivatable ACs. These cytosolic enzymes convert ATP into cAMP when exposed to light. There are two in popular use, one coming from a eukaryote and the other coming from a prokaryote (Iseki et al., 2002; Stierl et al., 2011). The prokaryotic photoactivatable AC (bPAC) has already proven to be smaller and more sensitive than its eukaryotic counterpart. It’s the ideal tool to produce cAMP cell-specifically, and its advantages are multiplied when synergistically combined with a high-precision microscope.

1.7 Microscopy

To fully take advantage of the features of PAC, it was necessary to use a microscope with high precision. To that end, conventional confocal microscopes are inadequate. The reason they are inadequate is that, although they are able to block light outside the focal plane when it comes time to image, they cannot block the excitation of regions outside of the focal plane within the sample itself. To address this challenge, I used a two-photon microscope.
1.7.1 Two-photon Microscopy

The two-photon effect was first theorized by Goppert-Mayer (1931) and by 1990, there was a working microscope prototype making use of the effect (Denk et al., 1990). The working principle is that it uses excitation light that is about twice the normal excitation wavelength to activate a photoreactive molecule. If the concentration of light is high enough, the photoreactive molecule will absorb two of these low-energy photons instead of one high-energy photon. To make this effect useful for microscopy, the light intensity must be very high. And to make this effect useful for biology, the intensity must not be so high that the sample is damaged. The solution is to take advantage of an extremely fast (femtosecond) pulsing laser, which is able to deliver a large amount of energy in a small timeframe, which minimizes any damage in the sample. Our particular set-up has the added advantage of using dual two-photon lasers, which allows for the independent delivery of two wavelengths of pulsed light to independently manipulate two photoreactive molecules at the same time.

There are several advantages to using a two-photon microscope. Firstly, the wavelength of light that is used is typically in the IR part of the spectrum. This long wavelength travels through biological tissue more efficiently, meaning less light is lost to scattering. This is a desirable trait when looking at thick tissues like networked neurons ex vivo as the efficiency gain means deeper regions can be imaged. Secondly, the excitation light is only intense enough to affect molecules in the focal plane, so there is no need to block unfocused light as in a confocal setup (Figure 4). By removing the need to block unfocused light, highly sensitive photodetectors can be used closer to the sample. It is using the technologies of optogenetics and two-photon microscopy that allowed me to study cAMP’s role in LTP in a new way.
Figure 4. Two-photon microscope principle.

In a conventional microscope (right), excitation light is produced both above and below the focal plane, which is filtered as the light returns to the viewer. In a two-photon microscope (left), there is still excitation light outside of the focal plane, but it is roughly double the wavelength and therefore does not excite photoactivatable molecules, including proteins.
1.7.2 Förster Resonance Energy Transfer microscopy

Förster Resonance Energy Transfer (FRET) microscopy was first theorized by German scientist Dr. Theodor Förster but the idea to use FRET to measure the distances between molecules came later (Forster, 1948; Stryer, 1978; Tron et al., 1987). The principle of FRET is essentially to chain a fluorescent protein’s emission (the donor fluorophore) to the excitation of a second fluorescent protein (the acceptor fluorophore). In reality FRET is a radiationless transfer of the excited state of one fluorophore to a second fluorophore, provided they are in close proximity (<10 nm). The rate of energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely distance-dependent. This distance scale matches quite closely the scale of protein-protein interactions, and so it’s unsurprising that this was one of the earliest applications of FRET microscopy.

Two-molecule FRET is sensitive to stoichiometric differences in the proteins of interest, but single-molecule FRET is not. By placing two fluorescent proteins on either side of a protease target (and thus in FRET distance), researchers have been able to directly measure protease activity during apoptosis (Mahajan et al., 1999). In addition to measuring protease activity, it has been possible to use FRET to detect conformational changes in proteins that accept ligands, thus being able to detect protein/ligand interactions. One example of this is a family of single molecule cAMP FRET sensors that exploit the interaction between cAMP and the cAMP-binding domain of Exchange protein activatable by cAMP (Epac; Violin et al. (2008)). It is through this type of approach that I propose to study the spatiotemporal dynamics of cAMP during LTP.
Chapter 1

Determining the spatiotemporal role of postsynaptic cAMP in structural synaptic plasticity

The tiny postsynaptic protrusion on the dendrite of excitatory neurons, the spine, is a crucial site for synaptic plasticity. Strong synaptic stimulation induces the influx of Ca\(^{2+}\) in the spines through NMDA receptors and the resultant activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) triggers long-term potentiation (LTP) in synapses of hippocampal pyramidal neurons (Lisman et al., 2002). The induction of potentiation also leads to postsynaptic structural enlargement, called structural long-term potentiation (sLTP) of dendritic spines (Kim et al., 2015; Matsuzaki et al., 2004). Reorganization of the actin cytoskeleton and the interplay of the enzymatic and structural functions of CaMKIIβ is crucial not only for the structural changes but also for functional potentiation (LTP), demonstrating a close link between the postsynaptic molecular machinery for both functional and structural potentiation (Kim et al., 2015; Okamoto et al., 2009; Okamoto et al., 2004; Okamoto et al., 2007).

In addition to Ca\(^{2+}\)/CaMKII signaling, the major intracellular messenger cAMP is involved in late phase of postsynaptic long-term potentiation (L-LTP) in the mammalian brain (Berkowitz et al., 1989; Deisseroth et al., 1996; Enna and Karbon, 1987; Frey et al., 1993; Greengard et al., 1991; Kandel, 2012; Walton et al., 1999). The cAMP-dependent protein synthesis mechanism is regulated in an input-specific manner, such that the new proteins
affect only the synapses that were activated, known as the “synaptic tag hypothesis” (Frey and Morris, 1998).

Pharmacological application of a potent adenylyl cyclase (AC) activator (forskolin) induces structural enlargement of dendritic spines (postsynaptic structures) along with CaMKII accumulation during chemically-induced potentiation in rodent hippocampal pyramidal neurons (Otmakhov et al., 2004), suggesting a role for cAMP in regulating synaptic structure. However, how postsynaptic cAMP mediates the structural changes of dendritic spines in synaptic potentiation and whether the structural mechanism is regulated in an input-specific manner remains elusive.

Current pharmacological and genetic approaches for studying cAMP signaling, including specific inhibitors and genetic manipulations (such as knockout mice), are limited by their lack of spatial specificity such as at the synapse, and do not permit rapid temporal manipulation of cAMP (Abel et al., 1997; Barad et al., 1998; Govindarajan et al., 2011; Wong et al., 1999). This makes it difficult to evaluate the dynamic spatiotemporal role of postsynaptic cAMP in structural synaptic plasticity. Therefore, there is a need for novel tools and approaches to specifically control and observe local cAMP levels within synapses.

To systematically assay postsynaptic cAMP function, I first established two-photon live imaging of endogenous postsynaptic cAMP dynamics by a FRET cAMP probe to study cAMP function during structural synaptic plasticity (Section 1.8). To further elucidate cAMP function during this process, I developed a method to optogenetically manipulate cAMP by using the two-photon excitation of a blue light sensitive enzyme, PAC (Photoactivatable Adenylyl Cyclase), which enables deep light penetration into brain tissue and highly
localized photoactivation within the volume of a single targeted dendritic spine (Denk et al., 1990; Judkewitz et al., 2006; Stierl et al., 2011). This allows precise temporal and spatial activation of adenylyl cyclase to synthesize cAMP by light at targeted dendritic spines in neurons deep within brain tissue (Section 1.9). Finally, using this novel two-photon optogenetic manipulation of cAMP, I demonstrated a novel intra- and inter-synaptic role of postsynaptic cAMP in the structural potentiation of dendritic spines, which could underlie a fundamental spatiotemporal interaction mechanism between plasticity at nearby spines (Section 1.10).

1.8 Observation of endogenous cAMP spatiotemporal dynamics and effect on structural synaptic plasticity

Synaptic activation of the axons of rodent hippocampal CA3 pyramidal neurons by tetanic stimulation increases cAMP levels within the CA1 region in an NMDA receptor-dependent fashion (Chetkovich et al., 1991). However, the spatiotemporal dynamics of cAMP at the synapse level remain elusive. To visualize and study the function of endogenous postsynaptic cAMP during synaptic potentiation in living rodent hippocampal neurons, 1) I developed two-photon cAMP probes for live imaging in hippocampal brain tissue by optimizing genetically-encoded cAMP FRET/FLIM probes (Section 1.8.1). 2) I observed endogenous postsynaptic cAMP dynamics at dendritic spines on hippocampal CA1 pyramidal neurons during synaptic potentiation by two-photon live imaging (Sections 1.8.2 & 1.8.3). 3) Finally, I examined the cAMP effect during structural synaptic potentiation at dendritic spines by two-photon live imaging (Section 1.8.4).
1.8.1 Development of genetically engineered cAMP FRET probes for use in living hippocampal tissue during two-photon live imaging

To monitor postsynaptic cAMP dynamics in living neurons using two-photon FRET/FLIM imaging, I first tested several previously reported cAMP FRET probes for their ability to detect cAMP *in vitro* and at the neuron level. These probes use the cAMP binding domain of exchange protein activated by cAMP (EPAC), modified to be catalytically dead and cytosolic (Violin et al., 2008) sandwiched between two fluorescent proteins (Figure 5). These cAMP probes were codon optimized for mammalian expression and biolistically transfected in the rat hippocampal CA1 pyramidal neurons in organotypically cultured hippocampal slices to test for neural expression quality. The probes responded to cAMP *in vitro*, however I found almost all cAMP probes aggregated in neurons of hippocampal slices (Figure 5). Among the cAMP probes, I found cAMP FRET probe ICUE2 was expressed homogeneously in the neurons without aggregation. ICUE2 is composed of a cAMP binding domain of EPAC in between a cyan fluorescence protein (ECFP) and a yellow fluorescence protein (Citrine) which has an EC$_{50}$ of 16.83 μM respectively (Violin et al., 2008).

To improve cAMP sensitivity and brightness for synapse level cAMP imaging in hippocampal tissue, I prepared a cAMP probe (CEY) by replacing ECFP from ICUE2 with a brighter CFP (mTurquoise2) and optimized the linker length (Figure 6A). When CFP-specific excitation light (433 nm) was applied to cell lysates containing CEY *in vitro*, the emission spectrum showed a distinct YFP FRET peak (527 nm) in the fluorescence spectrometry assay, which decreased upon addition of 8-Br-cAMP and concomitantly dequenched the CFP signal, indicating a cAMP-dependent FRET change (Figure 6B).
Trypsin cleaved the probe’s CFP from YFP, and completely abolished the YFP peak confirming the YFP signal was due to FRET. The cAMP FRET was changed by more than 20% upon the addition of 100 μM 8-Br-cAMP with an EC$_{50}$ of 1.68 μM, which is roughly an increase of 7.5 times over its predecessor (Figure 6C). Also, CEY did not respond to cGMP, indicating that it is specific for cAMP (Figure 6D).
Figure 5. Schematic domain structure and neuron expression properties of various cAMP FRET and FLIM probes.

Schematic of cAMP FRET probes. Note that EPAC* is a truncated version of Epac which contains the cAMP binding domain. Colours in the fluorescence proteins correspond to their emission wavelengths. DarkVenus acts as an energy acceptor for EGFP, but almost no fluorescence will be emitted (photoquencher). For neuron expression of the probes, a punctate probe distribution in the neurons is indicated here as "aggregates" while a homogenous distribution is "soluble". H-series probes and GEmCH come from van der Krogt et al. (2008) and ICUE2 comes from Violin et al. (2008).
Figure 6. *In vitro* characterization of the optimized cAMP FRET probe CEY.

(A) Schematic drawing of cAMP-dependent FRET change of cAMP by CEY. (B) Average emission spectra of HEK293 cell lysate expressing CEY before (Baseline) and after application of 100 µM 8-Br-cAMP (+cAMP). Trypsin digest was added after the application of cAMP to separate CFP and YFP (+Trypsin; n = 3). (C) Representative time course of CEY to the application of cAMP (100 µM 8-Br-cAMP). (D) The dose-dependent FRET change (CFP/YFP ratio) to various concentrations of 8-Br-cAMP (+cAMP; n = 6) or 8-Br-cGMP (+cGMP; n = 6). EC$_{50}$ of CEY to cAMP is 1.68 µM. Data in (B) and (D) are mean ± SEM.
1.8.2 Observation of endogenous cAMP dynamics induced by tetanic stimulation in living neurons

To monitor endogenous cAMP dynamics during synaptic plasticity, I biolistically transfected cAMP FRET probe (CEY) in CA1 pyramidal neurons of organotypically cultured hippocampal slices and observed cAMP-dependent FRET change using two-photon FRET ratiometric imaging as described previously (Okamoto et al. (2004); Figure 7A). Local tetanic stimulation was delivered at the Schaffer collateral-CA1 pyramidal neuron synapses through a glass electrode, which typically induces the late phase of LTP (L-LTP; Figure 7B). This caused a FRET change immediately in the dendrite and dendritic spines, and gradually returned to baseline (τ = 5.36 min; Figure 7C). In contrast, the control FRET probe (directly fused CFP and YFP), did not show this FRET change after tetanic stimulation, confirming that CEY responded to cAMP in the neurons (Figure 7D). The cAMP FRET change was blocked by the potent NMDA receptor inhibitor APV (+APV), indicating the cAMP increase was NMDA receptor-dependent (Chetkovich et al., 1991; Chetkovich and Sweatt, 1993). This suggests that the influx of Ca$^{2+}$ through activation of NMDA receptors may activate Ca$^{2+}$/Calmodulin-dependent adenylyl cyclase to increase postsynaptic cAMP during potentiation.
Figure 7. Observation of endogenous cAMP dynamics in living neurons following tetanic stimulation

(A) Schematic drawing of the cultured hippocampal slices. A cAMP FRET probe (CEY) or a control probe (Ctrl) was biolistically transfected in CA1 pyramidal neurons of the cultured slices. Their FRET changes were observed by FRET ratiometric two-photon live imaging. (B) Schematic of local tetanic stimulation from a glass electrode activating the Schaffer collateral axons of CA3 pyramidal neurons which form synapses onto dendrites in CEY-expressing CA1 pyramidal neurons. (C) Tetanic stimulation induced a transient cAMP increase in dendrites and spines. Left: representative cAMP ratiometric FRET (CEY) pseudo-coloured images of a dendrite before and after tetanic stimulation. Red colours indicate higher levels of cAMP. Right: Time-course of the postsynaptic cAMP FRET change before
and after tetanic stimulation (decay time $\tau = 5.36$ min, $n = 10$; exponential fit). Black arrow indicates the time point of tetanic stimulation (100 Hz, 1 sec every 10 sec x 3). (D) Quantification of cAMP FRET changes by cAMP probe CEY (CEY, $n = 10$) immediately after tetanic stimulation relative to baseline. A control FRET probe was also separately expressed in neurons (Ctrl: fused only CFP and YFP directly, $n = 13$). D-APV (50 µM) was bath applied in neurons expressing CEY for inhibition of NMDA receptors during tetanic stimulation (+APV, $n = 11$). NS, $p > 0.05$; ***, $p < 0.001$; Tukey’s Test. All data are mean ± SEM.
1.8.3 Observation of endogenous cAMP dynamics in living neurons using two-photon caged glutamate uncaging

In contrast to tetanic stimulation, a two-photon uncaging protocol of caged glutamate (Figure 8A, B) induces both functional early phase-LTP (E-LTP) and the related structural potentiation at single dendritic spines (Kim et al., 2015; Matsuzaki et al., 2004). Such early phase potentiation is typically triggered by NMDAR-dependent Ca$^{2+}$ signaling (such as with CaMKII activation) but not cAMP. The LTP induction protocol of two-photon caged glutamate uncaging resulted in enlargement of targeted dendritic spines from CA1 pyramidal neurons (Figure 8C). However, the cAMP probe CEY did not respond to a cAMP change in dendritic spines, even when assessed at higher temporal resolutions during uncaging itself (Figure 8D). These results indicate that caged glutamate uncaging did not increase postsynaptic cAMP to ~1 μM, which is the middle of the probe’s dynamic range (Figure 6D). Taken together with cAMP imaging results from the tetanic stimulation experiments (1.8.2), I found that there is a dynamic cAMP increase throughout the dendrite upon tetanic stimulation in a NMDAR-dependent manner during synaptic potentiation, but there is no cAMP increase detected resulting from uncaging.
Figure 8. Visualizing cAMP dynamics in living neurons during two-photon caged-glutamate uncaging.

