Multicolour optogenetics for studying the roles of cAMP and cGMP in synaptic plasticity and memory

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Abstract

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are major interactive signalling molecules involved in a variety of physiological systems. However, due to a lack of available techniques for investigating their spatiotemporal interactions, the functions of their signalling cascades are not entirely known. To study the interactive roles of cAMP and cGMP in the nervous system, I established a multicolour optogenetic method for selectively activating cAMP/cGMP signalling by utilizing the combination of blue light-sensitive adenylyl cyclase (PAC) and green light-sensitive rhodopsin guanylyl cyclase (RhGC). By two-photon photoactivation at target synapses, I revealed bidirectional regulation of structural plasticity by cAMP/cGMP signalling. Also, I demonstrated suppression of novel object recognition memory by cGMP, but not cAMP, using photoactivation in freely behaving mice. Thus, this novel optogenetic technique enables direct investigation of spatiotemporal cAMP/cGMP functions and will serve as a valuable tool for studies of their signalling in broad fields of research.
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List of Abbreviations

AAV  adeno-associated virus
AC   adenylyl cyclase
AD   Alzheimer’s disease
ATP  adenosine triphosphate
BlgC blue light-activated guanylyl cyclase
BLUF blue light using FAD
cAMP cyclic adenosine monophosphate
CaMKII calcium$^{2+}$/calmodulin-dependent kinase II
cGMP cyclic guanosine monophosphate
ChR  channelrhodopsin
cNMP cyclic nucleotide monophosphate
CNS  central nervous system
CRE  cAMP response element
CREB cAMP-responsive element-binding protein
DG   dentate gyrus
ELISA enzyme-linked immunosorbent assay
GC   guanylyl cyclase
GFP  green fluorescent protein
GTP  guanosine triphosphate
HEK  human embryonic kidney
NpHR Halorhodopsin from Natronomonas
LED  light emitting diode
LTD  long-term depression
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>nIR-AC</td>
<td>near-infrared adenylyl cyclase</td>
</tr>
<tr>
<td>nIR-GC</td>
<td>near-infrared guanylyl cyclase</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NORT</td>
<td>novel object recognition test</td>
</tr>
<tr>
<td>PAC</td>
<td>photoactivated adenylyl cyclase</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RhGC</td>
<td>rhodopsin guanylyl cyclase</td>
</tr>
<tr>
<td>sLTP</td>
<td>structural long-term potentiation</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1 Introduction

1.1 Memory

The neurobiology of learning and memory has evolved as a significant theme of research in the neurosciences. Learning refers to the process by which new information is acquired by the nervous system and memory refers to the encoding, storage and retrieval of learned information (Purves et al., 2007). Thus, learning and memory are tightly correlated and are vital for day-to-day living. Impairments in memory can lead to debilitating conditions such as the inability to form new memories, as in the case of Alzheimer’s disease, or difficulty in suppressing memories of unpleasant experiences, as in post-traumatic stress disorder.

Various approaches have been used to understand the neural processes underlying memory. Until recently, studies of human memory were largely confined to the observation of patients with behavioural deficits following trauma to the brain. Experimentation with animals have not only supported the findings from human studies, but also enable the use of methods which could not be performed on humans. Furthermore, the recent development of advanced technologies have welcomed a new era of investigation into the cellular and molecular underpinnings of memory processes.

1.1.1 Human studies of memory

Although the brain has a normal ability to forget information, pathological forgetfulness, or amnesia, is defined as the inability to form new memories (anterograde amnesia) or retrieve old ones (retrograde amnesia) (Purves et al., 2007). Interestingly, early studies of amnesiacs have been especially meaningful for understanding the anatomical organization of memory. Perhaps the best-known case is that of patient H.M., whose memory deficiency was a subject of study for over five decades until his death in 2008 (Scoville & Milner, 1957; Corkin, 1984). After a bicycle accident at the age of 7, H.M. suffered minor seizures since age 10 and major seizures since age 16. He eventually became so incapacitated by his seizures, that in 1953 at age 27, he underwent experimental surgery to resect his bilateral medial temporal lobes. This included removal of the amygdala, uncus, hippocampal gyrus, and anterior two-thirds of the hippocampus. Following surgery, H.M. continued to have seizures, though less debilitating than before, and
exhibited profound memory loss in the absence of any deficiencies in intelligence, perception or abstract thinking. Specifically, he could not form new memories (anterograde amnesia) or access memories from the three years leading up to his surgery (partial retrograde amnesia). Before his case, it was widely thought that memory functions were distributed across the cortex and were integrated with cognitive and perceptual abilities. However, formal testing of H.M., as well as the 9 other patients described by Scoville and Milner (1957), lead to the inaugural view that memory is a distinct cerebral function for which the medial temporal lobe is of vital importance.

Another critical case of human memory impairment was that of R.B (Zola-Morgan et al., 1986). At age 52, R.B. suffered an ischemic stroke during cardiac bypass surgery. Upon recovery, a severe memory deficit (anterograde amnesia) became apparent with no evidence of intellectual or cognitive disabilities. During the 5 years until his death in 1983, his amnesia persisted and was well documented with formal memory testing. Detailed histological examination of his brain post-mortem revealed a bilateral lesion in the entire CA1 region of the hippocampus. Since minor pathology was found elsewhere, R.B.’s case provided one of the first pieces of evidence highlighting the importance of the hippocampus for memory. This was notably supported by extensive neuropsychological and neuropathological analyses.

1.1.2 Animal studies of memory

Consistent with evidence from the cases of human patients, studies of animals with experimentally-induced lesions to the hippocampus also demonstrate impairment of memory. For developing an animal model of human amnesia in the monkey, the delayed matching- and nonmatching-to samples tasks were typically used to assess the animal’s memory (Gaffan, 1974; Mishkin, 1978). In these tasks, experimenters would present a sample object to the animal. After a delay period (from 0.5 s to 10 min), the animals would have to choose an object from one or more others that either matches the sample object presented before (in the matching test) or does not match the sample object (in the nonmatching test) in order to obtain a food reward. It was found that animals with lesions to hippocampal-amygdaloid areas showed memory impairments at delays greater than 30 s while animals with lesions to the adjacent temporal stem did not (Mishkin, 1978; Zola-Morgan et al., 1982). Additionally, while animals with lesions to the hippocampus also produced memory impairments, those with combined hippocampal-amygdaloid lesions produced a larger deficit (Zola-Morgan & Squire, 1985; Zola-Morgan &
Squire, 1986). These results were the first to implicate the role of the hippocampus and its connections in memory using a nonhuman primate model.

Similarly, lesion studies in rodents have corroborated these findings with both monkeys and human patients. One such example is that by Eichenbaum, Stewart and Morris (1990) who employed the Morris water maze task (Schenk & Morris, 1985) to study hippocampal lesions in rats. In this task, rats are placed in a circular pool surrounded by visual landmarks. Rats must learn to escape from submersion by swimming to a hidden platform. Rats with intact hippocampi found the hidden platform no matter their starting point in the pool. Rats with hippocampal lesions, however, repeatedly failed to learn to find the platform. This suggested that the ability to remember the location of the platform relative to the visual cues is dependent on the same neural structures (i.e. the hippocampus) critical for memory formation in humans.

1.1.3 New methods and models for studying memory

Findings from early lesion studies indicate the integral role of the hippocampus for memory formation in both humans and animals. More recently, technological advances have provided investigators with new and non-invasive ways to study the brain and its capacity to encode memories. For example in humans, functional imaging shows that the human hippocampus is activated during certain types of memory, mainly semantic memory (general knowledge) and episodic memory (personal experiences and events) (Wagner et al., 1998; Eichenbaum, 2004). In both rodents and humans, single and multi-cell recordings combined with virtual navigation have been used to study spatial memories and the contribution of the spatially selective place cells (John O’Keefe & Dostrovsky, 1971; Ekstrom et al., 2003; Harvey et al., 2009). Finally, genetic manipulations have become a widely adopted method for characterizing the role of specific genes in both vertebrate and invertebrate animals. The latter, such as Drosophila and C. elegans, offer numerous advantages as model systems such as simple and accessible nervous systems and high-throughput behavioural paradigms. Thus, decades of neuroscience research have employed the use of diverse techniques and animal systems to study memory at the neural, cellular and molecular levels. Current efforts aim to uncover the specific mechanisms underlying these complex memory processes.
1.2 Synaptic plasticity

In addition to his well-known work illustrating the neural architecture of the central nervous system (CNS), Santiago Ramon y Cajal provided several insights into the cellular basis of memory formation. In the late 19th century, he speculated that memories did not require the formation of new neurons but rather, were formed by changing the strength of connection between existing neurons (Cajal, 1894). It is currently understood that these activity-dependent changes in neuron communication or transmission occur through a specialized structure called the synapse, where the presynaptic axon terminal of one neuron meets the postsynaptic dendritic spine of another. Depolarization of the axon terminal causes the presynaptic release of neurotransmitters into the synaptic cleft. The neurotransmitters migrate across the cleft and bind to their receptors located on the membrane of the postsynaptic dendritic spine. The binding of neurotransmitters to their associated receptors can initiate a series of molecular events that propagate the signal forward.

The idea that synapses could change over time in response to increases or decreases in their activity was theorized by Canadian psychologist Donald Hebb (1949). Popularly known as the Hebbian learning rule, Hebb postulated that coordinated and persistent activity of a presynaptic terminal and a postsynaptic spine would strengthen the synaptic connection between them. This was originally proposed as a model for learning and memory, however, at that time, very little was known about the mechanisms responsible at the cellular and molecular levels.