(A) Schematic structure of MNI-caged glutamate before and after uncaging by light. Uncaged glutamate serves as a neurotransmitter that activates postsynaptic functions in excitatory neurons. (B) Schematic of two-photon caged-glutamate uncaging in front of a target dendritic spine. The uncaged glutamate was released only in the focal plane of the two-photon laser, mimicking the synaptic activity-dependent release of glutamate from presynaptic vesicles. This release occurs in front of targeted dendritic spines to activate NMDA receptors on the surface of the postsynaptic dendritic spine, which typically induces the structural potentiation that corresponds to early LTP (E-LTP). (C) Failure to detect
cAMP dynamics by glutamate uncaging which typically induces the structural potentiation that corresponds to E-LTP. Left: CEY ratiometric cAMP FRET probe pseudo-colour images of a dendrite before and after glutamate uncaging at a target dendritic spine (blue circle). Red colours indicate higher levels of cAMP. Right: Time-course of cAMP probe CEY two-photon FRET change in targeted dendritic spines (n = 16; linear fit). Black arrow indicates time of glutamate uncaging. (D) CEY two-photon FRET change collected at higher temporal resolution in target dendritic spines during glutamate uncaging (black bar; n = 12; linear fit). All data are mean ±SEM.
1.8.4 Effect of endogenous postsynaptic cAMP in structural potentiation of dendritic spines

To examine whether postsynaptic cAMP is involved in the structural potentiation of dendritic spines, I induced cAMP production by tetanic stimulation in GFP-expressing CA1 pyramidal neurons in hippocampal cultured slices (Figure 9A). Tetanic stimulation induced dendritic spine enlargement at some spines, indicating successful direct stimulation of the axons (Figure 9B, magenta). Some “neighbouring” spines were not enlarged, presumably because their presynaptic axons were not activated by tetanic stimulation (Figure 9B, black). Since the early LTP induction protocol of glutamate uncaging induces structural enlargement of dendritic spines (Kim et al., 2015; Matsuzaki et al., 2004) without producing cAMP (Figure 8C), I hypothesized that cAMP may serve to maintain the size of the already enlarged spine. To test this hypothesis, I used tetanic stimulation to stimulate the production of cAMP in the postsynaptic cell as previously observed (Figure 7C). Casual observation of spine structure after such a stimulus reveals that some dendritic spines are enlarged after tetanic stimulation, indicating those are the stimulated spines (Figure 9A, B). However, spines that have not enlarged after tetanic stimulation are also readily observable (Figure 9B, black arrowheads). These unenlarged spines are often in close proximity to enlarged spines, and can be thought of as “neighbouring” spines. To isolate and evaluate the effect of endogenous postsynaptic cAMP on structural potentiation, I induced structural potentiation using the glutamate uncaging protocol at these “neighbouring” spines after tetanic stimulation (Figure 9C). This was done so that cAMP produced in the tetanus-stimulated spines would spread to the neighbours, and thus affect the structural potentiation induced by caged-glutamate uncaging. I was able to induce structural potentiation on the spines (0-1 min post-uncaging), as shown
by the enlarged size of the dendritic spines with (magenta) or without (cyan) a prior remote tetanic stimulation (Figure 9D). However, there was enhanced structural potentiation in spines 20-30 min after uncaging if those spines had a prior remote tetanic stimulation. Interestingly, the enhanced structural potentiation appeared to be at a similar level to the plasticity in the directly stimulated spines by tetanic stimulation (Figure 9B, magenta). The potentiation effect of a prior remote tetanic stimulation was blocked in the presence of a protein kinase A (PKA) inhibitor (KT5720), but did not alter structural potentiation by uncaging alone, suggesting that remotely synthesized postsynaptic cAMP signals could serve as a positive modulator in the maintenance of spine structure (Figure 9D, cyan). These results also suggest the existence of a cAMP-mediated inter-synaptic crosstalk function for the interaction of structural remodeling between nearby spines during synaptic plasticity. This suggests that there may be a distinct inter-spine cAMP mechanism of structural potentiation in addition to its role in protein synthesis-dependent functional potentiation.
Figure 9. Effect of endogenous postsynaptic cAMP on structural potentiation of dendritic spines.

(A) Schematic of local tetanic stimulation and endogenous cAMP production. (B) Effect of structural potentiation induction by tetanic stimulation. Left: representative images of a dendrite before and after tetanic stimulation. Arrowheads indicate dendritic spines which are stimulated by tetanic stimulation (magenta) or not (black). Timestamp is in minutes, with 0 meaning immediately after tetanic stimulation. Right: Time course of structural potentiation after tetanic stimulation. Directly stimulated dendritic spines increased in size (Direct tetanus; magenta; \( n = 17 \)) while their neighbours located more than 5 \( \mu m \) away did not (Neighbours; black; \( n = 10 \)). (C) Schematic drawing of tetanic stimulation and glutamate uncaging experiment. After tetanic stimulation, structural potentiation was induced by glutamate uncaging at spines in the vicinity of directly enlarged spines by the tetanic stimulation. Note that glutamate uncaging does not produce a detectable cAMP increase (see
Figure 8) but does induce structural potentiation spines. (D) Left: representative GFP images of a dendritic spine before and after uncaging with remote tetanic stimulation. Right: Time-course of structural potentiation after tetanic stimulation (remote) and uncaging. Uncaging alone (black; \( n = 11 \)), uncaging with remote tetanic stimulation (magenta; \( n = 10 \)), and uncaging with remote tetanic stimulation in the presence of PKA inhibitor (KT5720; cyan; \( n = 11 \)). ***, \( p < 0.001 \); Tukey’s Test. All data are mean ± SEM.
1.9 Role of spatiotemporal cAMP mechanisms for regulating structural potentiation of dendritic spines

Next, to dissect the dynamic role of postsynaptic cAMP in structural potentiation of dendritic spines, I established a novel two-photon photoactivation method of producing cAMP. I took advantage of a photoactivatable adenylyl cyclase (PAC), which enabled me to directly assay the spatiotemporal function of cAMP with precision (Section 1.9.1 & 1.9.2).

This was needed since tetanic stimulation induces many other postsynaptic signalling events aside from cAMP (such as Ca\(^{2+}\)), making it difficult to specifically examine the effect of cAMP at dendritic spines. Utilizing the two-photon optogenetic approach to manipulate cAMP by light in combination with caged-glutamate uncaging to induce structural potentiation, I demonstrated cAMP is sufficient to enhance the maintenance of structural potentiation at dendritic spines (Section 1.9.3). This effect is dependent on protein kinase A (PKA) and is independent of protein synthesis (Section 1.9.4). I also show the temporal (Section 1.9.5) and spatial function (Section 1.9.6) of cAMP on structural potentiation. These results provide the first direct look at intra- and inter-spine cAMP function in structural potentiation.

1.9.1 Characterization of photoactivatable adenylyl cyclase *in vitro*

Bacterial PAC contains an adenylyl cyclase domain linked to a Blue Light receptor Using FAD (BLUF) domain that can be activated by blue-light (Stierl et al. (2011); Figure 10A). To confirm the photoactivation *in vitro*, I measured cAMP levels produced by human codon optimized PAC tagged with RFP (tdTomato; Shaner et al. (2004)). PAC-containing cell
lysates were photoactivated by an LED light (455 nm peak, 4.5 mW/mm$^2$) and the resulting cAMP was measured using an ELISA assay (Ryu et al., 2010) in vitro (Figure 10B). cAMP levels increased within seconds of light stimulation (Figure 10C) and removal of light stopped further detectable additional cAMP production (Figure 10D), indicating light-dependent photoactivation.

Next, to characterize PAC photoactivation using two-photon excitation light, which can deliver light in a focal volume similar in size to a dendritic spine (Figure 11A), I examined the two-photon excitation spectra between 700-1,025 nm (Figure 11B). The two-photon excitation wavelengths (700-800 nm) which roughly corresponded to twice the PAC single-photon excitation wavelength (Ryu et al., 2010; Stierl et al., 2011; Yoshikawa et al., 2005) efficiently activated PAC in vitro (up to 1,000 nm). To minimize photodamage and scattering in brain tissue caused by shorter wavelengths of two-photon light, I used 1,000 nm two-photon excitation and confirmed it can rapidly activate PAC (0.456 µmol cAMP/nl/ms/µmol PAC: Figure 11C).
Figure 10. In vitro characterization of photoactivatable adenylyl cyclase (PAC) using single-photon excitation light.

(A) A schematic diagram of PAC photoactivation. PAC consisted of a blue light receptor using FAD (BLUF) light sensor domain linked to the catalytic domain of adenylyl cyclase. (B) Schematic of the photoactivation of PAC in vitro using a LED light source (455 nm, 4.5 mW/mm²). (C) Time-course of 100 μL PAC solution photoactivation measured by a cAMP ELISA (n = 3). (D) Activity of PAC in the dark following 30 seconds of photoactivation (black bar; n = 6). The cAMP amount produced during 30 seconds was set as 0. All data are mean ± SEM.
Figure 11. Characterization of photoactivatable adenylyl cyclase using two-photon light.

(A) Schematic of two-photon photoactivation of PAC in 100 µl of solution (note: the two-photon photoactivation volume is ~1µm³). (B) Two-photon (30 mW, 700 – 1,025 nm, 15 min) excitation spectra of PAC activity. Peak cAMP level was set as 100 % (n = 9; cubic fit). (C) Time-course of two-photon (1,000 nm, 75 mW) PAC photoactivation. Speed of cAMP production in the two-photon focal volume is 0.456 µmol/nl/ms/µmol protein (n = 4; logistic fit). All data are mean ± SEM.
1.9.2 Validation of photoactivatable adenylyl cyclase photoactivation with a cAMP FLIM probe *in vitro*

To observe photoactivation of PAC in living neurons, I prepared a genetically-encoded cAMP FLIM (Fluorescence Lifetime Imaging Microscopy) probe by modifying the cAMP FRET probe CEY (Figure 7). To avoid photobleaching of the donor fluorescent protein by blue light from photoactivation of PAC (414 ± 21 nm, mercury arc lamp), I used YFP (Citrine; Heikal et al. (2000)) and RFP (tdTomato; Shaner et al. (2004)) as donor and acceptor, respectively (REY; Figure 12A). I expressed REY in HEK 293 cells, collected the cell lysates, and characterized its sensitivity to cAMP under a two-photon FLIM microscope (900 nm excitation). The dose-dependent change of the fluorescence lifetime of REY (donor: YFP) in response to 8-Br-cAMP was rapid (Figure 12B) and showed an EC\textsubscript{50} of 1.81 µM (Figure 12C), which is a comparable to the cAMP probe CEY (EC\textsubscript{50} of 1.68 µM; Figure 6D). I also demonstrated that the cAMP-dependent change in REY lifetime was between the lifetime range of REY without cAMP and YFP (without acceptor; Figure 12D), confirming the ability of the probe to detect cAMP by the FLIM change under the two-photon FLIM microscope *in vitro*. 
Figure 12. Characterization of a cAMP FLIM probe REY in vitro.

(A) A FLIM probe (REY) for cAMP detection. (B) Representative response of REY to 100 µM of 8-Br-cAMP. Under a two-photon FLIM microscope (900 nm excitation). (C) Dose-dependent change of the fluorescence lifetime of REY with an EC_{50} = 1.81 µM (n = 9; Hill fit). (D) Quantification of the lifetime before and after 100 µM 8-Br-cAMP, and YFP (donor) alone. REY (n = 9), REY + cAMP (n = 9), and YFP (n = 10). **, p < 0.01; ***, p < 0.001; Tukey’s test. All data in (C) and (D) are mean ± SEM.
To detect a light-dependent cAMP increase by photoactivation of PAC using the FLIM probe REY, I coexpressed REY with PAC in HEK293 cells and assayed the lifetime change of the probe upon PAC photoactivation (414 ± 21 nm, mercury arc lamp) in cell lysates (Figure 13A). Upon photoactivation of PAC to produce different amounts of cAMP, I observed a proportional increase in the lifetime of REY, indicating that the probe can detect cAMP produced by PAC (Figure 13B). To confirm the FLIM probe REY is detecting a PAC photoactivation-dependent increase in cAMP, I directly measured the cAMP concentration with an ELISA and measured the REY lifetime change from these same samples. In the absence of PAC expression, the photoactivation did not affect either REY fluorescence lifetime or cAMP concentration, but I found that both REY fluorescence lifetime and cAMP concentration were increased upon photoactivation of PAC (Figure 13C,D), demonstrating the ability of the cAMP FLIM probe REY to detect PAC photoactivation in vitro.

To induce cAMP by photoactivation of PAC and observe the dynamics in neurons, I utilized a cAMP FLIM probe REY (Figure 12A) and biolistically transfected REY with/without PAC in CA1 pyramidal neurons in cultured hippocampal slices (Figure 14A). Two-photon FLIM time-course imaging itself did not affect the fluorescence lifetime of REY in neurons (Figure 14B, No light). Upon PAC photoactivation (414 ± 21 nm, mercury arc lamp, 60 sec), the fluorescence lifetime increased and was maintained for at least 10 minutes in the neurons (Figure 14B, +PAC). In contrast, the lifetime was not significantly altered after the same photoactivation protocol in neurons without PAC (Figure 14B, -PAC). This quantitative assay indicates that the FLIM change was due to the light-dependent cAMP increase (Figure 14C).
Figure 13. Detection of PAC photoactivation with a cAMP FLIM probe REY in vitro.

(A) Schematic for the REY FLIM detection of cAMP produced by PAC. (B) Lifetime change of REY after photoactivation of PAC over increasing photoactivation time ($n = 10$; Hill fit). (C, D) Detection of PAC photoactivation using fluorescence lifetime change of REY (C) and a cAMP ELISA (D). cAMP was measured from REY transfected HEK293 cell lysate with or without PAC (PAC+/−), and with or without light (light +/−). Light source was 414 ± 21 nm mercury arc lamp, 11 mW for 5 sec. (C) PAC+/Light− ($n = 15$), PAC-/Light+ ($n = 9$), and PAC+/Light+ ($n = 15$). (D) PAC+/Light− ($n = 5$), PAC-/Light+ ($n = 5$), and PAC+/Light+ ($n = 5$). NS, $p > 0.05$; ***, $p < 0.001$; Tukey’s test. All data are mean ± SEM.
Figure 14. Detection of single-photon PAC photoactivation using a cAMP FLIM probe (REY) in living neurons.

(A) Schematic of single-photon excitation in cultured hippocampal slices. The CA1 pyramidal neurons were biolistically transfected with the cAMP FLIM probe REY. (B) Increase of cAMP level by PAC photoactivation in living neurons. Left: The cAMP pseudo-colour REY FLIM images of a dendrite before and after photoactivation (414 ± 21 nm mercury arc lamp, 11 mW, 60 sec). Red colours indicate higher cAMP levels. Right: Time-course of the fluorescence lifetime change in neurons expressing REY either with (+PAC, magenta, n = 11) or without PAC (-PAC, cyan, n = 10). No light: no PAC photoactivation during FLIM imaging in neurons expressing REY and PAC (No light, black, n = 15). Black bar represents PAC photoactivation (+Light). (C) Detection of PAC cAMP production by REY with and without PAC and also with and without light. PAC+/Light- (n = 15), PAC-/Light+ (n = 10), and PAC+/Light+ (n = 11). NS, p > 0.05; ***, p <0.001; Tukey’s test. All data are mean ± SEM.
Next, I applied two-photon excitation light to photoactivate PAC in individual dendritic spines (Figure 15A). Upon two-photon photoactivation (1000 nm, 30 sec) at target dendritic spines, the fluorescence lifetime of REY increased not only in the target spines, but also spread into the dendrite region, and gradually returned to baseline over time ($\tau = 4.9$ min; Figure 15B, +PAC). The cAMP decay time constant was comparable to that observed with tetanic stimulation (Figure 7C). In contrast, no significant change was found in fluorescence lifetime after the mock photoactivation in the absence of PAC (Figure 15B, -PAC). These quantitative results demonstrate a dynamic cAMP increase by two-photon PAC photoactivation in living neurons (Figure 15).
Figure 15. REY ex vivo detects cAMP produced by PAC resulting from two-photon photoactivation.

(A) Schematic of PAC two-photon photoactivation and cAMP detection by REY using a two-photon FLIM microscope. (B) Two-photon PAC photoactivation at single dendritic spines. Left: cAMP pseudo-colour images of a dendrite before and after two-photon PAC photoactivation (1,000 nm, 11 mW, 30 sec) at the dendritic spine (blue circle). Right: Time-course of the lifetime change at target spines expressing REY with (+PAC; n = 12) or without PAC (-PAC; n = 11). The cAMP decay time was τ = 4.9 min (exponential fit). (C) Detection of PAC cAMP production by REY with and without PAC and also with and without light. PAC+/Light- (n = 12), PAC-/Light+ (n = 11), and PAC+/Light+ (n = 12). NS, p > 0.05; ***, p <0.001; Tukey’s test. All data are mean ± SEM.
1.9.3 Effect of light-dependent postsynaptic cAMP in structural potentiation of dendritic spines

Using the two-photon optogenetic approach, I next determined if the increase of postsynaptic cAMP is sufficient to enhance the maintenance of structural potentiation. I biolistically coexpressed RFP-tagged PAC and GFP (as volume filler) in CA1 pyramidal neurons, and measured the size of target dendritic spines during structural potentiation (Figure 16A). I then combined the postsynaptic two-photon PAC activation (Figure 15B) with glutamate uncaging to trigger structural potentiation. I found PAC photoactivation with uncaged structural potentiation also induced a significant prolonged spine enlargement (Figure 16B) similar to the remote tetanic stimulation effect (Figure 9D). As a control, I biolistically coexpressed a mutant version of PAC that produces cGMP in response to light rather than cAMP (BlgC; Ryu et al. (2010)) along with GFP as a volume-filler. Photoactivation of BlgC together with glutamate uncaging did not show the enhanced structural effect (Figure 16C, +BlgC), indicating the enhancement of structural potentiation with PAC photoactivation is specific to cAMP and not cGMP or the effect of two-photon excitation light itself. Also, two-photon photoactivation of PAC without glutamate uncaging did not show the prolonged enhancement of spine structure, indicating that cAMP signaling itself is not sufficient to induce structural potentiation of the spine and needs to couple with another mechanism which initiates structural potentiation to exert its effect (Figure 16D).
Figure 16. Light-dependent induction of postsynaptic cAMP enhances structural potentiation.

(A) Schematic of two-photon photoactivation of PAC or BlgC at target dendritic spines (1,000 nm, 11 mW, 30 sec) with glutamate uncaging. (B) Photoactivating PAC enhanced structural potentiation. Left: Representative images of dendritic spines expressing GFP + PAC before and after glutamate uncaging (±sLTP) with or without photoactivation of PAC (±PAC stim). Time stamp is in minutes. Green time stamp indicates immediately after glutamate uncaging. Right: Time-course of structural potentiation expressing GFP and PAC following glutamate uncaging with (magenta; n = 13) and without (black; n = 11) PAC photoactivation. Spines were stimulated with glutamate uncaging immediately after PAC photoactivation. (C) Effect of mock stimulation or BlgC stimulation on structural potentiation with GFP as a volume-filler. Left: Representative spine images of the combination of two-photon photoactivation (1,000 nm, 11 mW, 30 sec) and glutamate
uncaging at a target dendritic spine with and without expressing mutant PAC (BlgC). Right: Time course of structural potentiation in neurons expressing GFP following only uncaging (-PAC stim; -BlgC; grey; $n = 13$), uncaging with photoactivation in the absence of PAC (+PAC stim.; -BlgC; blue; $n = 11$), and uncaging with photoactivation in the presence of BlgC (+PAC stim. +BlgC; green; $n = 11$). (D) Photoactivated cAMP does not induce structural potentiation on its own. Left: Representative images of dendritic spines before and after two-photon photoactivation of PAC without glutamate uncaging at a target dendritic spine. Right: Time course of dendritic spine size changes in neurons expressing GFP following PAC photoactivation only (with PAC expression; magenta; PAC stim. Only $n = 11$). ***, $p < 0.001$; Tukey’s test. All data are mean ± SEM.
1.9.4 Determining downstream postsynaptic cAMP targets to enhance structural potentiation of dendritic spines

To determine the downstream cAMP target that regulates structural potentiation, I induced the cAMP-dependent structural potentiation in the presence of specific inhibitors for cAMP targets (Beavo and Brunton (2002); Figure 17A,B). The structural effect of cAMP was specifically mediated by PKA (inhibited by KT5720) but not HCN (hyperpolarization-activated cyclic nucleotide) channels (inhibited by ZD7288; Pape (1996)), and did not require new protein synthesis (inhibited by anisomycin; Figure 17C), suggesting a distinct cAMP/PKA downstream pathway for structural potentiation. In contrast, the initial enlargement of spine structure during structural potentiation was not affected in the presence of specific inhibitors (Figure 17D). These results suggest that postsynaptic cAMP plays an important role in maintaining spine structural potentiation rather than in induction.

1.9.5 Time-dependent cAMP effect during structural potentiation

To determine the timing of cAMP to enhance structural potentiation, I optogenetically increased postsynaptic cAMP at several time intervals after uncaging, and compare the spine enlargement (Figure 18A). The cAMP effect was time-dependent and it was necessary to activate PAC within ~1 minute after glutamate uncaging (Figure 18B). This suggests that the intracellular signaling critical for cAMP-dependent modification of structural potentiation lasts for less than one minute after uncaging.
Figure 17. Representative targets of postsynaptic cAMP and their effect on structural potentiation.

(A) Schematic of the major postsynaptic cAMP pathway for synaptic potentiation in CA1 pyramidal neurons. Components outlines in magenta indicate the target molecules for the inhibitors. (B) List of the specific inhibitors for target molecules in (A). (C) Effect of the specific inhibitors for cAMP-dependent structural potentiation. Spines were stimulated with glutamate uncaging immediately after PAC photoactivation in the presence of inhibitors and their sizes were compared after 10 min. No inhibitor (Ctrl; n = 13), HCN channels blocker (50 μM ZD7288; n = 10), protein synthesis inhibitor (50 μM anisomycin; n = 12) and PKA inhibitor (1 μM KT5720; n = 14). The black dotted line shows the level of structural potentiation by only glutamate uncaging (Figure 16B, black). (D) The spine size changes
immediately after uncaging did not show a difference in the presence of inhibitors. NS, $p > 0.05$; *, $p < 0.05$; Steel’s test. All data are mean ± SEM.
Figure 18. Timing-dependent effect of postsynaptic cAMP during LTP.

(A) Schematics of the timing-dependent cAMP experiments. Target spines were PAC photoactivated at various time intervals after glutamate uncaging. Their spine sizes were measured after 10 min of structural potentiation induction by uncaging. (B) Timing-dependent cAMP effect on structural potentiation. Delay intervals were: 0 min (uncaging immediately after PAC photoactivation; n = 13), 30 sec (PAC photoactivation after uncaging; n = 10), 1 min (n = 14), 5 min (n = 14), and Ctrl (no PAC photoactivation; n =11). Spine size of the interval 0 min was set as 1.0, and baseline was set to 0 for all delay conditions. NS, p > 0.05; **, p < 0.01; ***, p < 0.001; Steel’s test. All data are mean ± SEM.
1.9.6 Spatial-dependent cAMP effect on structural potentiation

I finally tested whether the crosstalk of cAMP between neighbouring spines is sufficient to enhance structural potentiation. The photoactivation of PAC at a nearby spine (3 – 25 µm away) induced significantly enhanced structural potentiation of the target spines after uncaging (Figure 19A). The efficacy of this crosstalk by cAMP was half as effective as compared to that of direct PAC photoactivation of the same spines (Figure 16B), however the effect was maintained even in spines located at greater than 20 µm distances (Figure 19B), indicating a strong spatial crosstalk function of cAMP in modification of structural potentiation.
Figure 19. Spatial effect of postsynaptic cAMP between dendritic spines for structural potentiation.

(A) Spatial-dependent cAMP effect on structural potentiation. Left: schematic drawing of PAC photoactivation at target spines in combination with uncaging of neighbouring spines. Right: Time-course of structural potentiation with (magenta; \( n = 45 \)) or without (black; \( n = 13 \)) two-photon photoactivation of PAC at neighbouring spines. The grey dotted line shows the level of cAMP-dependent structural potentiation in the same target spines (Figure 16B magenta).

(B) Distance-dependent effect of cAMP in structural potentiation between spines for PAC photoactivation and uncaging. The PAC photoactivation effect was plotted by distances between the spines (\( n = 45 \)). Magenta line indicates the average effect (linear fit). All data are mean ± SEM. ***, \( p < 0.001 \); Tukey’s test.
1.10 Determining the cAMP signalling pathway that regulates structural potentiation of dendritic spines

Postsynaptic CaMKII (Ca$^{2+}$/calmodulin dependent protein kinase II) is a pivotal molecule controlling the actin cytoskeleton in an activity-dependent manner for structural and functional synaptic potentiation (Kim et al., 2015). CaMKII consists of α and β subunits with the β subunit possessing the F-actin binding function in addition to an enzymatic function (Section 1.1.3). CaMKII activity is negatively regulated by PP1 (Protein Phosphatase 1) which is also downregulated by the cAMP/PKA-dependent phosphorylation pathway in pyramidal neurons of the CA1 region of the hippocampus (Blitzer et al. (1998); Figure 17A).

Since I found that the cAMP/PKA pathway is critical for the regulation of structural potentiation of dendritic spines, I hypothesized that cAMP/PKA may phosphorylate PP1 after the induction of structural potentiation, resulting in the inactivation of PP1, and thereby prolong CaMKIIβ activation. Extended CaMKIIβ activation would permit prolonged postsynaptic structural enhancement during structural potentiation. To examine the cAMP pathway, I utilized a CaMKIIβ FRET probe (Section 1.10.1) and monitored the indirect cAMP effect on postsynaptic CaMKIIβ activity during structural potentiation in neurons using two-photon CaMKIIβ FRET imaging (Section 1.10.2).

1.10.1 Characterization of CaMKIIβ FRET probe in vitro

To monitor CaMKIIβ activity in living neurons, I first validated a previously reported CaMKIIβ FRET probe (Camüiβ) in vitro (Takao et al. (2005); Figure 20A). When the probe was illuminated with CFP-specific excitation (433 nm), the emission spectrum showed a
distinct YFP FRET peak (527 nm), which decreased upon addition of Ca$^{2+}$, calmodulin, and ATP (to activate CaMKIIβ) and concomitantly dequenched the CFP signal, indicating a FRET change corresponding to CaMKIIβ activation (Figure 20B). The subsequent addition of the calcium chelator EGTA resulted in a decrease in FRET ratio, but did not return to baseline, indicating the autophosphorylation-dependent activity of CaMKIIβ (Figure 20B-D). Trypsin cleaved the probe’s CFP from YFP, and completely abolished the YFP peak confirming the YFP signal was due to FRET. These results confirmed the detection of CaMKIIβ activity using the CaMKIIβ FRET probe (Camüiβ).
Figure 20. Characterization of the CaMKIIβ FRET probe Camüβ in vitro.