1.2.1 Long-term potentiation

In 1973, Bliss and Lomo gave the first demonstration of synaptic plasticity through the discovery of long-term potentiation (LTP). They placed electrodes in the hippocampus (perforant pathway) of anesthetized rabbits and found that by delivering a few seconds of high-frequency stimulation to the presynaptic axonal layer, they could produce a prolonged and potentiated response in the postsynaptic dendritic layer. In this study, potentiation of synaptic transmission (LTP) lasted for several hours. Other studies have shown that LTP can last for more than a year and occurs at other synapses of the hippocampus as well as synapses in other brain regions (Abraham, 2002). Additionally, long-term depression of synaptic transmission (LTD) has also been identified and found to occur when synapses undergo low-frequency stimulation for long durations (10 to 15 minutes) (Ito & Kano, 1982). LTD can also depress synapse activity for
sustained periods and when applied at a common site, can reverse the effect caused by LTP and vice versa (Lynch et al., 1977; Barrionuevo et al. 1980; Staubli & Lynch, 1990).

Early pharmacological studies using acute slices of the hippocampus have provided key advances in our understanding of LTP at the molecular level. For example, in 1983, Collingridge et al. found that the selective NMDA receptor antagonist AP5 prevented LTP in the hippocampal CA1-CA3 synapse, implicating the NMDA receptor as an essential component of LTP. Application of other inhibitors such as MK-801 (Coan et al., 1987) and 7-Cl KYNA (Bashir et al., 1990) were also shown to block LTP induction, however, NMDA activation alone was never sufficient to induce LTP (Collingridge et al., 1983; Kauer et al., 1988). The reason for this was later attributed to the unique biophysical properties of the NMDA receptor, in which it is permeable to calcium but is blocked by physiological concentrations of magnesium. For the receptor channels to open and thus induce LTP, the postsynaptic cell must be sufficiently depolarized to expel Mg\(^{2+}\) and also, glutamate must bind to induce their opening (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). The rapid influx of calcium that results from NMDA receptor activation has shown to be critical for NMDA receptor-dependent LTP and LTD induction (Lynch, 1983; Mulkey & Malenka, 1992)

1.2.2 Long-term potentiation and memory

One of the first studies to link LTP to memory was by Morris et al. in 1986. They found that in rodents, pharmacological inhibition of NMDA receptors with AP5 not only blocked LTP induction in the hippocampus, but also prevented animals from forming spatial memories. This was shown using the Morris water maze task where rats treated with NMDA antagonist consistently failed to locate the hidden escape platform, a task that was previously shown to be dependent on the hippocampus (Eichenbaum et al., 1990). Interestingly, pharmacological inhibition of NMDA receptors has also been shown to destabilize the place fields of hippocampal place cells over extended periods of time (Kentros et al., 1998).

Supporting evidence for LTP as a biological substrate for memory also comes from studies using genetically manipulated mice. For example, Tsien et al. (1996) generated a mouseline in which the NMDA receptor was knocked out in the CA1 region of the hippocampus. They reported that the animals exhibited impaired spatial memory (nonspatial memory remained intact) as well as a deficit in LTP. Mice genetically lacking the NR2A subunit of the NMDA receptor also display
impaired spatial learning and LTP (Sakimura et al., 1995; Bannerman et al., 2008) while animals overexpressing the NMDA NR2B subunit display enhanced LTP and memory (Tang et al., 1999; Wang et al., 2009).

In humans, Alzheimer’s disease (AD) research has been central to understanding how disease pathology is implicated in LTP and memory. Evidence from many laboratories suggests that the accumulation of amyloid-β protein (Aβ), which manifests as insoluble amyloid plaques, is responsible for the development of Alzheimer’s disease. Interestingly, injection of soluble Aβ has been shown to inhibit LTP in the rodent hippocampus (Lambert et al., 1998; Wang et al., 2002; Klyubin et al., 2005). Injection of antibody to Aβ prevented Aβ-induced inhibition of LTP and also reduced memory impairments in AD mouse models (Klyubin et al., 2005; Janus et al., 2000; Morgan et al., 2000). These findings suggest immunotherapy against Aβ as an effective treatment for Alzheimer’s disease and also provide a link between LTP and memory in humans.

1.2.3 Structural synaptic plasticity

In addition to changes in synaptic strength during synaptic plasticity, synapses can undergo changes in physical structure, called structural plasticity. Structural plasticity requires reorganization of actin, a major cytoskeletal protein, for alterations in the number and size of postsynaptic dendritic spines (Okamoto et al., 2004). For example, in hippocampal CA1 neurons, LTP induction causes dramatic dendritic spine enlargement within 60 seconds of the LTP-inducing stimulus (Matsuzaki et al., 2004; Okamoto et al., 2004). We previously showed that the dynamics of actin reorganization in dendritic spines is regulated by the postsynaptic protein calcium$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) (Okamoto et al., 2007; Kim et al., 2015). CaMKII is activated by the Ca$^{2+}$ influx upon postsynaptic depolarization and NMDA receptor activation during LTP. Not only does it play a signalling role by phosphorylating AMPA receptors and other postsynaptic proteins, but also a structural role in maintaining dendritic spine structure. Additionally, pharmacological antagonists of CaMKII blocked LTP induction (Malinow et al., 1989; Otmakhov et al., 1997) and genetically modified mice with mutated CaMKII show impaired memory function (Giese et al., 1998; Cho et al., 1998; Frankland et al., 2001).

The alterations in synapse structure are closely linked to changes in synaptic strength. Not surprisingly, aberrant synaptic signalling and dendritic spine morphology are characteristic of
many psychiatric and neurologic disorders (van Spronsen & Hoogenraad, 2010). Studies of both patients and animal models of Fragile X syndrome report abnormally long, thin and immature filopodia-like spines (Pfieffer & Huber, 2009). In mouse models for Alzheimer’s, highly dysmorphic spines and decreased spine number are observed in the vicinity of amyloid plaques (Spires-Jones et al., 2007; Tsai et al., 2004). Autism spectrum disorders are also associated with mutations in synaptic adhesion and scaffolding proteins, such as neurexins, neuroligins and Shank proteins (Abraham & Geschwind, 2008; Sudhof, 2008). Overall, these studies suggest a crucial link between synapse morphology and synapse function. Investigating the underlying structural elements of synaptic plasticity may deepen our understanding of synaptic dysfunction in cases of neuronal injury and disease.

1.3 Cyclic nucleotide signalling pathways

3’5’-cyclic adenosine monophosphate (cAMP) and 3’5’-cyclic guanosine monophosphate (cGMP) are intracellular second messenger molecules involved in a number of physiological processes in the mammalian system (Duman & Nestler, 1999; Zaccolo & Movesesian; 2007). They are composed of three components: a nitrogenous base (adenine in cAMP and guanine in cGMP), a 5-carbon ribose sugar and a phosphate group, which makes two separate bonds to the ribose sugar thus forming a cyclic ring. cAMP is synthesized from ATP while cGMP is synthesized from GTP. These reactions are mediated by the enzymes adenylyl (AC) and guanylyl cyclase (GC), respectively, which exist in both membrane-bound and soluble forms in a variety of tissue types. Meanwhile, the degradation of cAMP and cGMP is mediated by a superfamily of phosphodiesterases (PDEs) in which several subfamilies and isoforms exist. Some are selective for cAMP (PDEs 4, 7, 8) or cGMP (PDEs 5, 6, 9), while others are non-selective and capable of degrading both cAMP and cGMP (PDEs 1, 2, 3, 10, 11).

cAMP and cGMP act as second messengers within eukaryotic cells. The binding of an extracellular first messenger (such as a hormone or neurotransmitter) to its receptor protein on the target cell membrane can directly or indirectly stimulate cyclase activity. The newly synthesized second messengers (i.e. cAMP or cGMP) can then act intracellularly by binding to specific proteins at a cyclic nucleotide-binding domain. cAMP/cGMP-binding proteins include kinases, transcription factors and ion transporters, and cAMP/cGMP binding causes a conformational change that affects the protein’s activity. Since different cells contain different...
amounts and types of protein, the end result of a change in cAMP/cGMP concentration can dramatically differ from cell to cell.

1.3.1 cAMP

In 1971, Earl Sutherland was awarded the Nobel Prize for his discovery of cAMP, the “original second messenger” and its role in glycogen metabolism in the liver (1958). Soon after, it was shown to mediate the actions of several hormones and neurotransmitters (e.g. dopamine, glutamate, serotonin), thus taking part in a diverse array of cellular functions and capabilities. In neurons, the role of cAMP (and its downstream effectors) in synaptic plasticity was famously studied by Eric Kandel, to whom a Nobel Prize was also awarded. He found that the marine mollusk *Aplysia californica* exhibited a form of learning called sensitization and identified many of the molecular signalling components necessary for mediating its response (Pinsker et al., 1973; Kandel 2001; Kandel, 2012). These included cAMP, the cAMP-dependent protein kinase A (PKA) and cAMP response element binding protein (CREB) (Kandel, 2012). The canonical signalling pathway involves activation of AC, which increases intracellular cAMP. cAMP then activates PKA by promoting dissociation of its regulatory subunits from its catalytic subunits. The catalytic subunits can then phosphorylate its target proteins, one of which is CREB. Phosphorylated CREB can translocate into the nucleus where it affects the transcription of genes that are regulated by the cAMP response element (CRE) binding sequence. This sequence of events is thought to be critical for the long-term, protein synthesis-dependent aspect of memory formation in several species.