(A) Schematic of detection of CaMKIIβ activity with FRET (Camüβ) in vitro. (B) Emission spectra of a CaMKIIβ FRET probe (Camüβ) with CFP-specific excitation (433 nm). Before (Baseline) and after addition of 200 µM CaCl₂/1 µM Calmodulin/50 µM ATP (+Ca²⁺), 400 µM EGTA (+EGTA) and 1% Trypsin digestion (+Trypsin). (C) Average time-course of the CaMKIIβ FRET change stability in the presence or absence of CaCl₂/Calmodulin/ATP (Ca²⁺; n = 3). Chelating Ca²⁺ by EGTA did not totally reverse the FRET change due to the presence of CaMKIIβ autophosphorylation (EGTA; n = 3). (D) Quantification of activation-dependent...
CaMKIIβ FRET (CFP/YFP ratio) changes after the addition of CaCl₂/ Calmodulin/ATP (+Ca²⁺) and EGTA (+EGTA; n = 3). ***, p < 0.001; Tukey’s test. All data are mean ± SEM.
1.10.2 The indirect effect of cAMP in CaMKIIβ activity during structural potentiation of dendritic spines

To explore the downstream cAMP signaling pathway for structural potentiation, I measured the postsynaptic cAMP effect on CaMKIIβ activity during structural potentiation using two-photon CaMKIIβ FRET imaging in the presence of specific inhibitors (Figure 21A, B). After structural potentiation induction by uncaging with mock two-photon photoactivation (in the absence of PAC), CaMKIIβ activity rapidly increased and lasted around 1 min ($\tau = 82$ sec: Figure 21C) which was similar to that of CaMKIIα (Lee et al., 2009; Lisman et al., 2012). Structural potentiation induction with PAC photoactivation showed prolonged CaMKIIβ activity, which was blocked in the presence of a PKA inhibitor (KT5720). I also found that a bath applied PP1 inhibitor (tautomycetin) prolonged CaMKIIβ activity during structural potentiation without PAC photoactivation, suggesting the effect of cAMP on CaMKII activity is mediated through cAMP/PKA/PP1 signaling. The induction of structural potentiation in the presence of a PP1 inhibitor (tautomycetin) induced an enhanced increase in spine size (Figure 21D) which was similar to the effect of PAC photoactivation (Figure 16B), suggesting a signaling mechanism of structural modification through the cAMP/PKA/PP1/CaMKIIβ pathway.
Figure 21. Postsynaptic cAMP signalling pathway for structural potentiation modification.

(A) Schematic of the postsynaptic cAMP signalling pathway involving cAMP and CaMKII. Magenta circles indicate the targets for specific inhibitors. (B) List of inhibitors for the target molecules in (A). (C) Time-course of postsynaptic CaMKIIβ activity during structural potentiation by two-photon CaMKIIβ FRET imaging (Camüiβ). Glutamate uncaging with mock PAC photoactivation (black, n = 14, τ = 82.8 sec, exponential fit), PAC photoactivation with glutamate uncaging (magenta, n = 12), and in the presence of a PKA inhibitor (KT5720, blue, n = 10), or in the presence of a PP1 inhibitor without PAC photoactivation (tautomycetin, green, n = 10). (D) Effect of a PP1 inhibitor on structural potentiation (without PAC photoactivation). Left: Representative images of dendritic spines expressing only GFP.
before and after glutamate uncaging in the presence or absence of a PP1 inhibitor (5 nM tautomycetin). Time stamp is in minutes. Green time stamp indicates uncaging. Right: Time-course of structural potentiation following only glutamate uncaging (black; \(n = 11\)) and in the presence of tautomycetin (magenta; \(n = 10\)). A black arrow indicates the time point for glutamate uncaging. The grey dotted line shows the level of structural potentiation with PAC photoactivation at 20-30 minutes post-uncaging (Figure 3B magenta). ***, \(p < 0.001\); Tukey’s test. All data are mean ± SEM.
1.11 Discussion

By establishing two-photon optogenetic approaches for cAMP at single synapses, I revealed a mechanism in which postsynaptic cAMP serves as a modulator for structural potentiation of dendritic spines (Figure 22). Furthermore, activation of this mechanism at targeted spines exhibits crosstalk with neighbouring spines. Under nominal structural potentiation induction, we have previously demonstrated that activation of CaMKII (α/β hetero-oligomer) by postsynaptic Ca\(^{2+}\)-influx induces changes in spine structure by detaching CaMKII from F-actin, which creates a time window for remodeling of the actin cytoskeleton during structural potentiation (Kim et al., 2015). In this study, I found that strong synaptic stimulation leads to postsynaptic cAMP increases in addition to activation of CaMKII by Ca\(^{2+}\) influx, triggering the cAMP/PKA pathway (Figure 22). This prolongs the activation time of CaMKIIβ during synaptic potentiation through PP1 inactivation (Figure 21C). This results in an enhancement of the maintenance of structural potentiation probably by extending the duration of actin cytoskeleton remodeling (Figure 21D).

The time window for the cAMP-dependent modification is short. In the optogenetic experiment, which allows for timing-dependent cAMP production in spines, cAMP is effective within the first minute after the induction of structural potentiation (Figure 18B). However, PAC-produced cAMP alone could not induce structural changes in spines (Figure 16D), indicating other signals (such as Ca\(^{2+}\)/CaM) are necessary to trigger structural potentiation for cAMP-dependent structural enhancement to be effective. Considering that the short time window of cAMP is comparable to that of CaMKIIβ activity, and that cAMP indirectly regulates CaMKIIβ activity through PKA/PP1 (Figure 21C), cAMP may be present
Figure 22. A proposed model for intra- and inter-spine cAMP function during structural potentiation.

Role of intra- and inter-spine cAMP as an interaction mechanism that regulates structural potentiation in dendritic spines. Left spine: Strong stimulus activates Ca\(^{2+}\)/CaM-dependent AC (adenylyl cyclase) in addition to CaMKII and results in the activation of the cAMP/PKA signaling pathway. Active PKA indirectly phosphorylates PP1 which inactivates dephosphorylation function of PP1 resulting in prolonged CaMKII activity and enhanced structural potentiation. Right spine: Glutamate uncaging-dependent structural potentiation, which is typically triggered by activation of CaMKII (α/β hetero-oligomer) through Ca\(^{2+}\)-influx (Kim et al., 2015; Okamoto et al., 2007), does not require/produce cAMP but is enhanced by the crosstalk of cAMP/PKA signals without new protein synthesis (induced by strongly stimulated spines). This allows interaction of nearby spines for structural potentiation by cAMP crosstalk.
during activation of CaMKIIβ to modify structural potentiation. Also, a PKA inhibitor blocks the cAMP-dependent modulation but not nominal structural potentiation (Figure 9D, Figure 17C), indicating cAMP/PKA signaling may provide a positive feedback mechanism for CaMKIIβ activity to modify potentiation.

Furthermore, I demonstrated the ability of cAMP to modify structural potentiation at nearby spines (Figure 9, Figure 19), suggesting a spatial crosstalk mechanism between synapses for potentiation. The cAMP effect is protein synthesis independent (Figure 17), therefore distinct from cAMP-dependent protein synthesis machinery for LTP which is input-specific at individual synapses (Kandel, 2001, 2012). Because the observed cAMP crosstalk function has a reduced effect even on structural potentiation (Figure 19), its concentration may be insufficient to induce the protein synthesis machinery at neighbouring spines. In addition, the optogenetic experiments indicate that an increase of exogenous cAMP is sufficient to modify structural potentiation (Figure 16). This suggests that other cAMP signaling such as G-protein coupled adenylyl cyclase pathways (Tang and Gilman, 1991) could also modify potentiation. As we have demonstrated that the signaling pathway for structural potentiation is closely linked to the induction of functional LTP (Kim et al., 2015), it is therefore of great interest to understand how various cAMP signaling pathways are intra- and intersynaptically coordinated for the modification of functional synaptic potentiation.

Thus, two-photon optogenetics, along with photoactivatable enzymatic proteins, will provide novel and powerful tools to study spatiotemporal signaling pathways in an unprecedented way. Previous neuron-level PAC studies showed that activation of PAC led to changes in the spike and current in both Aplysia sensory neurons (Nagahama et al., 2007) and – with coexpressed exogenous CNG channels – rat CA1 pyramidal neurons (Stierl et al., 2011),
indicating rapid light-dependent cAMP increase by PAC activation in neurons. My study extends these cellular level PAC applications by using two-photon excitation which enables the photoactivation at subcellular compartments, such as dendritic spines, thus allowing the analysis of spatiotemporally localized effect for synaptic potentiation within living neurons.

In summary, I revealed a mechanism of cytoskeletal regulation mediated by postsynaptic cAMP, essential to spatiotemporally modify dendritic spine structure in glutamatergic excitatory synapses. The structural role of cAMP is protein synthesis-independent, and critical to prolong CaMKIIβ activity during synaptic potentiation. The function of cAMP not only modifies potentiation within targeted synapses but also in neighbouring synapses, suggesting cooperative cAMP-driven mechanisms for synaptic potentiation are at work. Thus, my studies illustrate that cAMP mediates a rapid mechanism for synaptic modulation that exhibits spatiotemporal interactions that could underlie synaptic interplay as a fundamental feature of learning and memory.
Chapter 2

The rapid effect of postsynaptic cAMP in neural potentiation
and related short-term learning

In Chapter 1, I revealed novel intra- and inter-synaptic cAMP mechanisms which indirectly regulate CaMKIIβ signaling for the modulation of structural potentiation independently of cAMP-mediated protein synthesis. This suggests that postsynaptic cAMP may be able to enhance potentiation not only in strongly activated synapses (which produce cAMP), but also at the neural circuit level by affecting the potentiation of nearby synapses which are weakly activated. As we previously found that the postsynaptic CaMKIIβ/actin interaction is crucial to regulate not only structural potentiation but also functional potentiation (Kim et al., 2015), I hypothesized that rapid and protein synthesis independent cAMP also has a functional role at the neural circuit level. To elucidate the function of cAMP at the neural circuit level, I studied the spatiotemporal effect of cAMP in functional potentiation at the medial perforant path (PP) synapses of dentate gyrus (DG) granule neurons, which plays a critical role in learning and memory (Gilbert et al. (2001); Moser et al. (1998); Section 2.1). Since protein synthesis-independent potentiation is correlated with short-term learning (Quevedo et al., 2004), I next examined a novel rapid cAMP function in the related object recognition learning (Saab et al., 2009) by establishing optogenetic approaches to precisely manipulate cAMP levels by light in targeted brain regions of freely behaving mice (Section 2.2). These results demonstrate a rapid and protein synthesis-independent cAMP function at the neural circuit level during functional synaptic potentiation and the related learning processes.
2.1 Spatiotemporal effect of postsynaptic cAMP on protein synthesis-independent synaptic potentiation

To examine the rapid and protein synthesis-independent cAMP function at the neural circuit level, I studied the spatiotemporal effect of cAMP in functional potentiation at the medial perforant path (PP) synapses of dentate gyrus (DG) granule cell neurons. These synapses exhibit post-synaptic NMDA receptor-dependent long-term synaptic potentiation (LTP), and postsynaptic cAMP is involved in the protein synthesis dependent late phase of potentiation (L-LTP; Brandon et al. (1995); Nguyen and Kandel (1996)). However, it is still unknown what functional effects cAMP has on protein synthesis-independent potentiation. Since pharmacological (such as with inhibitors) and genetic (such as with knockouts) approaches don’t have the spatiotemporal resolution to manipulate postsynaptic cAMP at PP-DG synapses, I again utilized a photoactivatable adenylyl cyclase (PAC) optogenetic approach which light-dependently increases cAMP. To best utilize PAC, I established a mouse line which highly expresses PAC in hippocampal DG granule neurons (Section 2.1.1). Using this transgenic mouse I investigated the effect of cAMP on functional plasticity using electrophysiology, which has high temporal specificity, in collaboration with the labs of Dr. John Roder and Dr. Graham Collingridge at the Lunenfeld-Tanenbaum Research Institute (Section 2.1.2). I also investigated the effect of cAMP on the distribution of functional plasticity using a combination of electrophysiology and voltage-sensitive dye imaging, which has high spatial specificity, in collaboration with the lab of Dr. Takashi Tominaga at Tokushima Bunri University in Japan (Section 2.1.3). Using these techniques, I investigated the role of postsynaptic cAMP in functional potentiation using acute hippocampal slices of transgenic mice which highly express PAC in hippocampal DG granule cells.
2.1.1 Generation of a PAC expressing transgenic mouse line

In order to manipulate postsynaptic cAMP levels by light at hippocampal PP-DG synapses, we generated a transgenic mouse line which highly expresses PAC in DG granule neurons. To express PAC in the mouse DG, we prepared a transgene construct in which the CaMKII promoter drives the expression of a red fluorescence protein (RFP)-tagged PAC in excitatory neurons (Figure 23A). The Centre for Phenogenomics (TCP) Transgenics Core injected C57B/6J embryos with this transgene and then implanted them into pseudo-pregnant recipient mice. We confirmed the presence of the transgene in twelve independent mouse lines by genotyping (Figure 23B). I found a line which showed robust RFP expression in the hippocampal DG region (Figure 23C). To confirm PAC had enzymatic function, I photoactivated a lysate prepared from hippocampal slices with 30 seconds of blue light exposure (455 nm peak, 4.5 mW/mm²). The expressed PAC showed a light-dependent cAMP increase, indicating enzymatic functionality (Figure 23D).
Figure 23. Generation of a transgenic mouseline that highly expresses PAC in the DG of the hippocampus.

(A) Schematic representation of the PAC transgene construct. (B) Genotyping of PAC transgenic mice. The specific PCR amplification of an 859 bp band for PAC in transgenic mice was detected on the agarose gel. TG (PAC transgenic mice), WT (wild type mouse).

(C) Distribution of the expressed RFP (tdTomato)-PAC in the hippocampal slice. The RFP fluorescence from the hippocampal slice was imaged by a confocal microscope and merged with the transmitted light images. Top: WT (wild type) Bottom: TG (PAC transgenic mouse).

(D) Detection of light-dependent cAMP production by WT (WT) and PAC transgenic (TG) mouse hippocampal slices. The hippocampal slice lysate was illuminated by a 455 nm LED (4.5 mW/mm², 30 seconds) and the resulting cAMP was measured by an ELISA (n = 3). **, p < 0.01, Unpaired t-test. All data are mean ± SEM.
2.1.2 Effect of postsynaptic cAMP in PP-DG post-tetanic potentiation

To confirm that the mere expression of PAC (without photoactivation) at PP-DG synapses does not affect their basal function, I examined the basal synaptic transmission at perforant path to dentate gyrus (PP-DG) synapses by measuring the field excitatory postsynaptic potentials (fEPSPs) in acute hippocampal slices (Figure 24A). The input/output (I/O) curves and DG-specific short-term depression (measured by paired-pulse ratio (PPR)) were similar between TG and WT, indicating that the PAC transgenic mice have comparable synaptic responses without photoactivation (Figure 24B, C). To test the effect of PAC photoactivation on basal synaptic responses in PP-DG synapses, I illuminated slices with an LED (480 ± 15 nm, 1.5 mW, 60 sec) and recorded the fEPSPs every 20 sec during photoactivation (Figure 24D). Photoactivation of PAC alone did not affect basal synaptic transmission at PP-DG synapses, indicating no immediate effect of cAMP without tetanic stimulation.
Figure 24. Baseline synaptic transmission of PAC transgenic mice and wild-type littermates.

(A) Schematic of PAC photoactivation experiments in hippocampal slices. PAC is highly expressed in DG granule cells (magenta) and was photoactivated by blue light (480 ± 15 nm). fEPSPs were measured in the PP-DG synapse region using paired-pulse stimulation every 20 sec. (B) Input/output relationship within dentate synapses with (TG, n = 16 slices/9 mice) and without (WT, n = 7 slices/3 mice) PAC. (C) Comparison of paired-pulse ratio in the PP-DG between WT and TG. Paired-pulse activity was calculated from the ratio of the second fEPSP slope to the first, at different interpulse intervals (WT n = 12 slices/4 mice, TG n = 15 slices/7 mice). (D) Quantification of synaptic response with/without PAC photoactivation.
Slices were photoactivated (480 ± 15 nm, 1.5 mW) for 60 sec during baseline recording under a fluorescence microscope (TG n = 11 slices/7 mice, WT n = 13 slices/3 mice). NS, $p > 0.05$, unpaired t-test. All data are mean ± SEM.
To examine the rapid postsynaptic cAMP effect independent of protein synthesis, I first applied tetanic stimulation without blocking the activity of inhibitory neuronal inputs (maintaining endogenous conditions). The induction of functional potentiation by tetanic stimulation in PAC TG hippocampal slices without photoactivation showed a short fEPSP increase that returned to baseline within 1-2 minutes (Figure 25B, C). In contrast, when photoactivation of PAC was delivered alongside tetanic stimulation in PAC TG hippocampal slices, I observed a robust enhancement of potentiation that lasted for around 30 minutes (Figure 25B). This effect was not seen in WT littermates using the exact same light and tetanic stimulation treatment (Figure 25B, C). In addition, the presence of the potent protein synthesis inhibitor anisomycin did not affect cAMP-dependent enhanced potentiation, indicating a protein synthesis-independent cAMP function (Figure 25B). To confirm that this cAMP effect is postsynaptic, I measured the PPR of TG and WT slices before and after tetanic stimulation either with (+Light) or without (-Light) PAC photoactivation (Figure 25D). I found all responses showed paired-pulse depression, with no significant difference in the PPR before and after tetanic stimulation, indicating a postsynaptic cAMP effect. Taken together, these results demonstrate that the postsynaptic cAMP effect transiently enhances postsynaptic functional potentiation at the PP-DG pathway, independent of protein synthesis.
Figure 25. Photoactivation of PAC enhances functional potentiation at PP-DG synapses.

(A) Representative traces of the fEPSP response before (Baseline; black) and after (+Tet; magenta) tetanic stimulation (100 Hz, 3x pulse width, 4 times, 20 seconds apart) over time. Blue arrows indicate time of paired-pulse stimulation from the stimulation electrode. (B) PAC photoactivation enhanced the post-tetanic potentiation induced by tetanic stimulation at PP-DG synapses. The potentiation was induced through a strong tetanic stimulation (4 x 100Hz, 3x pulse width) in TG animals with (+Light, TG; magenta; n = 11 slices/7 mice), and without (-Light, TG; black; n = 10 slices/3 mice) blue light (480 ± 15 nm, 5 min, magenta bar). Also tested were WT littermates in the presence of blue light (+Light, WT; cyan; n = 13 slices/3 mice) and TG animals in the presence of blue light and the protein synthesis inhibitor
anisomycin (+Light, TG, Anis; grey; n = 10 slices/3 mice). Different letters (a, b) correspond to significantly different groups (p < 0.01; Tukey’s test), while the same letter corresponds to not significantly different groups (p > 0.05; Tukey’s test). Data is compared as averages of 25-30 min after tetanic stimulation (black line). (C) Comparison of post-tetanic potentiation within the boxed region in (B) showing the fEPSP slope in detail of WT littermates in the presence of blue light (cyan) and TG animals without blue light (black). (D) Comparison of paired-pulse ratio before (-10 to 0 min, black) /after tetanic stimulation (10 to 20 min, white) ± blue light in both TG and WT from (B). NS, p > 0.05, paired t-test. All data are mean ± SEM.
2.1.3 Postsynaptic cAMP enhances spatial potentiation in the PP-DG neuronal circuit

To examine the spatial effect of postsynaptic cAMP on the activity of PP-DG neural synapses in response to functional potentiation, I employed fluorescent voltage-sensitive dye (VSD; di-4-ANEPPS) imaging in addition to fEPSP recording in collaboration with Dr. Takashi Tominaga (Figure 26). Using VSD imaging, I monitored spatially the optically detected depolarization that spread throughout the PP-DG neural circuit region before and after tetanic stimulation of the pathway both with (Figure 27) and without (Figure 28) photoactivation of PAC in acute hippocampal slices.
Figure 26. Properties of the voltage-sensitive dye di-4-ANEPPS.

(A) Top: Schematic chemical structure of the voltage-sensitive dye di-4-ANEPPS. Bottom: Di-4-ANEPPS intercalates in the plasma membrane of cells and becomes fluorescent. Adapted from Loew et al. (1992) (B) Fluorescent response of di-4-ANEPPS following voltage change. Adapted from Knopfel et al. (2006). (C) Excitation (blue) and emission (red) spectra of di-4-ANEPPS. Adapted from Loew et al. (1992). (D) Schematic drawing of VSD imaging on a hippocampal slice. A stimulation electrode (Stim) was placed in the PP (green). The VSD fluorescent signals allowed for the measurement of synaptic activation of the DG granule cells (magenta) with simultaneous fEPSP monitoring in the molecular layer with a recording electrode (Rec). The faint blue circle indicates the light spot that activates PAC in the hippocampal slices of a transgenic mouse that highly expresses PAC in DG granule cells. Dotted rectangle indicates a ROI region for the VSD imaging.
Figure 27. Tetanic stimulation-induced changes in the VSD signals in the hippocampal DG region with PAC photoactivation.

(A) Schematic setup for measuring VSD fluorescence signal and fEPSP on the PAC TG hippocampal slices. Stim: stimulation electrode. Rec: recording electrode. (B) Schematic time-course diagram of VSD imaging (VSD) with fEPSP recording before and after photoactivation of PAC (PAC) and tetanic stimulation. VSD fluorescence changes were recorded as an average of 8 images (400 msec duration each) taken every 20 sec before and after tetanic stimulation (1 train, 100 x 0.05 msec duration pulses, 0.5 mA). (C) Sample trace of the VSD signal after tetanic stimulation with PAC photoactivation. The black arrow (Stim) shows the time of delivery of electrical stimulation which produces the optical signals (at 100 msec). Grey box (Imaging): time window (100-120 msec) used for the measurement of fluorescence changes during basal stimulation and after tetanic stimulation. (D) Time-lapse
pseudocolour images of the VSD fluorescence signal after the electric stimulation with photoactivation of PAC. Red colours indicate higher fluorescence intensities compared to blue colours, suggesting strong depolarization.
Figure 28. Tetanic stimulation-induced changes in the VSD signals in the hippocampal DG region without PAC photoactivation.

(A) Schematic setup for measuring VSD fluorescence signal and fEPSP on the PAC TG hippocampal slices. Stim: stimulation electrode. Rec: recording electrode. (B) Schematic time-course diagram of VSD imaging (VSD) with fEPSP recording before and after tetanic stimulation. VSD fluorescence changes were recorded as an average of 8 images (400 msec exposure each) every 20 sec before and after tetanic stimulation (1 train, 100 x 0.05 msec duration pulses, 0.5 mA). (C) Sample trace of the VSD signal after tetanic stimulation without PAC photoactivation. The black arrow (Stim) shows the time of delivery of electrical stimulation which produces the optical signals (at 100 msec). Grey box (Imaging): time
window (100-120 msec) used for the measurement of fluorescence changes during basal stimulation and after tetanic stimulation. (D) Time-lapse pseudocolour images of the VSD fluorescence signal after the electric stimulation without photoactivation of PAC. Red colours indicate higher fluorescence intensities compared to blue colours, suggesting strong depolarization.
I observed the area of optically-detected depolarization throughout the DG along the perforant path (PP) by tetanic stimulation of the PP (Figure 29A). The photoactivation of PAC followed by tetanic stimulation immediately showed a cAMP-elicited potentiation in the intensity of fEPSPs (Figure 29B). This corresponded to an increase in fluorescence intensity in the DG compared with control (Figure 29C, +Light). Interestingly, tetanic stimulation in the absence of PAC photoactivation did not enlarge the fluorescence area compared to baseline (Figure 29D, -Light). However, this fluorescence area was significantly enlarged by tetanic stimulation with photoactivation of PAC (Figure 29D, +Light).

A three-dimensional (3D) map of the averaged VSD fluorescence changes also indicated an overall increase of both intensity and area of activity in the DG after tetanic stimulation of the PP in the presence of cAMP (photoactivation of PAC, Figure 30) compared with tetanic stimulation only (Figure 31). These results demonstrate that postsynaptic cAMP enhances the area and intensity of fEPSPs in the DG region. The cAMP-dependent enlarged region of VSD fluorescence change may indicate an increased number of activated synapses on the dendrites (Figure 32).
Figure 29. Postsynaptic cAMP enhances spatial potentiation in the PP-DG synapse region.

(A) Representative VSD images in DG area after tetanic stimulation with (+Light) or without (-Light) PAC photoactivation. The VSD fluorescence signal changes are displayed in pseudo colour at the peak change time (106.4 msec). Red-shifted colors indicate more depolarization compared to blue-shifted colors. (B-D) Quantification of the fEPSP changes after tetanic stimulation (B), the changes in the averaged peak fluorescence intensity (C), and area size (D) both during 0.4 – 160 sec after tetanic stimulation (8 x 20 msec imaging every 20 sec) with (+Light, n = 8 slices/2 mice) or without (-Light, n = 7 slices/2 mice) PAC
photoactivation normalized to baseline (before tetanic stimulation). *, $p < 0.05$; **, $p < 0.01$; unpaired t-test. All data are mean ± SEM.
Figure 30. 3D visualization of tetanic stimulation-induced potentiation with PAC photoactivation.

(A) Pseudo-colored three-dimensional graph of the averaged peak VSD fluorescence changes in the DG before (Baseline) and after (+Tetanus) tetanic stimulation with PAC photoactivation (n = 8 slices/2 mice). (B) The differences of three-dimensional averaged distribution of VSD fluorescence changes in the presence of photoactivation of PAC. The 3D fluorescence images were calculated as the ratio of the fluorescence changes between before and after tetanic stimulation in the presence of PAC photoactivation. (C) Fluorescence profiles across the lines in the 3D fluorescence distribution (white box from (A)) fitted with a parabola. Black, before tetanic stimulation (Baseline); magenta, after tetanic stimulation with photoactivation of PAC (+Tet/+Light).
Figure 31. 3D visualization of tetanic stimulation-induced potentiation without PAC photoactivation.

(A) Pseudo-coloured three-dimensional graph of the averaged peak VSD fluorescence changes in the DG before (Baseline) and after (+Tetanus) tetanic stimulation without PAC photoactivation (n = 7 slices/2 mice). (B) The differences of three-dimensional averaged distribution of VSD fluorescence changes in the absence of photoactivation of PAC. Fluorescence images were calculated as the ratio of the fluorescence changes between before and after tetanic stimulation in the absence of PAC photoactivation. (C) Fluorescence profiles across the lines in the 3D fluorescence distribution (white box in (A)) fitted with a parabola. Black, before tetanic stimulation (Baseline); magenta, after tetanic stimulation without photoactivation of PAC (+Tet/-Light).
Figure 32. A model for the spatial effect of cAMP in functional potentiation.

A schematic of PP axons (green) and DG dendrites (blue) in the hippocampal DG region. Synapses at the DG-PP receive inputs of varying strength (different sized green arrows), and cAMP enhances the potentiating effect (even of subthreshold inputs).
2.2 Role of the rapid function of cAMP in short-term object recognition learning

Synaptic plasticity such as potentiation and depression is a cellular model for learning and memory, where protein synthesis independent plasticity is tightly correlated with short-term memory (Bliss and Collingridge, 1993). The murine hippocampal DG region is involved in short-term memory (Kesner, 2007). Studies of mouse models of psychiatric disease that have defects in the cAMP pathway suggest that cAMP may affect not only long-term memory but also short-term memory of object recognition (McGirr et al., 2016; Saab et al., 2009).

Postsynaptic cAMP is critical for protein-synthesis-dependent LTP in PP-DG synapses and is tightly correlated to long-term memory; however the cAMP-dependent machinery for short-term memory remains elusive (McGirr et al., 2016; Wu et al., 1993). To directly elucidate the function of cAMP in short-term learning, I utilized the photoactivatable enzymes I previously established to light-dependently manipulate cAMP (increase and decrease) in mouse hippocampal DG granule cell neurons. These optogenetic approaches allowed for spatiotemporal manipulation of cAMP levels by light in target regions of freely behaving mice, which has not been possible using pharmacological or genetic methods. In this way, I was able to elucidate a rapid function of cAMP during short-term memory. In collaboration with the lab of Dr. John Roder (LTRI), I learned and employed two mouse object recognition memory tests, which are DG-dependent tests of short- and long-term memory (Ennaceur and Delacour, 1988). By photoactivation of light-sensitive enzymes in the freely behaving mice, I behaviourally assayed the role of cAMP on short-term learning using object recognition and discrimination tests.
2.2.1 Targeted optogenetic control of cAMP in the mouse DG region

To examine the effect of cAMP on short-term object recognition learning in freely behaving mice, I implanted bilateral optical fibers fed by LEDs into the hippocampal DG region of PAC (photoactivatable adenylyl cyclase) transgenic mice (Figure 33). The LEDs connect to a battery and wireless control unit, which allows for the remote photoactivation of PAC by light.
Figure 33. Wireless control of DG PAC photoactivation in freely behaving mice.