Single gene mutations in *Drosophila melanogaster* have implicated the cAMP signalling pathway in learning and memory (Davis, 1996; Lee, 2015). For example, mutant flies that lack either the PDE4 *dunce* or adenylyl cyclase *rutabaga* genes, were shown to perform worse at olfactory learning and memory tasks compared to controls. However, flies that were mutant for both *dunce* and *rutabaga* showed a significantly larger decrease in performance, despite their opposite influences on intracellular cAMP levels (Tully & Quinn, 1985). This indicates the importance of maintaining cAMP homeostasis in regulating plasticity and memory in *Drosophila* CNS. Furthermore mutants for *amnesiac*, a neuropeptide that stimulates AC activity and thus cAMP synthesis, were found to learn normally, but forget four times as quickly as wildtype flies
Transgenic flies expressing a peptide inhibitor of PKA have also demonstrated impaired olfactory memory (Drain et al., 1991).

In rodents, the cAMP pathway is also known to regulate hippocampal synaptic plasticity and memory (Nicoll & Malenka, 1995; Nguyen & Woo, 2003; Abel & Nguyen, 2008). Chemical application of forskolin, an AC activator, can increase neuronal excitability and enhance transmission in hippocampal slices (Pockett et al., 1993; Huang et al., 1994). This effect can be pharmacologically blocked by PKA inhibitors (Greengard et al., 1991). For demonstrating the role of cAMP in memory, genetic manipulations have been especially informative and have the advantage of regional specificity in intact mice. This was first demonstrated in 1995 when Wu et al. disrupted the gene encoding AC1 in the brains of mice. Mice lacking AC1 had reduced levels of cAMP synthesis, attenuated hippocampal LTP and also failed to find the hidden platform in the Morris water maze task. Similarly, Abel et al. (1997) created the R(AB) transgenic mouse line, which expresses an inhibitory form of the regulatory subunit of PKA. Mice with R(AB) expression in the hippocampus exhibited decreased LTP in the CA1 and behavioural deficits in spatial memory and long-term fear conditioning memory. Conversely, knockout mice for specific PDE subtypes have been shown to display increased cognition and memory enhancement in the radial arm maze, water maze and object recognition tasks (Li et al., 2011; McGirr et al., 2016). Rolipram, a PDE4 inhibitor, was also shown to enhance memory of wildtype mice (Barad et al., 1998) and reverse the memory impairments in aged mice (Bach et al., 1999; de Lima et al., 2008) and in mouse models of mental retardation and Alzheimer’s disease (Bourtchouladze et al., 2003; Gong et al., 2004).

1.3.2 cGMP

The second messenger actions of cGMP have been more difficult to identify compared to cAMP (Duman & Nestler, 1999). cGMP exists in lower concentrations in most tissues and is produced more slowly. Thus, it is thought to may play a less widespread role in cellular functions. The cGMP signalling pathway involves stimulation of guanylyl cyclase by nitric oxide (NO), which increases intracellular concentration of cGMP. The classical targets of cGMP include PDEs, cyclic nucleotide-gated cation channels and the cGMP-dependent protein kinase (PKG), the latter of which can phosphorylate synaptic proteins such as CREB, thus acting in parallel with the cAMP-PKA pathway.
Several lines of evidence suggest that cGMP is involved in long-term potentiation in the hippocampus. In rodent hippocampal slices, Zhuo et al. (1994) showed that inhibitors of PKG blocked LTP induction while activators of PKG produced long-lasting enhancement. It has also been shown that cGMP-PKG signalling is important for the induction of LTD in both the hippocampus and cerebellum (Zhuo et al., 1994; Boulton, 1994; Fiel et al., 2003). Its effects are thought to occur primarily through a presynaptic mechanism where NO acts as a retrograde messenger mediating neurotransmitter release via cGMP-PKG (Gartwaithe et al., 1988; Prast & Philippu, 2001). However, the molecular mechanisms of the NO-cGMP-PKG pathway and functional relevance of its dual role in both LTP and LTD are not entirely known (Fiel & Kleppisch, 2008).

Interestingly, many studies have identified CaMKII, as a target for the NO-cGMP-PKG signalling pathway, despite not being directly phosphorylated by PKG. Presynaptic injection of a CaMKII inhibitor with NO donors or PKG activators blocked hippocampal LTP (Ninan & Arancio, 2004). Interaction between CaMKII and the NO-cGMP-PKG pathway has also been demonstrated in the cerebellum at the Purkinje cells during LTD (Kawaguchi & Hirano, 2013). They showed using FRET measurements that CaMKII causes sustained increase of cGMP during LTD. However, NO-induced activation of the cGMP-PKG pathway can also support LTD induction independent of CaMKII. This suggests that interaction between NO and CaMKII can facilitate LTD and this may occur through CaMKII-mediated downregulation of PDE1, which can degrade both cGMP and cAMP (Kawaguchi and Hirano, 2013).

Recent findings support an essential role for cGMP in memory formation. In *Drosophila*, Kaun et al. (2007) tested reward learning and memory in allelic variants of the *foraging (for)* gene, which encodes PKG. Genotypes associated with high activity displayed greater olfactory memory acquisition and retention compared to genotypes with low PKG activity. Pharmacological inhibition of PDE5 has also been shown to enhance memory in both aged mice and transgenic mouse models of Alzheimer’s disease (Zhang et al., 2013; Palmeri et al., 2013). In rats, PDE5 inhibitors sildenafil and vardenafil increased object recognition performance when injected directly after learning (Prickaerts et al., 2002b; Rutten et al., 2007b). In comparison, PDE4 inhibitor rolipram only showed improvement when injected 3 hours later (Rutten et al., 2007; Bollen et al., 2014). These results suggest that cAMP and cGMP signalling are involved in different phases of memory consolidation.
1.3.3 cAMP and cGMP signalling crosstalk

Crosstalk between the cAMP and cGMP signalling pathways has been well characterized in the cardiovascular system (Zaccolo & Movsesian, 2007; Stangherlin & Zaccolo, 2012). In the heart, cGMP acts as a regulator of cAMP hydrolysis through the activity of cAMP-hydrolyzing PDEs. Dual specific PDE2 and PDE3 degrade both cAMP and cGMP, but with different affinity and catalytic rate. Binding of cGMP to PDE2 increases cAMP hydrolysis by 10-fold (Rosman et al., 1997). Conversely, cGMP acts as a competitive inhibitor for cAMP hydrolysis through PDE3 (Zaccolo & Movsesian, 2007). These dynamic interactions of cAMP and cGMP signalling are known to contribute to a number of cardiac cell mechanisms, such as potentiation of L-type Ca\(^{2+}\) channel currents in frog ventricular myocytes (Frace et al., 1993) and modulation of β-adrenergic-mediated cardiac ionotropy in rat neonatal myocytes (Mongillo et al., 2006).

The interplay between cAMP and cGMP signalling is less characterized in the nervous system. cGMP-mediated regulation of cAMP by PDE2 has been shown in thalamic (Hepp et al., 2007) and striatal neurons (Polito et al., 2013). cAMP and cGMP dynamics are also known to be involved in developing neuronal circuits. For example, simultaneous imaging of cAMP and cGMP FRET signals revealed spatially asymmetric cAMP and cGMP in non-stimulated, freely deviating growth cones (Kobayashi et al., 2013). In stimulated growth cones, high cAMP/cGMP ratios led to axonal attraction to the attractive guidance cue netrin-1, while low cAMP/cGMP ratios led to axonal repulsion (Nishiyama et al., 2003).

Strong expression of PDE2 in neurons of the hippocampus suggests that regulation of cAMP and cGMP is important for neuronal plasticity and memory formation. Pharmacological inhibition of PDE2 enhanced LTP in hippocampal slices (Boess et al., 2004). Inhibition of PDE2 also improved the performance of rats in social and object recognition memory tasks (Boess et al., 2004) and of mice with pharmacologically-induced memory deficits (van Donkelaar et al., 2008; Reneerkens et al., 2013). Furthermore, hippocampal administration of selective inhibitors for PDE4 and 5 revealed that cGMP-PKG signalling enhances early-phase LTP and early memory consolidation while cAMP-PKA signalling enhances late-phase LTP and late memory consolidation (Bollen et al., 2014). This study also showed that the memory-enhancing effects of PDE5 inhibitor could be blocked by co-administration of a PKA inhibitor, but not vice versa. This suggests that early cGMP-PKG signalling requires late-phase cAMP-PKA signalling to
enhance LTP and memory. However, how these cAMP- and cGMP-mediated processes are linked to each other remain elusive.

1.4 Optogenetics

Optogenetics is a recently developed technology most commonly used for studying neuronal circuits. It is a combination of genetic and optical methods that allows targeted and fast control of precisely defined events in biological systems (Deisseroth, 2011; Deisseroth 2015). This is achieved through cell specific expression of proteins that can modify the activity of the cell, or within the cell, when exposed to light. These proteins, or optogenetic actuators, may induce action potentials, suppress neural activity or modulate biochemical signalling pathways over a range of timescales. Various types have been identified in nature and have since been optimized for controlling neuron activity and complex animal behavior by light. However, the field has also expanded to non-neuronal research fields, such as cardiac physiology, with an increasing number of tools being developed that possess a diversity of functional properties useful for a range of different applications.

Progress towards developing optogenetics as a method for studying neuronal circuits was primarily driven by the desire of scientists to functionally characterize the thousands of neuronal cell types that exist in the human brain. Even though electrical methods can achieve temporal precision within a given tissue volume, they lack specificity for cell type. Pharmacological agents can be specifically designed to perturb molecular signalling cascades within cells. However, depending on the mode of administration, they may cause widespread changes throughout the brain and can take several minutes or hours to display an effect. Genetic manipulations have recently become a popular technique for studying single genes in target cell populations, but the method lacks the ability to manipulate single cell or synapse level functions. Thus, taking into account the significant limitations associated with techniques classically used to study brain function, optogenetics has become a technological breakthrough allowing scientists temporally precise and noninvasive control of intact neuronal systems as complex as freely behaving animals.
1.4.1 Single-colour optogenetics

In 1971, decades before the first demonstration of an optogenetic system in mammalian neurons, microbial biologists Dieter Oesterhelt and Walther Stoechenius discovered the first microbial opsin protein, bacteriorhodopsin from Halobacteria. Bacteriorhodopsin is a light-driven proton pump that can be activated by visible light photons to create an electrical current across the cell membrane. Unlike the rhodopsin proteins that mediate phototransduction in the vertebrate eye, scientists were intrigued that bacteriorhodopsin was a single-gene, single-component system that could directly and rapidly activate in response to light. However, the neuroscience community was hesitant to consider the strategy for probing neuronal function, as neuroscientists were doubtful that such foreign membrane proteins were nontoxic to the fragile mammalian nervous system and that the opsin cofactor, retinal, was present in sufficient amounts in vertebrate tissues (Deisseroth, 2011). Nonetheless, further investigation over the ensuing decades has led not only to a deeper understanding of bacteriorhodopsin but also to the discovery of new microbial opsin subfamilies, including the membrane bound ion pumps and channels such as the halorhodopsins and the channelrhodopsins.

Microbial opsin genes are found in prokaryotes, algae and fungi (Zhang et al., 2011). Like animal opsin proteins, microbial opsins have seven-transmembrane structures and require retinal, a vitamin A-related cofactor that serves to capture photons. In functional opsin proteins, retinal is bound and can isomerize upon absorption of a photon. This leads to a sequence of conformational changes within the opsin that acts as a trigger for its light-dependent activity. Naturally occurring microbial opsins serve a variety of purposes for their host microorganisms. These include navigation towards sources of energy and away from hazardous environments, and maintenance of the intracellular concentrations of different ions. After bacteriorhodopsin, additional microbial opsin variants were identified or engineered with a vast set of new functions but similar homologies. These include halorhodopsin, a yellow light-gated chloride pump (Matsuno-Yagi & Mukohata, 1977) and channelrhodopsin, a blue light-activated cation channel (Nagel et al., 2002). Both opsin proteins are now widely employed by the neuroscience community to suppress or induce neural activity, respectively. However, it would take several years since their discovery for neuroscientists to develop and apply these proteins to control genetically-defined neurons by light in vivo.
In August of 2005, the laboratory of Karl Deisseroth published the first introduction of microbial opsins into cultured hippocampal neurons, making them responsive to light (Boyden et al., 2005). They used Channelrhodopsin-2 (ChR2), the blue light-gated cation channel from algae, delivered via lentiviral vectors, which resulted in highly reliable and precise millisecond-timescale control of neuronal spiking. Numerous reports followed soon after, including those focused on optimizing expression and photocurrent in mammalian systems (Lin, 2011). In December of that same year, Nagel and colleagues showed that ChR2 could be an effective tool for in vivo studies of behaving animals, specifically when expressed under cell specific promoters (Nagel et al., 2005). For example, in C. elegans, ChR2 evoked strong and simultaneous muscle contractions when expressed in muscle wall motor neurons. Meanwhile, in mechanosenory neurons, light activation caused withdrawal reflexes that are normally observed in response to mechanical stimuli. ChR2 has also been employed for targeted control of neuronal behaviours in Drosophila and zebrafish using the GAL4/UAS expression system (Zhang et al., 2007b; Douglass et al., 2008). The most widely published optogenetic model organism, however, has been the mouse. Transgenic mice allow selective opsin expression in defined sets of neurons through a promoter-transgene assembly. The first transgenic opsin-expressing mouse model was generated using the Thy1 promoter to drive ChR2 (Arkeniel et al., 2007). This was first used to study cortical and olfactory circuits (Arkeniel et al., 2007; Wang et al., 2007) and later to investigate a myriad of neuronal processes including sleep rhythms, fear circuitry, and motor control following stroke (Kim et al., 2012; McCullough et al., 2016; Anenberg et al., 2014)

Other methods for achieving opsin expression in the mouse nervous system include virus microinjections, site-specific recombination and gene knock-in approaches, all of which have demonstrated considerable success in expressing different optogenetic tools (Fenno et al., 2011; Allen et al., 2015).

### 1.4.2 Multi-colour optogenetics

By 2010, channelrhodopsin, bacteriorhodopsin and halorhodopsin all have proved capable of controlling neurons in response to diverse colours of light (Deisseroth, 2011). In addition, new opsins have been identified and/or generated with varied functions and optimized kinetic and spectral properties. Specifically, much effort has been focused on developing opsins with shifted excitation spectra and the reason for this is twofold (Guru et al., 2015). The first is that longer wavelength-sensitive opsins (i.e. red-shifted opsins) would enable deeper penetration of light
into tissues with minimal scattering. The second is that opsins with separated action spectra may be combined to achieve either bidirectional control of the same neuronal population or independent control of different populations. These strategies can be incredibly useful for decoding complex neural circuitries and have been previously demonstrated in only a few reports.

For instance, Zhang and colleagues (2007) found that the light-driven chloride pump, NpHR, with an excitation maximum of 580 nm, could be paired with ChR2, which has an excitation maximum of 460 nm. They predicted that the spectral separation would allow independent activation of the opsins thus enabling bidirectional modulation of membrane potential. To test this, they co-delivered NpHR and ChR2 to mouse pups via lentivirus injection and prepared acute cortical slices a few days later. Illumination of the slices with either blue or yellow light resulted in differential calcium transients as measured by fura-2 calcium imaging. Blue light alone evoked intracellular $[\text{Ca}^{2+}]$ transients while simultaneous illumination with both blue and yellow light (and also yellow light alone) had no detectable effect on intracellular $[\text{Ca}^{2+}]$. To test if the same strategy could also be applied to modulate behaviour, they expressed the NpHR/ChR2 system in either muscle or cholinergic motor neurons of *C. elegans*. In both transgenic animals, blue light activation of ChR2 resulted in muscle wall contractions. When NpHR was photoactivated simultaneously, the contractions stopped and only resumed again when NphR photoactivation was ceased.

To examine whether wavelength-shifted excitatory opsins could be applied to gain independent control of multiple neuronal populations, many groups have tried combining blue-sensitive ChR2 with red-shifted opsins. However, a fundamental limitation of these combinations is the large overlap between their excitation spectra, due to the intrinsic blue light absorption of the retinal chromophore. As an alternative to redesigning existing channelrhodopsins, which had previously resulted in slower variants, Klapoetke and colleagues (2014) turned to nature and performed *de novo* transcriptome sequencing of over 100 different species of alga. They identified 61 new channelrhodopsin homologs and functionally characterized each of them in cultured neurons according to photocurrent, wavelength sensitivity, kinetics and trafficking. From these, they identified and optimized the first yellow-peaked channelrhodopsin, Chrimson (590 nm spectral peak), and the blue-green light-sensitive channelrhodopsin, Chronos. The latter has faster kinetics and higher light sensitivity than any other channelrhodopsin previously reported. In
opsin-expressing neurons from acute mouse brain slices, 625 nm red light elicited spikes in Chrimson-expressing cells (≥1 mW/mm² light power) while 470 nm blue light drove spikes in only Chronos-expressing cells (0.05 to 0.5 mW/mm² light power). When Chronos and Chrimson were expressed in separate sets of neurons within the same cortical microcircuit, activation of each opsin by different wavelengths could reliably drive distinct postsynaptic responses in downstream non-opsin-expressing neurons. This approach was later adapted to establish bimodal control of avoidance behaviours in C. elegans, where channelrhodopsin pairs Chrimson and CoChR were expressed and activated in different mechanosensory neurons of the same living animal (Schild & Glauser, 2015).

1.4.3 Optogenetics for controlling cAMP and cGMP

Besides optogenetics actuators that function to induce or suppress neural activity, there also exist actuators that modulate the activity of intracellular signalling pathways. Optical control of intracellular signalling, such as the cAMP and cGMP pathways, are highly desirable because they mediate a variety of neuronal functions. OptoXR are opsin-receptor chimeras engineered to enable optical control, with high spatiotemporal precision, over G protein-coupled receptor-mediated biochemical signalling in mammals. Airan et al. (2009) replaced the intracellular loops of G<sub>t</sub>-coupled bovine rhodopsin with those of specific adrenergic receptors (AR), including G<sub>s</sub>-coupled hamster β<sub>2</sub>AR and also G<sub>q</sub>-coupled human α<sub>1</sub>AR. The result was a new set of light-sensitive proteins that could selectively recruit distinct, targeted signalling pathways in response to light. Specifically, opto-β<sub>2</sub>AR yielded significant production of cAMP in opto-β<sub>2</sub>AR-expressing HEK cells when illuminated with 504 nm green light, while stimulation of opto-α<sub>1</sub>AR-expressing cells led to increased production of inositol triphosphate (IP3). Importantly, upregulation of signalling molecules by optical stimulation was comparable to that achieved by pharmacological treatment of HEK cells expressing the wildtype receptors.