(A) Schematic cross section of optical fibre placement in the mouse’s brain. Transgenic mice (PAC expressed highly in DG granule neurons) have optical fibres inserted bilaterally into their DG. Light (470 nm) is emitted by an LED unit on the top of the skull and channeled into the DG through the fibres directly. The assembly is held firmly in place by fixing it to the skull with dental cement. The LED units have detachable batteries and a wireless remote control module. (B) Photograph of the implanted mouse.
2.2.2 Short-term memory test for mouse object recognition

To determine the effect of cAMP on short-term learning, I utilized an object recognition memory test (Ennaceur and Delacour, 1988). The test takes advantage of the innate exploratory nature of the mice (novelty-seeking) and does not require external reinforcement (reward or pain) for the mice to perform the task. Specifically, I used a displaced object test (DOT) and a novel object test (NOT) for the short-term object recognition memory tests (Figure 34A). In the displaced object test, one of the objects in the area is moved after habituation, and the mice are tested to see if they can recognize this change within 5 minutes, reflecting spatial short-term memory (Ennaceur and Delacour, 1988). In the novel object test, the moved object (from the displaced object test) is replaced with a new one and returned to the original position from the habituation phase. The novel object test, in contrast to the displaced object test, reflects the ability to recognize a novel object which requires memory of object properties. Mouse behaviour was tracked using a video camera and the time they spent with each object was automatically recorded (Figure 34B). The mice are given a 5 min habituation stage (training), in which they first recognize the original position and properties of two identical objects (typically short-term learning). During this habituation stage I photoactivated enzymes to increase cAMP levels in the DG, then evaluated the formed memory by object recognition tests compared to no photoactivation controls. This allowed me to examine the cAMP effect on short-term object recognition learning.
Figure 34. Design of the object recognition test for short-term memory.

(A) A timeline for object recognition memory. Top: The mice are placed in an area with two objects (circles) and habituate either with (Light ON) or without (Light OFF) photoactivation of PAC for short-term object recognition learning. Middle: One object is then moved, and the mice are placed back into the area for five minutes (the displaced object test). Bottom: Finally, the displaced object is changed to a novel object, returned to its habituation position, and the mice are placed back into the arena for another five minutes (the novel object test). (B) Sample traces of mouse behaviour in the object recognition tests for short-term learning with (Light ON) and without (Light OFF) photoactivation of PAC during habituation. The time each mouse spends at the objects is tracked during the experiment using a video camera.
2.2.3 The effect of increased cAMP on the formation of short-term object recognition memory

To examine the role of cAMP in the hippocampal DG for the formation of short-term object recognition memory, I photoactivated PAC (photoactivatable adenylyl cyclase) during the 5 min habituation phase. In the displace object test for short-term memory, mice did not show discrimination between displaced and stationary objects (Figure 35A). However, mice which had PAC photoactivated during the habituation phase (Light ON mice) spent more time with the displaced object compared to the stationary object in the displacement phase, indicating a cAMP-dependent preference for the displaced object (Figure 35B). I repeated the displaced object test but with a 24 hour delay after the initial 5 minutes of PAC photoactivation (instead of a 1.5 min delay). These 24 hour delayed mice did not show a preference for either the stationary or displaced object. This suggests that cAMP promotes the short-term learning of displaced objects.

In the novel object test for short-term memory, the mice in both light conditions (Light ON/OFF) strongly preferred to spend time with the novel object in the novel object phase, suggesting a distinct object recognition memory mechanism from the DOT (Figure 36B, C) that was not affected by additional cAMP (Figure 36D). As freely behaving mice have diverse synaptic activities in response to environmental input, the endogenous cAMP level in the DG may be sufficient for recognizing the novel object in the novel object phase.
Figure 35. Short-term displaced object recognition learning with or without PAC photoactivation.

(A) Top: summary schematic of displaced object test results without PAC photoactivation. Bottom: displaced object preference ratio without PAC photoactivation ($n = 28$). (B) Top: summary schematic of displaced object test results with PAC photoactivation. Bottom: displaced object preference ratio with PAC photoactivation. Mouse DG photoactivation was during the habituation phase (5 min duration) before being assayed in this displaced object testing phase ($n = 28$). (C) Top: summary schematic of displaced object test results with PAC photoactivation and 24 hours between the habituation phase and displaced object testing phase. Bottom: displaced object preference ratio 24 hours after hippocampal DG PAC photoactivation during habituation ($n = 14$). NS, $p > 0.05$; **, $p < 0.01$, unpaired t-test. All data are mean ± SEM.
Figure 36. Short-term novel object recognition learning with or without PAC photoactivation.

(A) Top: summary schematic of novel object test results without PAC photoactivation. Bottom: novel object preference ratio without PAC photoactivation (n = 23). (B) Top: summary schematic of novel object test results with PAC photoactivation. Bottom: novel object preference ratio with PAC photoactivation. Mouse DG photoactivation was during the habituation phase (5 min duration) before being assayed in this novel object testing phase (n = 23). (D) Preference for the novel object with (+Light) and without (-Light) PAC photoactivation (n = 23). NS, p > 0.05; *, p < 0.05, unpaired t-test. All data are mean ± SEM.
2.2.4 The generation of a light-activated phosphodiesterase 4 (LAPD4) expressing mouse

To determine endogenous cAMP function in short-term object recognition, I utilized an optogenetic approach which light-dependently hydrolyzes endogenous cAMP using a light-activated phosphodiesterase 4 (LAPD4) in the mouse brain. LAPD4 was developed and established by Mrs. Fiona Bergin the rapid light-dependent cAMP specific activity in vitro and in vivo (Figure 37).

To examine the effect of endogenous cAMP suppression on short-term object recognition learning in freely behaving mice, lentiviral particles carrying GFP-LAPD4 were microinjected into the hippocampal DG of wild-type mice using a stereotaxic microinjection system (Figure 38A). In order to photoactivate LAPD4 in the DG, we utilized a wireless LED device, identical to the PAC TG mice (Figure 38B). For the mouse behaviour assays, I photoactivated LAPD4 to decrease endogenous cAMP during the 5 min habituation phase to determine the effect of cAMP suppression on short-term recognition memory in the displaced and novel object tests, similar to the PAC TG behavioural assay (Figure 34A). Expression and function of LAPD4 was confirmed by confocal imaging of coronal slices from transfected animals (Figure 39A). The ability of the expressed LAPD4 to light-dependently catalyze the hydrolysis of cAMP was confirmed by an in vitro ELISA assay of a cell lysate prepared from the brains of transfected mice (Figure 39B).
Figure 37. LAPD4 hydrolyzes cAMP upon exposure to blue light.

(A) Schematic of LAPD4, composed of the light-sensitive phytochrome domain (PGP) fused to the catalytic domain of PDE4. Upon photoactivation by blue light, LAPD4 catalyzes the conversion of cAMP into AMP. (B) Time course of LAPD4 activity *in vitro*. Photoactivity was measured with (magenta, $n = 4$) and without (black, $n = 3$) photoactivation in the presence of cAMP, and photoactivation in the presence of cGMP (cyan, $n = 3$). (C) The off response of LAPD4 following 5 sec of light (black bar, $n = 4$). All data are mean ± SEM.
Figure 38. Photoactivation of LAPD4 in hippocampal DG granule cells in freely behaving mice.

(A) Schematics of lentiviral LAPD4 microinjection in the DG in the mouse. GFP-LAPD4-encoding lentiviral particles were stereotaxically injected into the DG and driven by the CaMKII promoter. Injection path is represented by a red dotted line. (B) An LED device with optical fibres was implanted into the mouse’s brain to enable the wireless control of LAPD4 activation by light, similar to the transgenic PAC mice.
Figure 39. Generation of mice that highly express LAPD4 in the DG of the hippocampus by lentivirus injection.

(A) Representative image of GFP-LAPD4 expression in the DG. The GFP fluorescence image (green) was merged with the bright field image (grey) in the acute brain coronal section slice. (B) Detection of light-dependent activation of LAPD4 in the same brain slices. The slices were homogenized and the lysates were illuminated by light (455 nm LED, 4.5 mW/mm², 30 sec). Degradation of cAMP was measured by an ELISA (n = 9). **, p < 0.01, unpaired t-test. All data are mean ± SEM.
2.2.5 The effect of endogenous cAMP suppression with LAPD4 on short-term object recognition memory

In the displaced object test, no significant object preference was found with (Light ON) or without (Light OFF) LAPD4 photoactivation (Figure 40). In the novel object test, there was a preference for the novel object both with (Light ON) and without (Light OFF) LAPD4 photoactivation (Figure 41A, B). However, the suppression of endogenous cAMP during the 5 min habituation phase decreased the magnitude of the mouse’s preference for the novel object (Figure 41C). This suggests the involvement of endogenous cAMP for short-term learning in novel object recognition.
Figure 40. Short-term displaced object recognition learning with or without LAPD4 photoactivation.

(A) Top: summary schematic of displaced object test results without LAPD4 photoactivation. Bottom: displaced object preference ratio without LAPD4 photoactivation ($n = 10$). (B) Top: summary schematic of displaced object test results with LAPD4 photoactivation. Bottom: displaced object preference ratio with LAPD4 photoactivation. Mouse DG photoactivation was during the habituation phase (5 min duration) before being assayed in this displaced object testing phase ($n = 10$). NS, $p > 0.05$; unpaired t-test. All data are mean ± SEM.
Figure 41. Short-term novel object recognition learning with or without LAPD4 photoactivation.

(A) Top: summary schematic of novel object test results without LAPD4 photoactivation. Bottom: novel object preference ratio without LAPD4 photoactivation (n = 12). (B) Top: summary schematic of novel object test results with LAPD4 photoactivation. Bottom: novel object preference ratio with LAPD4 photoactivation. Mouse DG photoactivation was during the habituation phase (5 min duration) before being assayed in this novel object testing phase (n = 12). (C) Preference for the novel object with (+Light) and without (-Light) LAPD4 photoactivation (n = 12). *, p < 0.05; **, p < 0.01; unpaired t-test. All data are mean ± SEM.
In summary, I found a novel cAMP function for short-term object recognition learning using optogenetic cAMP manipulation methods in the hippocampal DG of freely behaving mice. In the displaced object test, which is involved in spatial memory recognition, an increase in cAMP promoted short-term learning. In contrast, decreasing cAMP in the displaced object test did not affect short-term learning (Figure 42). These results suggest endogenous cAMP levels were not sufficient to form short-term memory of displaced objects and that additional cAMP may be necessary.

In the novel object test, which has a properties component, an increase in cAMP did not affect the preference for the novel object but suppression of endogenous cAMP decreased the novel object preference (Figure 43). This suggests that endogenous cAMP is sufficient and involved in the formation of short-term memory for novel object recognition. Taken together, these results suggest that cAMP is a promoter of short-term learning for object recognition memory in the hippocampal DG region.
Figure 42. Summary of the cAMP effect in the displaced object test.

(A) Comparison of the effect of cAMP manipulation for short-term learning in displaced object recognition. Data are taken from Figure 35 and Figure 40. (B) Schematic of the effect of cAMP. *, p < 0.05; unpaired t-test. All data are mean ± SEM.
Figure 43. Summary of the cAMP effect in the novel object test.

(A) Comparison of the effect of cAMP manipulation for short-term learning in novel object recognition. Data are taken from Figure 36 and Figure 41. (B) Schematic of the effect of cAMP. ***, p < 0.001; unpaired t-test. All data are mean ± SEM.
2.3 Discussion

By establishing optogenetic approaches for manipulating cAMP levels in target granule neurons of the murine hippocampal DG region, I revealed that postsynaptic cAMP serves as a rapid and protein synthesis-independent promoter of functional potentiation at medial perforant path synapses of dentate gyrus granule neurons (PP-DG) in hippocampal slices.

A postsynaptic cAMP/PKA pathway is crucial for protein synthesis-dependent L-LTP at PP-DG synapses (Brandon et al., 1995; Nguyen and Kandel, 1996). However, due to the presence of strong inhibitory circuits on PP-DG, tetanic stimulation induces a brief (~5min) potentiation of fEPSP (post-tetanic potentiation) when inhibitory neurons are not blocked (Maroun and Richter-Levin, 2002; Matsuyama et al., 2008; Saab et al., 2009). The optogenetic application of cAMP enhanced post-tetanic potentiation immediately after tetanic stimulation (Figure 25). This potentiation lasted within 30 minutes and was independent of cAMP-mediated protein synthesis. Also the increase of cAMP alone did not potentiate the synaptic activity, indicating that other signaling pathways (such as Ca\(^{2+}\)) are necessary for the induction of the potentiation, with cAMP serving as a postsynaptic modifier.

Furthermore, increasing cAMP enlarged the activation area of the DG along the perforant path (PP) during tetanic stimulation of the PP-DG pathway (Figure 29). The enlarged region mainly consists of the dendrites of granule cells forming synapses with the perforant path (PP) axons, suggesting a cAMP-dependent increase in the number of activated synapses. The rapid cAMP function may enhance the subthreshold synaptic activation at PP-DG synapses in addition to facilitate the fully activated synapses by tetanic stimulation.
These structural (Chapter 1) and functional plasticity results that suggest that there is cAMP signaling from nearby synapses and possibly other areas within the broader context of the neuron, such as hormone related cAMP signaling. This may serve to facilitate protein synthesis independent potentiation by lowering the threshold that inputs need to reach in order to create association. Rapid postsynaptic cAMP functions may play important roles in integrating nearby synaptic activity for learning and memory.

Since new protein synthesis independent potentiation is believed to correlate with short-term memory (Frey et al., 1988; Krug et al., 1984), I studied the effect of cAMP in short-term learning utilizing murine object recognition memory tests (Zolamorgan et al., 1989a). Spatial-dependent object recognition (DOT: displaced object test) showed an enhancement of short-term learning after an optogenetic increase in cAMP in the hippocampal DG (Figure 35), suggesting a cAMP-dependent formation of displaced object recognition memory. The increased cAMP activity may facilitate the encoding of spatial memory information by enhancing spatiotemporal DG synaptic plasticity (post-tetanic potentiation; Figure 44). This also suggests that the broad activation of cAMP signaling in the hippocampal DG during the recognition memory could strengthen learning.

In contrast, endogenous DG neural activity was sufficient to form short-term memory in the novel object recognition test (NOT), but was suppressed by a decrease of endogenous cAMP (Figure 41). This indicates the involvement of endogenous cAMP in the formation of novel object recognition memory. If novel object recognition requires a limited number of synapses and neurons in the hippocampal DG compared with displacement, endogenous cAMP levels
could be sufficient to form short-term memories for novel objects (Figure 45). By establishing a dual photoactivation protocol of PAC (cAMP increase) and LAPD4 (cAMP decrease) in the same mouse brain, it would be possible to understand the time window for the cAMP effect in object recognition memory.

In summary, I revealed a cAMP-dependent function for protein synthesis independent synaptic plasticity and its related short-term object recognition memory. The presented work illustrates the new role for cAMP for rapid synaptic potentiation that could underlie a fundamental mechanism of learning and memory formation.
Figure 44. Model of the effect of cAMP on displaced object recognition.
Figure 45. Model of the effect of cAMP on novel object recognition.
Conclusion

Postsynaptic cAMP pathways are thought to invoke protein synthesis dependent synaptic potentiation and the related long-term memory and disease (Beavo and Brunton, 2002; Maurice et al., 2014); however their immediate actions and role in rapid synapse structural and functional potentiation and related short-term learning remained elusive. My project elucidated a rapid cAMP function for the regulation of activity-dependent structural potentiation of synapses, and how it regulates functional potentiation and short-term learning in the brain.

By employing optogenetic approaches that light-dependently manipulate cAMP levels in combination with live imaging, electrophysiology, and mouse behaviour assays, I revealed that rapid and protein synthesis independent intra- and inter-postsynaptic cAMP signaling facilitates structural synaptic long-term potentiation (sLTP) (Chapter 1). Furthermore, I demonstrated that cAMP rapidly enhanced functional synaptic potentiation and the related short-term object recognition learning (Chapter 2). Thus, my project provided the first direct look at how protein synthesis independent cAMP signaling regulates synaptic functions and hippocampal memory formation from the single synapse level, the circuit level, and all the way up to the living mouse brain. This work revealed fundamental aspects of structural and functional synaptic potentiation and advanced our understanding of the molecular basis of learning and memory formation.

In addition, cAMP metabolic enzymes have been actively targeted for drug discovery for neuropsychiatric and other disorders including Alzheimer’s disease (AD; Maurice et al. (2014)). The medial perforant path synapses of DG granule neurons in the hippocampus that
I used in this project are known to be related to memory impairment in human AD patients and AD mouse models (Palmer and Good, 2011), and the latter of which are actively used for AD research (Jacobsen et al., 2006; Roy et al., 2016).

Since cAMP optogenetic approaches established in this project enable us to noninvasively manipulate target spatiotemporal cAMP functions in an unprecedented way, long-term optogenetic cAMP manipulation during the development or maintenance of neural circuits in combination with mouse models of disease could provide new therapeutic targets and methods to combat cognitive disorders such as AD and autism spectrum disorder.
Materials and Methods

3.1 Expression vectors

PAC (optimized for human expression, addgene ID 28134; Stierl et al. (2011)) was fused with RFP (tdTomato; Shaner et al. (2004)) as a red fluorescent marker at the N-terminal and subcloned into pCAG plasmid vector (Niwa et al., 1991). To modify PAC into photoactivatable guanylyl cyclase (BlgC), three amino acids involved in the ATP binding site of PAC were changed for GTP binding (K197E, D265K, T267G; Ryu et al. (2010)). The cAMP FLIM probe (REY) was constructed by replacing CFP from ICUE2 (Violin et al., 2008) with RFP (tdTomato), and subcloned into the pCAG plasmid vector. Since I detected some aggregation of the probes from neurons in the cultured hippocampal slices using other RFPs including mCherry, mRFP and mStrawberry (Shaner et al., 2004) as an acceptor, I used tdTomato which did not show any aggregation of the probe in neurons. For cAMP FRET probe (CEY), I replaced CFP from ICUE2 with mTurquoise2 (Goedhart et al., 2012). The cGMP FLIM probe (cGiR) was constructed from the Gln^{79} – Tyr^{345} in cGMP-dependent protein kinase I (cGKI; Russwurm et al. (2007)) of Cygnet 2.1 (addgene ID 19737; Honda et al. (2001)) and replaced CFP with RFP (tdTomato), and subcloned into the pCAG plasmid vector. GFP (EGFP with A206K mutation to prevent dimerization) for a volume filler was subcloned into the pCAG plasmid vector (Zacharias et al., 2002). The CaMKIIα FRET probe was identical to a previously reported probe (Camüi; Takao et al. (2005)). The CaMKIIβ FRET probe (Camüiβ) was prepared by swapping the CaMKIIα domain in Camüi with CaMKIIβ. The gene encoding the catalytic domain of Homo sapiens cAMP-specific phosphodiesterase 4 (HsPDE4B; UniProt PDE4B_HUMAN: amino acids 630-1971) was
synthesized (GenScript, NJ, USA). Light activated phosphodiesterase 4 (LAPD4) constructs were generated by fusing the PDE4B (amino acids 630-1971) with N-terminus of LAPD phytochrome (DrBPhy; UniProt BPHY_DEIRA: amino acids 1-506) 1. For in vitro cell lysate assay, LAPD4 was subcloned into the pCAGGS plasmid vector with a C-terminal EGFP tag (Clontech, CA, USA).

3.2 Biochemistry

5 x 10^5 HEK293 cells were plated into each well of 6-well plates and transfected with PAC-containing, LAPD4-containing, or empty plasmids (2.5 µg) using Lipofectamine 2000 (Life Technologies, Grand Island, NY). The HEK293 cells were harvested after 48 hours of transfection, and homogenized in a buffer (40 mM HEPES/Na, pH 8.0, 0.1 mM EGTA, 5 mM magnesium acetate, 1mM DTT, and 0.01% Tween-20) by sonication, and centrifuged at 16,000 g for 15 min to clear large cell debris. The supernatant was then isolated and used for further analysis. For PAC and LAPD4 in vitro assays, these steps were done in darkness. The RFP tagged enzyme concentrations were measured by RFP ELISA kit (Cell Biolabs, Inc, CA, USA). In the coexpression experiments using photoactivatable enzymes with the corresponding FLIM probes (PAC and REY), HEK293 cells were cotransfected with the plasmids at a ratio of 1:1.

3.3 In vitro cAMP measurements by ELISA

Cell lysates were excited with a 455 nm LED (4.5 mW/mm^2; ThorLabs, NJ, USA) on a plastic paraffin film (Parafilm M®, Bemis, USA) covered glass slide at room temperature, and cAMP was measured by a cAMP ELISA kit (Enzo Life Sciences, NY, USA). The
reaction solution (100 µl) of cell lysates contained 40 mM HEPES/Na, pH 8.0, 0.1 mM EGTA, 5 mM magnesium acetate, 1mM DTT, 0.01% Tween-20 and either 100 µM Mg-ATP or 200 µM Mg-GTP. For in vitro PAC photoactivation assays under a microscope, the samples were excited with focal light from a mercury arc lamp (414 ± 21 nm, 11 mW; Olympus, Tokyo, Japan) under 60X objective lens (NA 1.0, LUMPlanFL N, FN26.5; Olympus, Tokyo, Japan) on a fluorescence microscope (BX51; Olympus, Tokyo, Japan). Excitation with two-photon laser light (30 mW, 700 – 1025 nm) was performed under the same 60X objective lens in a custom-made two-photon microscope (FV1000 MPE; Olympus, Tokyo, Japan) equipped with 2 two-photon lasers (Mai Tai HP DeepSee; Newport, CA, USA). Results from the blank vector controls were subtracted from the PAC or LAPD4 vector results. The two-photon focal photoactivation volume was calculated from Airy disk form of the Abbe diffraction limit:

\[
\text{radius} = 0.61 \times \frac{1000 \text{ nm} (\lambda)}{1.0(NA)} \times \frac{1}{\sqrt{2}}
\]

where the two-photon diffraction limit is equal to \(\frac{1}{\sqrt{2}}\) times the single-photon diffraction limit. The light-dependent reactions were stopped immediately by application of 0.1 M HCl following the ELISA kit protocol.

Hippocampal slices of PAC transgenic and WT control mice were homogenized in a buffer (40 mM HEPES/Na, pH 8.0, 0.1 mM EGTA, 5 mM magnesium acetate, 1mM DTT, and 0.01% Tween-20) by sonication, and centrifuged at 16,000 g for 15 min to clear large tissue debris. The supernatant was then isolated and I measured protein concentration using an RFP ELISA kit (Cell Biolabs, San Diego, CA, USA). The hippocampal slice lysates (100 µl) were
excited for 30 seconds with a 455 nm LED (4.5 mW/mm²; ThorLabs, NJ, USA) on a glass slide covered with plastic paraffin film (Parafilm® M, Bemis, USA) at room temperature. I then measured cAMP by a cAMP ELISA kit (Enzo Life Sciences, NY, USA).

3.4 FRET and FLIM assay

For in vitro assays, HEK293 cells were transfected with the FRET or FLIM probes and homogenized in the assay buffer containing 40 mM HEPES/Na, pH 8.0, 0.1 mM EGTA, 5 mM magnesium acetate, 1mM DTT, 0.01% Tween-20. The supernatant after centrifugation at 16,000 g for 15 min was used for the measurements. For fluorospectrometric measurement of FRET, CFP was excited at 433 nm and FRET was measured as a ratio of emission at 478 nm (CFP) to 525 nm (YFP) in which a higher value indicates less FRET, using a fluorescence spectrophotometer (LS 55, Perkin-Elmer, MA, USA) as described previously (Okamoto and Hayashi, 2006; Okamoto et al., 2004). Cyclic nucleotide analogs 8-Br-cAMP (Sigma-Aldrich, MO, USA) was used for in vitro cAMP FRET (CEY) assay to avoid the effect of endogenous phosphodiesterases in HEK293 cell lysates. For the in vitro CaMKII FRET (Camüi, Camüiβ) assay, the probes were stimulated by adding 0.2 mM CaCl₂ (~0.1 mM free Ca²⁺) in the presence of 1µM calmodulin and 50 µM ATP and the reaction was stopped by 0.4 mM EGTA (Takao et al., 2005). For two-photon FRET imaging of neurons, CFP and YFP images (800 nm excitation, 460-500 nm for CFP, 520-542 nm for YFP emission) were used for FRET ratiometric assay in a custom-made two-photon microscope (FV1000MPE; Olympus, Tokyo, Japan) equipped with a 60X objective lens (NA 1.0, LUMPlanFL N, FN26.5; Olympus, Tokyo, Japan) and analyzed by Metamorph software (Molecular Devices, CA, USA).
For the FLIM assay, the fluorescence lifetime of the FLIM probes (REY, cGiR) were measured with a time-correlated single photon counting (TCSPC) system (PicoHarp 300, FLIM upgrade kit for Olympus FV1000MPE, SymPhoTime software; Picoquant, Berlin, Germany) with 900 nm two-photon excitation (Mai Tai HP DeepSee; Newport, CA, USA) for 60 seconds (in vitro FLIM assay), or 30 seconds (neuron FLIM imaging) using single plane imaging in the same custom-made two-photon FRET/FLIM microscope as ex vivo FRET imaging. Fluorescence lifetime data were analyzed by SymPhoTime software (PicoQuant, Berlin, Germany; Hille et al. (2009)). For average time-domain fluorescence lifetime measurements, the lifetime decay curves were fit by a double exponential decay model using the tail-fitting analysis. The fluorescence lifetime pseudo-colour image of neurons was constructed using a pixel by pixel fitting function.

3.5 Neuron imaging

Organotypic slice cultures of hippocampus were prepared from postnatal day 6-7 rats as previously described (Okamoto et al., 2004). CA1 pyramidal neurons were biolistically transfected after 5 days in vitro, and the live imaging experiments were performed 3-5 days after transfection in the distal regions of the main apical dendrite while being continuously perfused with ACSF (artificial cerebrospinal fluid) solution containing 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 26.2 mM NaHCO3, 1 mM NaH2PO4 and 11 mM glucose at 30 °C equilibrated with 5% CO2/95% O2 (Okamoto et al., 2004). For the dendritic spine structural assay, the expression vectors of RFP-PAC (or RFP-BlgC) and GFP (volume filler) were cotransfected at a ratio of 4:1. Target neurons were excited with focal light from a mercury arc lamp (414 ± 21 nm, 11 mW, 60 sec; Olympus, Tokyo, Japan) under 60X
objective lens (NA 1.0, LUMPlanFL N, FN26.5; Olympus, Tokyo, Japan) for dendritic level photoactivation (PAC or BlgC). Due to low penetration of focal light into tissues, positive neurons near the surface of the cultured slices were photoactivated and imaged. To photoactivate postsynaptic PAC or BlgC, the target spines were excited at 1,000 nm two-photon laser light (point-scanning, 11mW, 30 sec). Uncaging ACSF for structural potentiation induction was 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl$_2$, 26.2 mM NaHCO$_3$, 1 mM NaH$_2$PO$_4$ and 11 mM glucose, 1 µM tetrodotoxin, and 2.5 mM 4-methoxy-7-nitroindoliny1 (MNI)-L-glutamate (Tocris, Bristol, UK). The two-photon glutamate uncaging (MNI-glutamate, Tocris, Bristol, UK) at 720 nm was applied near the targeted spine (~1 µm from the tip of the spine head, 10 mW, 4 msec, 1 Hz, 60 times), and subsequent images of dendritic spine enlargement were captured using two-photon GFP imaging (900 nm excitation, 495-540 nm emission). Spine size change was measured by summed GFP fluorescence intensity of the spines from 15-20 sections taken at 0.5 µm intervals of z-stack images of the dendrites using Imaris software (Bitplane, Zurich, Switzerland).

3.6 Animal care

Organotypic slice cultures of rat hippocampus were prepared in accordance with the guidelines of the university health network animal care committee approved protocol and oversight (Canada).

Acute mouse hippocampal slices were prepared in accordance with the guidelines of the university health network animal care committee (Canada) and Tokushima-Bunri University (Japan) approved protocol and oversight. Animal procedure for mouse behavioral assays
were approved by the university health network animal care committee (Canada) performed at The Centre for Phenogenomics (Toronto, ON, Canada).