Non-opsin-based light-activated approaches have also been developed for non-invasive and reversible control of intracellular cAMP and cGMP signalling. Photoactivated adenylyl cyclase (PAC) is a small nucleotidyl cyclase that has enzymatic activity that increases 300-fold in the light (Stierl et al., 2011). PAC from the bacterium Beggiatoa is regulated by a blue light using FAD (BLUF) sensory domain and has been shown to modify cAMP-mediated behavioural changes in the Drosophila nervous system (Ryu et al., 2010; Stierl et al., 2011). Mutation of
three amino acids relevant for nucleotide specificity enables the binding of GTP instead of ATP, thus producing a bacterial light-activated guanylyl cyclase (BlgC) (Ryu et al., 2010). BlgC catalyzes the synthesis of cGMP and has been used in male rats to evoke penile erection after blue light illumination of BlgC-transfected corpus cavernosum (Kim et al., 2015). Additionally, fusion of a phytochrome from *Rhodobacter sphaeroides* to a catalytic adenyl cyclase domain resulted in the near-infrared window light-activated AC (Ryu et al., 2014). Transgenic animals expressing the AC in *C. elegans* cholinergic neurons resulted in hyperactivity and increased frequency of body bends in response to red light.

A new type I rhodopsin fused to a guanylyl cyclase was recently identified in the aquatic fungus *Blastocladiella emersonii* (Schieb et al., 2015; Gao et al., 2015). Light illumination yielded >50 times more cGMP in RhGC-expressing oocytes than in BlgC-expressing oocytes (Gao et al., 2015). Meanwhile, when expressed in *C. elegans* sensory neurons, RhGC was shown to induce behavioural responses consistent with normal sensory function. The authors speculate that since RhGC is a naturally evolved light-triggered enzyme, it may be a more efficient optogenetic tool for light-dependent cGMP synthesis compared to the genetically engineered BlgC.

Along with the photoactivatable nucleotide cyclases that promote the synthesis of cAMP and cGMP, engineering of complementary light-activated phosphodiesterase (LAPD) has been achieved to regulate their degradation. Gasser et al. (2014) combined the photosensory module of *Deinococcus radiodurans* phytochrome with the catalytic domain of human PDE2. Red light absorption of LAPD upregulated the hydrolysis of cAMP and cGMP in eukaryotic cell cultures and zebrafish embryos. This was the first demonstration of a PDE that is directly regulated by light. By switching the catalytic domain of LAPD with that of human PDE4 and PDE5, we recently developed photoactivatable phosphodiesterase 4 (PhPDE4) and 5 (PhPDE5). PhPDE4 and PhDE5 are also driven by light and enable selective degradation of cAMP and cGMP, respectively. Unlike LAPD, they can be applied in neural systems to independently investigate the functions of cAMP and cGMP signalling pathways. However, as PhPDE4 and PhPDE5 are regulated by the same light-sensitive regulatory domain, they cannot be applied as a combination to independently target cAMP or cGMP signalling in the same neurons.
1.5 Thesis overview

The purpose of this project was to develop a new approach for investigating the spatial and temporal roles of both cAMP and cGMP signalling in synaptic plasticity and memory. cAMP and cGMP are ubiquitous second messengers that mediate a variety of physiological processes in the mammalian system. Decades of research have recognized the functional roles of cAMP and cGMP as integral for neuronal processes underlying synaptic plasticity and memory. However, the specific and interactive mechanisms by which they regulate their effects remain elusive. This is largely due to the lack of established techniques that can mimic or interfere with rapid intracellular signalling systems in subcellular microdomains or complex neuronal networks. Thus, an ultimate goal is to develop and validate a new approach that would enable non-invasive and precise investigation of cAMP and cGMP signalling underlying brain function and dysfunction in mammals.

Optogenetics is an emerging technique that allows non-invasive, reversible and spatiotemporally precise perturbation of biological function. In the past decade, the optogenetic toolkit for cAMP and cGMP signalling has expanded with the discovery and engineering of photoactivatable cyclic nucleotide cyclases and phosphodiesterases. These enzymes catalyze the synthesis and hydrolysis of cAMP and/or cGMP by light, respectively. However, it remains unclear whether multiple enzymes can be applied and controlled independently to investigate cAMP and cGMP spatial and temporal interactions (i.e. crosstalk) in the same biological system.

In the following chapters, I describe the development of a novel optogenetic technique that enables independent, spatial and temporal control of cAMP and cGMP signalling in the mammalian nervous system. By utilizing two distinct photoactivatable cyclases that selectively synthesize cAMP and cGMP by different wavelengths of light, I demonstrate the biological consequences of light-induced cAMP and cGMP synthesis on structural synaptic plasticity and memory formation. I will discuss how this optogenetic technique can be targeted to study single synapses and animal behaviours and also how it can be applied to further elucidate the functions of cAMP and cGMP signalling in both neuronal and non-neuronal systems.

The following work consists of unpublished data generated by myself with the assistance of my colleagues in the Okamoto Lab.
Chapter 2 Materials and Methods

2.1 Molecular biology

2.1.1 Expression vectors

Human codon optimized PAC (Addgene plasmid #28134) with a mutation (S27A: reduced dark activity) was subcloned into pCAG plasmid vector. To convert PAC into a 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). nIR-AC (Ryu et al., 2014) was codon optimized for mammalian cell expression and synthesized (GenScript, NJ, USA) and subcloned into pCAG plasmid vector. To modify nIR-AC into near-infrared light-sensitive guanylyl cyclase (nIR-GC), three amino acids involved in ATP binding were changed for GTP binding (K540E, D633K, T635G) (Ryu et al., 2010). RhGC (Avelar et al., 2014) was synthesized and human codon optimized for mammalian cell expression (GenScript, NJ, USA).

For protein purification, PAC, BlgC, nIR-AC, nIR-GC and RhGC were subcloned into pCAG plasmid vector with a N-terminal polyhistidine tag (6 x His). For in vitro cell lysate assays and expression in neurons, PAC and BlgC were fused with RFP (tdTomato) in their N-terminal, nIR-AC and nIR-GC were fused with GFP (EGFP) in their N-terminal, and RhGC was fused with GFP (EGFP) in the C-terminal and subcloned into pCAG plasmid vector.

2.1.2 Protein expression and purification

HEK293T cells were seeded in a six well plate with 2 mL of cell culture medium (DMEM + 10% FCS + 1% PS). The cells were transfected with plasmids using Lipofectamine 3000 (Life Technologies, Grand Island, NY, USA) and harvested 48 hours post-transfection. The cells were homogenized in buffer (40 mM HEPES/Na, pH 8.0, 0.1 mM EGTA, 5mM magnesium acetate, 1 mM DTT, ad 0.01% Tween-20) by sonication, and centrifuged at 16,000 g for 15 minutes. The supernatant was isolated and used for further in vitro experiments. The concentration of fluorescent protein fused to light-activated cyclase was measured with RFP or GFP ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA).
For purification, HEK293T cells were prepared as above and transfected with plasmids containing a N-terminal polyhistidine tag. 48 hours post-transfection, cells were harvested and homogenized, and for rhodopsin-containing fragments, extracts were solubilized in dodecyl maltoside (DDM). After binding to Ni-nitrilotriacetic acid resin column and washing with 50 mM imidazole, the protein fractions were collected with 500 mM imidazole (Capturem His-Tagged Purification Maxiprep Kit, Takara Bio USA, Inc., Mountain View, CA, USA). The fractions were then desalted and concentrated (Amicon Ultra 30-100 kD, Millipore, Danvers, MA, USA) in the same assay buffer as above. Protein concentration was measured using His Tag ELISA Detection Kit (GenScript, Piscataway, NJ, USA).

2.2 In vitro photoactivation experiments

2.2.1 LED (one-photon) photoactivation

The assays measuring cyclase activity were performed on a glass slide covered in plastic paraffin film (Parafilm® M, Bemis Company, Inc, Neenah, WI, USA) in darkness at room temperature. The reaction mixtures were 100 µL in volume and contained cell lysates (diluted in buffer) with either 100 µM ATP or 200 µM GTP. Mounted LEDs (Thorlabs, Inc., Newton, NJ, USA) were used to photoactivate the enzymes. The reactions were stopped by adding 60 µL aliquots to 540 µL 0.1 M HCl. The level of cAMP or cGMP was measured by Direct cAMP or cGMP ELISA Kit (Enzo Life Sciences, Inc., Farmindale, NY, USA). All independent experiments were assayed in triplicate.

2.2.2 Two-photon photoactivation

For two-photon excitation wavelengths 700-1025 nm, photoactivation experiments were performed under a 60X objective lens (NA 1.0, LUMPLFLN; Olympus, Tokyo, Japan) in a custom-made two-photon microscope (FV1000 MPE; Olympus, Tokyo, Japan) equipped with dual two-photon lasers (Mai Tai HP and MaiTai DeepSee; Spectra-Physics, Newport, CA, USA). For wavelengths 1025-1300 nm, experiments were performed at the Centre for the Neurobiology of Stress (CNS) at the University of Toronto Scarborough. Samples were photoactivated using a multiphoton microscope (FVMPE-RS; Olympus Tokyo, Japan) equipped with dual two-photon lasers (Insight DeepSee and MaiTai DeepSee; Spectra-Physics, Newport, CA, USA) and 40X objective lens (NA 0.8, LUMPLFLN; Olympus, Tokyo, Japan). Cell lysate reactions were prepared and measured for cAMP/cGMP concentration as above.
2.3  *In vivo* neuron experiments

2.3.1  Hippocampal slice preparation

The following protocol was approved by the Animal Care Committee of the University Health Network following the policies and guidelines set forth by the Canadian Council on Animal Care.

Organotypic hippocampal slice cultures were prepared from postnatal day 6 to 7 Sprague Dawley rats. Following decapitation, the brain was removed from the skull and placed in a 10 cm Petri dish containing filter paper covered with ice cold, oxygenated dissection medium (10 mM glucose, 4 mM KCl, 26 mM NaHCO₃, 248 mM sucrose, 5 mM MgCl₂, 1 mM CaCl₂ and 0.1% phenol red, equilibrated with 95% O₂ and 5% CO₂). The hippocampi were exposed and isolated, and cut into 400 µm thick transverse sections with McIlwain Tissue Chopper (Micke Laboratory Engineering, Gomshall, Surrey, UK). The sliced hippocampi were transferred to a 6 cm dish containing slice culture medium (MEM with Earle’s salts and L-glutamine, 20% horse serum, 1.2 g/L glucose, 5 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 30 mM HEPES, 0.5 mM ascorbic acid, 1 µg/mL insulin, pH 7.3, and 320-330 osmolarity) and further separated with forceps. The slices were then placed in a six well plate, with each well containing one 30 mm, 0.4 µm pore size Millicell membrane (Merck Millipore, Billerica, MA, USA) and 1 mL of warm slice culture medium. The plate was kept in an incubator at 35°C with 95% O₂ and 5% CO₂. Slice culture medium was exchanged every 48 hours until use.

2.3.2  Gene gun transfection and expression in neurons

After 5 to 6 days in culture, hippocampal slices were transfected with a Helios gene gun (Bio-Rad, Hercules, CA, USA) using 1.6 µg gold microcarriers coated with DNA plasmids. For making bullets, the amount of DNA and gold particles used for 25 inches of Tefzel tubing were 200 µg of RFP-PAC (or RhGC-GFP) + 100 µg of GFP (or RFP) + 15 mg of gold for neuron expression analysis and 200 µg of PAC + 200 µg of RhGC + 50 µg of GFP + 15 mg of gold for structural plasticity experiments. Live imaging was performed 3 to 5 days after transfection in the dendrites of CA1 pyramidal neurons. Slices were transferred to a recording chamber and perfused in ACSF containing 119 mM NaCl, 2.5 mM KCl, 4 mM CaC₂, 4 mM MgCl₂, 26.2 mM NaHCO₃, 1 mM NaH₂PO₄ and 11 mM glucose at 30°C and bubbled with 95% O₂/5% CO₂. Z-stack images of dendrites (neurons) were acquired via two-photon imaging (900 nm excitation)
through a 60X objective lens at 0.5 µm intervals. Post-processing of images and line scan measurements were performed using Imaris (BitPlane, Zurich, Switzerland) and MetaMorph (Molecular Devices, Sunnyvale, CA, USA) softwares, respectively.

2.3.3 Structural long-term potentiation (sLTP)

Slices were perfused with ACSF as above with 1 µm tetrodotoxin and 2.5 mM 4-methoxy-7-nitroindoliny1 (MNI)-L-glutamate (Tocris, Bristol, UK). sLTP was induced in target dendritic spines from CA1 neurons by two-photon glutamate uncaging 1 µm from the dendritic spine membrane. Uncaging was performed by photolysis of MNI-L-glutamate with a 720 nm pulse train of 4 ms duration, 1 Hz frequency and 5 mW power for 1 minute. To photoactivate postsynaptic PAC (cAMP), target spines were excited with 800 nm two-photon laser light (1 mW, 10 seconds) prior to uncaging. To photoactivate postsynaptic RhGC (cGMP), target spines were excited with 1,100 nm two-photon laser light (1 mW, 10 seconds). Images of dendritic spine enlargement were captured using time-lapse two-photon imaging (860 nm excitation). Subsequent spine size change was measured by mean GFP fluorescence intensity (over a fixed volume) of stimulated spines from 20-30 z-stack images using Imaris software (BitPlane, Zurich, Switzerland).

2.4 Mouse behavior assay

2.4.1 Generation of RFP-PAC transgenic mice

The RFP (tdTomato)-PAC gene, which is codon-optimized for human expression (Addgene plasmid #28134), was placed under the CMV enhancer-CamKIIα promoter. cDNA was subcloned into a custom plasmid vector (pMM403) for bacterial amplification. The amplified 5.2 kb plasmid was digested with SfiI and SalI to linearize the DNA and remove prokaryotic sequences. Transgenic mice were generated by injection of the purified insert into the pronuclei of C57BL/6J mice at The Centre for Phenogenomics (Toronto, ON, Canada). Genotyping was performed by PCR. Founder lines were backcrossed into C57Bl/6J mice for at least 6 generations.
2.4.2 Virus vector construction and production

pAAV-CaMKIIα-RhGC-EGFP was constructed by replacing the iRFP fragment from pAAV-CaMKIIa-iRFP (Addgene plasmid #47903) with RhGC-mGFP. High titer \(1.52 \times 10^{13}\) AAV particles (serotype 9) were produced by SignaGen Laboratories (Rockville, MD, USA).

2.4.3 Stereotaxic microinjection surgery

The following procedures were approved by the Toronto Centre for Phenogenomics Animal Care Committee and were performed in compliance with the Canadian Council on Animal Care.

Mice were anesthetized with isoflurane (2% vol/vol) and then transferred to a stereotaxic apparatus. Mice were secured to the apparatus by fixing ears bars to the head and insertion of the incisor adaptor. A nose cone was used to administer isoflurane during surgery to maintain anesthesia as confirmed by mild pinching of the tail. Mice were injected with 0.1 mL of meloxicam (0.5 mg/mL) and 0.1 mL of saline subcutaneously prior to surgery. Hair atop the scalp was removed with electric clippers and antiseptic solution was applied topically to clean the skin. Additionally, Tear-Gel (Alcon, TX, USA) was applied to lubricate the eyes and reapplied when necessary. A scalpel was used to make a midline incision and expose the bregma and lambda landmarks of the skull. After leveling the head, a drill was used to make two small holes 2 mm posterior from bregma and 1.3 or 1.6 mm lateral to the midline. Injection volumes were 2 µL per hemisphere and delivered at a rate of 0.1-0.2 µL per minute using a gastight syringe with a 26 gauge needle (Hamilton, Reno, NV, USA). Following injection, the syringe was kept in place for an additional five minutes to allow diffusion of the virus particles. The syringe was then slowly withdrawn from the head and the injection site was wiped clean. The skin was sutured and antibiotic ointment was applied over the wound. The mouse was removed from the stereotaxic apparatus and placed in a recovery cage under a heat lamp. Meloxicam was administered subcutaneously every 24 hours for 2 to 3 days following surgery. Mice were given at least 3 weeks of postoperative recovery before fluorescence imaging of protein expression.

2.4.4 Fluorescence imaging of mouse hippocampal slices

Acute mouse hippocampal slices of 300 µm thickness were prepared and bathed in ACSF equilibrated with 95% O₂ and 5% CO₂ at room temperature. RFP-PAC fluorescence imaging was conducted using a Nikon C2+ confocal laser scanning microscope with 4X objective lens.
(Nikon, Tokyo, Japan) and 543 nm laser excitation (585 nm emission). For imaging RhGC-GFP, 488 nm laser excitation (514 nm emission) was applied. Image analysis and processing was performed using NIS-Elements software (Nikon, Tokyo, Japan).

2.4.5 Bilateral LED design and insertion surgery

For behavioural experiments, a second surgical procedure was conducted for insertion of the bilateral LED into the skull immediately following viral injection as described above. The 1 g LED device was custom-made to include a bilateral LED attached to dual optic fibers. The cannulae surrounding the optic fibers were 0.5 mm in diameter, 3.0 mm in length and spaced 2.6 or 3.2 mm apart (Eicomm USA, San Diego, CA, USA). The device was slowly inserted through the holes previously drilled for virus injection and fixed to the skull using two kinds of adhesive resin (Bistite II DC; Tokuyama Dental Corporation, Tokyo, Japan; Unifast TRAD; GC America, Alsip, IL, USA). Mice were given at least 3 weeks of postoperative recovery before commencing behavioural testing.