### 3.7 Statistical analysis

Statistical methods are indicated in the figure legends. Data are presented as mean ± SEM. 
***, \( p < 0.001 \); **, \( p < 0.01 \); *, \( p < 0.05 \); NS, \( p > 0.05 \). Data were analyzed using a variety of tests including: one-way ANOVA Tukey-Kramer Test, paired and unpaired t-Tests, and Wilcoxon Rank Sum Test. Differences were considered statistically significant at \( p > 0.05 \).

### 3.8 Transgene construction and generation of PAC mice

The transgene contains CMV enhancer-CaMKII\(\alpha\) promoter (1.3kb); the coding region of RFP (tdTomato) fused with PAC, which is codon-optimized for human expression (addgene ID 28134; Stierl et al. (2011)) at the C-terminal; and a polyadenylation signal. The resulting cDNA were subcloned into a custom plasmid vector (pMM403), including a gene for ampicillin resistance, for bacterial amplification. A 5.2kb transgene was digested with SfiI + SalI to linearize the DNA and remove prokaryotic sequences. Transgenic mice were generated by injecting the purified insert into the pronuclei of C57BL/6J mice at The Centre for Phenogenomics (Toronto, ON, Canada). Genotyping was performed by PCR to make a fragment 859 bp long and using the following primers: 5’- TTCTCCGTTTGCACTCAGGAGC-3’ and 5’-GATGACGGCCATGTGTGTGT-3’. Founders were backcrossed into C57BL/6J mice for at least 3 generations.
3.9 Chromosomal mapping of the transgene using genomic walking

The insertion site of the transgene was identified by the genomic walking approach (GenomeWalker Universal Kit, Clontech, Takara Bio USA Inc., CA, USA). Briefly, the genomic DNA was prepared from PAC transgenic mice, digested with restriction enzymes (DraI, EcoRV, PvuII, StuI), and ligated with Clontech adaptor sequence. After primary and secondary PCR following the kit’s instructions, I obtained PCR products containing the DNA fragment of PAC and flanking sequences. The transgene insertion sites in chromosomes were determined with a BLAST search (www.ensemble.org). I also validated both 5’ and 3’ flanking sequences using PCR by genomic primers and transgene primers.

3.10 Electrophysiology

To electrically stimulate (100 Hz, 1sec, 3 trains by 10 sec intervals) cultured organotypic slices locally, a glass electrode filled with 0.5 M NaCl and fluorescent beads (1 μm diameter, FluoSphere, Molecular Probe) to aid placement within the sample, was placed within 5-15 μm of the target dendritic spines (Khan et al., 2014; Okamoto et al., 2004). The standard ACSF was replaced within 60 sec by uncaging ACSF for the combination experiment of the tetanic stimulation and glutamate uncaging.

Acute hippocampal slices were prepared as previously described (Henderson et al., 2001) from PAC transgenic mice and their wild-type littermates. After 1-2 hours recovery, slices were transferred to a chamber perfused with an aCSF solution containing 124 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM
glucose (pH 7.4, 30°C, 1.5 ml/min) equilibrated with 5% CO₂/95% O₂. Recordings of field excitatory postsynaptic potentials (fEPSPs) were conducted as previously described (Henderson et al., 2001) in the DG (perforant path/dentate synapses) without blocking inhibitory synaptic function. The stimulation electrode was positioned in the dorsal blade of the dentate molecular layer for medial perforant path stimulation. Paired field responses were evoked by stimulating with an intensity (0.05 msec pulses, 40 msec apart) that yielded fEPSPs that were 40% of the maximum spike-free fEPSP size. Responses were evoked and acquired every 20 sec throughout the experiment using an Axopatch 1D amplifier (Axon Instrument) digitized at 20 kHz and measured by slope (10–50% of fEPSP rising phase). The expressed PAC in DG granule cells were photoactivated using a blue LED light (1.5 mW, 5 min) under an objective lens (4X, NA0.1). Tetanus was induced with a bipolar tetanic stimulation (100 Hz, 0.15 msec pulses delivered in 4 trains of 0.5 sec duration, 20 sec apart) for the perforant path stimulation. In the case of photoactivation of PAC with tetanic stimulation experiments, tetanic stimulation was induced after the first minute of light exposure during the photoactivation. KT5720 (1 μM) was bath applied 30 minutes prior to tetanic stimulation. In the time course experiments, field responses were plotted by normalizing to the baseline fEPSP slope (average of the 10 minute period prior to tetanic stimulation).

3.11 Fluorescence imaging of RFP-PAC in hippocampal slices

The hippocampal slices of PAC transgenic mice were incubated with aCSF at room temperature in the microscope chamber. The RFP-PAC fluorescence imaging was conducted using a confocal scanning microscope (Nikon C2 equipped with a 4X objective lens) using
543 nm (excitation laser) and 575-630 nm (emission). The fluorescence image was combined across the z-stacks composed of 30-50 sections taken at 5 µm intervals, and merged with the transparent image (no red fluorescence emission filter).

### 3.12 Fluorescent voltage-sensitive dye (VSD) imaging

Acute hippocampal slices were prepared in accordance with the institutional guidelines of Tokushima Bunri-University (Japan) and conducted fluorescent VSD imaging as a previously reported (Tominaga and Tominaga, 2016). Briefly, hippocampal slices (400 µm thick) were prepared from adult (10-21 week-old) PAC transgenic mice and maintained in aCSF, containing 124 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose, pH 7.4, gassed with 95% O₂/5% CO₂. After incubating for 1h, each slice was stained for 15 min with 110 µl of VSD solution, containing 0.1 mM Di-4-ANEPPS (Molecular Probes) in 48.1% aCSF, 48.1% fetal bovine serum (Sigma), 2.5% ethanol, 1.17% distilled water, and 0.13% Cremophor EL (Sigma). After washout of the dye with aCSF, slices were incubated for >1h before start imaging.

For VSD imaging, slices (supported by Plexiglas ring) were placed in an immersion-type recording chamber. Slices were continuously perfused at a rate of 1 ml/min with aCSF at 31°C, gassed with 95% O₂/5% CO₂. The fluorescence VSD signals were recorded using MiCAM Ultima imaging system with THT-01 epifluorescence optics (BrainVision, Inc., Tokyo, Japan), consisting of a two lenses system: A custom-made objective lens (Olympus MYCAM 5x/0.6 WI) and a projection lens (PLANAPO, 1X, Leica Microsystems GmbH, Wetzlar, Germany). Excitation light was provided by a 150W halogen light source (MHF-G150LR) with an excitation filter (530 ± 10 nm) and an emission filter (> 590 nm) for VSD
imaging. The VSD fluorescence imaging was recorded for 400 msec during which time it recorded the fluorescence change following stimulation for optical recording (at 100 msec). Images were obtained by a mean of 8 images using 20 msec imaging duration after baseline electrical stimulation used for recording (every 20 sec and using 14 mW of excitation light). The VSD fluorescence recordings were taken at prior to (baseline) and immediately following tetanic stimulation (at time 0 msec) with a recording pulse (1 train, 0.05 msec duration) that yielded field excitatory postsynaptic potential (fEPSPs) that were 40% of the maximum spike-free fEPSP size. PAC was photoactivated using 15 x 4 sec duration pulses of excitation light (3.2 mW, 482/35 nm) every 10 sec immediately before tetanic stimulation using the same light path as imaging light. The imaging system provided a resolution of 18.2 x 18.2 µm at the objective plane (100 x 100 pixels resolution), and the temporal resolution was 0.2 msec/frame.

The fluorescence signal intensity prior to stimulation was averaged and used as the baseline reference intensity ($F_0$). The change in fluorescence [$\Delta F(t) = F(t) - F_0$] was normalized by $F_0$ ($\Delta F/F_0$) and used as the optical signal. Optical signals were then Gaussian filtered in time and space by 5 x 5 x 3 (horizontal x vertical x temporal; IgorPro; WaveMetrics Inc, OR, USA). These pseudo-colored optical voltage maps were superimposed on the initial (prestimulation) gray-scale fluorescent image of hippocampal DG area for a visual reference of the location. Since di-4-ANEPPS decreases in fluorescence when the membrane depolarizes, I chose to represent this change as a positive value to make the results more intuitive (Tominaga et al., 2002; Tominaga et al., 2000). The pseudo-colored 3D presentation of the VSD fluorescence changes were created using Origin Pro (OriginLab, MA, USA). The threshold levels of the signal/noise were set independently of the peak fluorescence changes, using a set threshold of
40% of the max signal intensity at baseline in order to determine the distribution area size of the fluorescence changes. For fitting the plots (Figure 3F, S3), I used OriginPro (OriginLab, MA, USA).

For simultaneous recording of fEPSPs in the perforant path/dentate synapses in addition to the optical signaling, the single dendritic responses were evoked by bipolar stimulation (1 train, 0.05 msec pulse, 20 sec apart) that yielded fEPSPs that were 40% of the maximum spike-free fEPSP size. Responses were evoked and acquired every 20 sec throughout the experiment using a Model 440 amplifier (Neurophase LLC, CA, USA) digitized at 10 kHz (ITC-18; InstruTech Inc., NY, USA) and measured by slope (10–50 % of fEPSP rising phase).

Tetanus was induced with a strong (100 Hz) bipolar tetanic stimulation (1 train, 100 x 0.05 msec duration pulses, 0.5 mA) using a borosilicate pipette (5 µm inner diameter; filled with aCSF) without blocking inhibitory synapse function. Tetanic stimulation was induced immediately after light exposure for PAC. The change of fEPSP was plotted by normalizing to the baseline slope (average of the 10 minute period prior to tetanic stimulation).

### 3.13 LED bilateral implant

PAC transgenic mice were anaesthetized with isoflurane and transferred to a stereotaxic apparatus. Mouse heads were kept rigidly in place by gently fixing ear bars to the head and placing the open snout in a stainless steel nose clamp. Placed in this way, the nose was thus in a small breathing chamber through which isoflurane and oxygen were provided throughout the surgery. The mouse was subcutaneously injected with 0.1 mL of saline to combat
dehydration and 0.1 mL of METACAM® (Boehringer Ingelheim GmbH., Germany) for analgesia. The mouse’s head was shaved between the ears from between the eyes to approximately 1.0 cm posterior to the ears, and betadine was applied topically to sterilize the shaved area. The mouse’s eyes were then covered with Tear-Gel (Alcon, TX, USA) to prevent coronal damage due to dryness and reapplied when necessary. After testing for analgesia, a scalpel was used to expose the skull. All tissue was pushed aside and the area swabbed to better examine skull morphology. A small hand-held drill attached to the stereotactic device was used to make two small 1 mm holes 2 mm posteriorly from bregma and +/-1.6 mm from midline. Into these holes, a bilateral 470 nm wavelength LED device (0.75 mm diameter, 20 mW, Eicom, CA, USA) was inserted slowly to minimize damage to brain tissue. The LED device was fixed to the skull using two kinds of dental cement (Bistite II DC, Tokuyama Dental America Inc., Japan, and UNIFAST Trad Liquid, GC AMERICA INC, USA) and cured. The mouse was then removed from the stereotaxic device and placed in a recovery cage under a heat lamp. Analgesia (METACAM) was continued by subcutaneous injection every 24 hours for 3 days post-surgery. At least 14 days were interspaced between surgery and behavioural testing.

3.14 Lentiviral construction

The pLenti-CaMKIIα promoter-EGFP-LAPD4 was constructed by replacing the ChR2-EYFP fragment from the pLenti-CaMKIIα-ChETA-EYFP (gift from Dr. Karl Deisseroth: Addgene plasmid # 26967; Gunaydin et al. (2010)) with EGFP-LAPD4 from the pCAGGS plasmid vectors. Lenti vectors were packaged and concentrated 1 × 10⁹ TU / ml (viral titres) at SignaGen laboratories (USA).
3.15 Lentiviral injection

Viral injections were done as described previously (Cetin et al., 2006). 2-4 month old male C57BL/6J mice were anesthetized with isoflurane for stereotaxic injection. The viral injections were targeted bilaterally into the DG (-2.0 mm AP (anterior-posterior), ± 1.3 mm ML (medial-lateral), 1.9 mm DV (dorsal-ventral)) and the injection volumes were 2 microliter per hemisphere at a rate of 0.1-0.2 microliter per minute using a 26G non-coring needle attached to a 10 µl Hamilton gastight syringe. After injection, the syringe was left in place for an additional 5 min to allow for diffusion of viral particles into the surrounding tissue. The syringe was then slowly removed and the LED was inserted as described.

3.16 Mouse behaviour

All experiments were approved by the local committee on animal care and conformed to the national guidelines (CCAC; https://www.ccac.ca). Animals were kept in tightly controlled environment in a 12 hour light/dark cycle starting at 7:00 AM. To minimize the effects of circadian rhythm on behaviour, all experiments were conducted between 7:30 AM and 3:00 PM. Animals were handled daily for a minimum of 3 days prior to experimentation (2 days), and were returned to their home cages for at least a week before retesting. All behavioral analysis were carried out in heterozygous mice from backcrossed PAC transgenic parents and aged 2-3 months.

Mice were placed in one of two identical closed white Plexiglas arenas (20 cm³) along with four identical objects (two per arena; 4 cm in diameter) under dim lighting (Figure 4B). Each object was initially placed in the upper right and upper left corners, 4 cm from each wall.
After 5 minutes of this habituation phase, mice were removed from the arenas and placed in their home cages for 1.5 minutes. During this time outside of the mouse’s view, the right object from each arena was moved to the lower right position, and mice were returned to the same arenas. After 5 minutes of this object displacement phase, mice were removed from the arenas and placed in their home cages for 1.5 minutes. During this time outside the mouse’s view, the displaced objects were removed. Two identical novel objects (6.5 cm in diameter) were each placed in the upper right position of each arena, and mice were again placed in the arenas. Finally, after 5 minutes of this novel object recognition phase, the mice were removed from the arena and placed in their home cages. Arenas and objects were cleaned with Clidox®-S (Pharmacal Research Laboratories Inc., CT, USA) between subjects to remove any traces of scent. Objects, object positions, and visual cues were kept constant throughout the experiment.

Implanted PAC transgenic mice were separated into two groups. Group 1 had their LED implant activated continuously during the entirety of the habituation phase, and Group 2 did not. After 24hrs, the experiment was run again with Group 2 having their LED implant activated continuously during the entire habituation phase, while Group 1 did not. Mice were timed using Ethovision 8.5 (Noldus Information Technology Inc., VA, USA) for their attention to the objects, which was roughly defined as the presence of the snout within a ~1.5x object diameter distance from the object centre.
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