2.4.6 Novel objection recognition test

Animals were maintained on a 12 hr light/dark cycle with food and water at the Toronto Centre for Phenogenomics. Experiments were conducted between 10 AM and 6 PM and mice were handled daily for 3 days prior to experimentation (2 days, every 2 weeks). Before testing, either a working receiver unit (for Light ON conditions) or a dummy unit (for Light OFF conditions) was attached manually to the LED fixed to the mouse’s skull. Mice were placed in one of two identical white Plexiglass arenas (40 cm³) with two identical objects positioned in each. At the start of the habituation phase, 385 nm (ultraviolet), 590 nm (yellow) or no light was continuously applied and controlled by wireless remote. Mice were given 5 minutes of exploration (habituation) time and their movements were recorded by video camera. After 5 minutes, the LEDs were switched off and mice were returned to their home cages for 2 minutes while one of the objects in each arena was replaced with a novel object in the same position. During the test phase, mice were placed back into the arenas and allowed another 5 minutes of exploration without additional light stimulation. At the end of the test, mice were returned to their home cages and the arenas, objects and receiver units were cleaned with a chlorine dioxide solution before repeating with new test subjects. Data analysis was performed using Ethovision 8.5 software (Noldus Information Technology Inc., Leesburg, VA, USA).
2.5 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; n.s., not significant. Analyses were performed using KyPlot 2.0 (Kyens Lab, Inc., Japan). Data were considered statistically significant at p < 0.05.
Chapter 3
Multicolour optogenetics for cAMP and cGMP

3.1 Introduction of Chapter 3

Photoactivatable enzymes for cAMP and cGMP signalling have been reported as optogenetic tools for manipulating cAMP and cGMP by light. Blue, green and red light-sensitive cyclases and phosphodiesterases have been shown to generate and degrade cAMP and cGMP, respectively. However, it remains unclear whether multiple enzymes can be applied and controlled independently in the same cells. This would enable further investigation of cAMP and cGMP functions such as their spatial and temporal signalling interactions.

By characterizing the optimized or engineered photoactivatable enzymes available for light-dependent synthesis of cAMP/cGMP, specifically their unique excitation spectra, I established a combination of photoactivatable enzymes to independently control cAMP and cGMP by light. Studies of cAMP and cGMP in the nervous system have employed pharmacological and genetic methods, such as chemical inhibitors or knockout mice, but have limited cellular specificity and spatiotemporal precision. The optogenetic approach provides unique advantages for neuroscience studies including spatiotemporal manipulation of cAMP/cGMP levels in the target cells of a specific brain region. Specifically, two-photon excitation light allows target single synapse photoactivation in deep brain tissues, enabling the study of spatiotemporal cAMP/cGMP function at the synapse level.

In this chapter, I validated the photoactive properties of the light-sensitive adenylyl and guanylyl cyclases and found a combination of cyclases to separately photoactivate cAMP and cGMP production by specific excitation wavelengths of light (Chapter 3.2). Furthermore, I characterized two-photon photoactive properties of these cyclases and optimized a combination of two-photon excitation wavelengths for dual photoactivation of adenylyl (cAMP) and guanylyl cyclases (cGMP) (Chapter 3.3). Also, I confirmed the cellular expression and distribution of photoactivatable enzymes in the living neurons of rodent hippocampal tissues (Chapter 3.4). These techniques will enable rapid and independent control of cAMP and cGMP synthesis for elucidating their interactive signalling dynamics and functions in an unprecedented way.
3.2 Characterization of photoactivatable cyclases

To establish dual photoactivation of light-sensitive cyclases to independently control cAMP/cGMP by different wavelengths of light, I characterized the photoactive properties of five light-sensitive adenylyl and guanylyl cyclases which have been optimized for mammalian cell expression.

For blue light-sensitive cAMP synthesis, I utilized the photoactivatable adenylyl cyclase (PAC) from the soil bacterium *Beggiatoa*. The catalytic adenylyl cyclase is linked to a regulatory blue light using FAD (BLUF) domain that is activated by blue light (Ryu et al., 2010; Stierl et al., 2011) (Figure 1). For blue light-sensitive cGMP synthesis, I tested a mutant PAC (BlgC) that has mutagenesis of three residues in the substrate-binding site enabling GTP binding instead of ATP (Ryu et al., 2010) (Figure 1).

To complement the blue light-sensitive enzymes, I validated a far-red light-activated adenylyl cyclase (nIR-AC), which was engineered by fusing a photosensory module (PAS-GAF-PHY) from a *Rhodobacter sphaeroides* phytochrome to an adenylyl cyclase catalytic domain from the *Nostoc* sp. CyaB1 protein (Ryu et al., 2014). By modifying this enzyme for cGMP utilizing a similar strategy for GTP binding, I also examined a far-red light-activated guanylyl cyclase (nIR-GC) (Figure 1).

Most recently, a new type I microbial rhodopsin was found in the aquatic fungus *Blastocladiella emersonii* (Scheib et al., 2015; Gao et al., 2015). The rhodopsin, directly fused with a guanylyl cyclase domain (RhGC), enables green light-driven cGMP synthesis (Figure 1). I validated its green light-dependent activation *in vitro*.

To establish independent synthesis of cAMP and cGMP levels by different excitation wavelengths, I compared the photoactivatable adenylyl and guanylyl cyclases variants *in vitro* using LED (one-photon) light (Chapter 3.2.1). I determined the optimal combination of cyclases and separate excitation wavelengths for multicolour light-dependent control of cAMP and cGMP (Chapter 3.2.2)
3.2.1 One-photon characterization of photoactivatable cyclases in vitro

To validate light-dependent activity, human codon-optimized enzymes (tagged with GFP or RFP) were expressed in HEK 293T cells and assayed for light-dependent cAMP/cGMP synthesis along with enzymatic concentration in the cell lysates by ELISA (Figure 2). Light-dependent cAMP/cGMP synthesis was measured upon exposure to light (Figure 3A) and also in the dark following exposure to light (Figure 3B).

Excitation of blue light-sensitive PAC with 455 nm blue LED light induced rapid synthesis of cAMP upon seconds of illumination (Figure 3C). Excitation of BlgC by the same blue light also resulted in rapid light-dependent synthesis of cGMP (Figure 3C). Importantly, the removal of light stopped further cAMP/cGMP production indicating their rapid turn off response. Similarly, excitation of far-red AC (nIR-AC) and GC (nIR-GC) with 660 nm red light caused production of cAMP and cGMP, respectively. However, levels of cAMP/cGMP were not detectable until minutes of illumination, indicating slower activity compared to PAC and BlgC (Figure 3D). In contrast, excitation of green light-activated RhGC with 565 nm green light led to rapid and robust elevation of cGMP and also inactivation, indicating tight regulation of light-dependent enzymatic activity (Figure 3E).

To precisely compare the photoactivation efficiency of each photoactivatable cyclases, I next determined their photoactivation kinetics using purified enzymes without their fluorescent tags.
Using His-tagged photoactivatable enzymes, I measured their enzymatic concentration and light-dependent cAMP/cGMP synthesis by ELISA assays. Upon illumination with the optimal wavelength of light (455 nm, 565 nm or 660 nm at 5 mW/mm²), blue light-sensitive enzymes PAC and BlgC displayed the strongest photoactivity (0.29 and 0.33 µmol cNMP/µmol enzyme/s/mW/mm²) while the far-red light-sensitive nIR-AC and GC barely revealed any activity (0.0038 and 0.0037 µmol cNMP/µmol enzyme/s/mW/mm²). Interestingly, the photoactivity of the green light-sensitive RhGC was higher than BlgC in cell lysates (Figure 3C,E) but was much lower than BlgC in the purified condition (0.042 µmol cNMP/µmol enzyme/s/mW/mm²). This may be due to the removal of functional membrane components from the RhGC enzyme by the His-tag purification process. In summary, PAC and RhGC showed most efficient cAMP and cGMP synthesis by different excitation wavelengths of light.

**Figure 2. Schematic of LED photoactivation of photoactivatable cyclases in vitro.**

Cell lysates expressing photoactivatable adenyllyl or guanylyl cyclases were combined with 100 µM ATP or 200 µM GTP, respectively, on a glass slide covered with paraffin film. Mounted LEDs were used to photoactivate the photoactivatable cyclases in vitro. Light-dependent cAMP/cGMP synthesis and protein concentration were measured by ELISA.
Figure 3. Photoactivation of light-dependent adenylyl and guanylyl cyclases by LED light.
(A) Schematic of photoactivation time course to measure light-dependent activity of cyclases upon exposure to light and (B) in darkness following exposure to light. HEK cell lysates expressing (C) blue light-sensitive PAC or BlgC, (D) far-red light-sensitive nIR-AC or nIR-GC, and (E) green light-sensitive RhGC were illuminated with LED light of specified wavelength. PAC/BlgC were fused with RFP (tdTomato) and nIR-AC/GC and RhGC were fused with GFP (EGFP) as fluorescence markers for in vitro experiments. Left: time course of the photoactivation of enzymes; right: time course of the off response of the enzymes after 30 seconds of illumination in (C) and (E) or 5 minutes in (D). For the off response, data are after subtraction of the initial photoactivation. For all experiments, photoactivation was measured in the presence of 100 µM ATP for adenylyl cyclase or 200 µM GTP for guanylyl cyclase. Light-dependent activity of cAMP or cGMP synthesis was measured from three independent experiments by ELISA and assayed in triplicate. Data are mean ± SEM.

Table 1. In vitro photoactivation properties of photoactivatable cyclases.

<table>
<thead>
<tr>
<th>Photoactivatable enzyme</th>
<th>cAMP/cGMP</th>
<th>$\lambda_E$ (nm)</th>
<th>One-photon photoactivity ($\mu$mol cNMP/μmol enzyme/s/mW/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC</td>
<td>↑ cAMP</td>
<td>455</td>
<td>0.29 ± 0.0058</td>
</tr>
<tr>
<td>BlgC</td>
<td>↑ cGMP</td>
<td>455</td>
<td>0.33 ± 0.024</td>
</tr>
<tr>
<td>RhGC</td>
<td>↑ cGMP</td>
<td>565</td>
<td>0.042 ± 0.015</td>
</tr>
<tr>
<td>nIR-AC</td>
<td>↑ cAMP</td>
<td>660</td>
<td>0.0038 ± 0.0013</td>
</tr>
<tr>
<td>nIR-GC</td>
<td>↑ cGMP</td>
<td>660</td>
<td>0.0037 ± 0.014</td>
</tr>
</tbody>
</table>

$\lambda_E$: Excitation wavelength; photoactivity is ± SEM.

3.2.2 Dual-colour optogenetic control of cAMP and cGMP in vitro

Since the blue light-sensitive adenylyl cyclase PAC and the green light-sensitive guanylyl cyclase RhGC displayed strong photoactivity upon light excitation in vitro and have similar excitation wavelengths to ChR2 and NpHR, I next examined if they could be used as a suitable enzyme combination for producing cAMP and cGMP independently by different wavelengths. To avoid overlap in their excitation spectra, I tested different wavelengths of light to separate the
light-dependent activity of PAC and RhGC (Figure 4A). I found that UV light (385 nm) could activate PAC while yellow light (590 nm) could activate RhGC, but not vice versa (Figure 4B,C). This suggests that by co-expression of both enzymes in vivo, 385 nm light excitation can induce cAMP synthesis by PAC while 590 nm light excitation can induce cGMP synthesis by RhGC (i.e. dual-colour control).

Using 385 nm and 590 nm wavelengths, I also examined the activity of PAC and RhGC in response to different light intensities (Figure 5). Upon photoactivation of each enzyme, cAMP/cGMP was detected from 1 mW/cm² and reached a plateau by 20 mW/cm². This power requirement suggests sufficient photoactivation of PAC and RhGC in deep brain regions using our custom-made optical fiber-coupled LEDs, which have comparable light intensity for photoactivation in the brain of freely behaving animals in vivo.

Figure 4. Excitation wavelength-dependent cAMP/cGMP synthesis by photoactivation of PAC and RhGC.

(A) Multicolour optogenetic strategy for wavelength-dependent photoactivation of PAC and RhGC. (B) Time course of light-dependent cAMP/cGMP synthesis by photoactivation of HEK293T cell lysates with UV light (385 nm peak) and (C) yellow light (590 nm peak). The cell
lysates expressing PAC (blue, n=3) or RhGC (green, n=3) were illuminated with UV or yellow LED light (10 mW/cm²) in the presence of 100 µM ATP or 200 µM GTP, respectively. Light-dependent activity of cAMP or cGMP synthesis was measured by ELISA and assayed in triplicate. Data are mean ± SEM.

**Figure 5. Light intensity-dependent activation of cAMP/cGMP synthesis by photoactivation of PAC and RhGC.**

(A) UV light intensity-dependent synthesis of cAMP by PAC cell lysates (n=3). (B) Yellow light intensity-dependent cGMP synthesis by 590 nm photoactivation of RhGC cell lysates (n=3). Photoactivation was applied by LED light for 10 seconds with the specified wavelength and light intensity in the presence of 100 µM ATP or 200 µM GTP, respectively. Peak cAMP or cGMP levels were set as 100%. Light-dependent activity of cAMP or cGMP synthesis was measured by ELISA and assayed in triplicate. Data are mean ± SEM.

### 3.3 Characterization of two-photon photoactivation of cAMP/GMP synthesis

Following the development of a dual-colour optogenetic strategy for independent control of cAMP and cGMP using one-photon (LED) light, I next aimed to establish a dual-colour strategy using two-photon excitation light in combination with PAC and RhGC. Two-photon microscopy uses excitation light that is about twice the normal (one-photon) excitation wavelength to activate a photoreactive molecule (Denk et al., 1990). The simultaneous absorption of two low-energy photons, instead of one high-energy photon, can excite electrons from their ground state to an excited state using their combined energies.
There are several advantages of using two-photon microscopy for photoactivation of optogenetic proteins. Firstly, the wavelength of light that is typically used is from the infrared spectral range. Long wavelengths of infrared right can penetrate biological tissues more efficiently, as less energy is lost to tissue scattering. This is desirable for optogenetic stimulation in thick tissues where two-photon light can penetrate deeply with reduced phototoxicity. Additionally, two-photon excitation only occurs at the focal point, where the probability of two-photon absorption is high. The focal point is less than 1 μm³ in volume, which is ideal for imaging or photoactivating small subcellular structures, such as synapses, with minimal background signal. Thus, to develop a dual-colour strategy for control of cAMP and cGMP synthesis by two-photon light, I first characterized the two-photon excitation wavelength spectra of PAC and RhGC and found an optimal combination of two-photon wavelengths for dual photoactivation of PAC and RhGC in vitro.

To examine the two-photon excitation wavelength spectra of PAC and RhGC, HEK293 cell lysates expressing each enzyme were excited with different wavelengths of two-photon excitation light under a two-photon microscope (Figure 6). PAC displayed the highest photoactivity of cAMP synthesis in response to shorter wavelengths of two-photon light (700 nm and 800 nm) (Figure 7A). In contrast, RhGC showed the highest photoactivity for cGMP synthesis with much longer wavelengths (1,100 nm and 1,200 nm) (Figure 7B). These wavelengths roughly correspond to twice the one-photon excitation wavelengths (blue light for PAC; green light for RhGC), suggesting the possibility of independent photoactivation using two-photon excitation light.

To further test the efficiency of two-photon photoactivation of PAC and RhGC, I illuminated the lysates with 800 nm and 1,100 nm light, respectively. cAMP and cGMP were produced within seconds of two-photon excitation, indicating rapid response with two-photon excitation light (Figure 8A,B). These findings suggest that in addition to one-photon LED excitation, dual two-photon laser excitation can be used to regulate cAMP and cGMP signalling in neurons. Due to the small excitation focal point of two-photon laser light (<1 μm³), this technique would be suitable for manipulating light-sensitive enzymes in small focal volumes (<1 μm³), such as single dendritic spines.
Figure 6. Schematic of two-photon photoactivation of photoactivatable cyclases in vitro.

Cell lysates expressing PAC or RhGC were combined with 100 µM ATP (for PAC) or 200 µM GTP (for RhGC), respectively, on a glass slice covered with paraffin film. The sample was placed under the objective lens of a two-photon microscope which was used to photoactivate the photoactivatable cyclases in vitro. Light-dependent cAMP or cGMP synthesis and protein concentration were measured by ELISA.

Figure 7. Two-photon wavelength-dependent photoactivation of PAC and RhGC.

Two-photon excitation wavelength spectra of (A) PAC (n=3) (700-1,000 nm, 15 mW, 15 minutes) and (B) RhGC (n=3) (800-1,025 nm, 15 mW, 15 minutes; 1,000-1,300 nm, 4 mW, 5
minutes) in the presence of 100 µM ATP (PAC) or 200 µM GTP (RhGC), respectively. Peak cAMP or cGMP levels were set as 100%. Data are mean ± SEM.

Figure 8. Time course of two-photon photoactivation of PAC and RhGC.

Two-photon time course of photoactivation by excitation of (A) PAC with 800 nm light (15 mW) (n=3) and (B) RhGC with 1,100 nm light (5 mW) (n=3). The two-photon photoactivation volume is ~1 µm³. Data are mean ± SEM.

3.4 Expression of PAC and RhGC in CA1 pyramidal neurons

To validate the expression and subcellular distribution of PAC and RhGC in living neurons, I biolistically co-transfected RFP-PAC with GFP or GFP-RhGC with RFP (DsRed2) in the CA1 pyramidal neurons of rat organotypic hippocampal slice cultures (Figure 9A,B). In both cases, the co-transfected soluble fluorescent protein (GFP or RFP) was used as a volume filler control. The two-photon fluorescence images showed that the RFP-tagged PAC was distributed homogeneously in the neuron which was similar to the GFP (Figure 9C). In contrast, the RhGC-GFP showed increased fluorescence intensity on the dendritic membrane, demonstrating the membrane localization of the RhGC (Figure 9D). These results show similar subcellular distribution with the endogenous photoactivatable enzymes (PAC, RhGC) in bacteria, suggesting their photoactivatable functions in living neurons.
Figure 9. Subcellular distribution of PAC and RhGC in hippocampal CA1 neurons.

(A) Schematic of biolistic transfection of the enzymes with volume marker. (B) Two-photon fluorescence imaging of transfected CA1 pyramidal neurons in organotypic slice cultures. (C) Representative two-photon fluorescence images. Left: RFP-tagged PAC with GFP (volume marker). Right: fluorescence intensity profile across the dendrite (indicated by white line). (D) Left: GFP-tagged RhGC with RFP. Right: fluorescence intensity profile of the white line on left merged image. Arrowhead indicates membrane localization of GFP-RhGC.

3.5 Summary of Chapter 3

This chapter described the characterization and development of a new multicore optogenetic technique for studying cAMP and cGMP function by light.

By characterizing the photoactivity of human codon-optimized blue, green and far-red light-sensitive adenylyl and guanylyl cyclases originally from bacteria or genetically engineered, I
found that the far-red light sensitive enzymes have much lower photoactivation efficiency compared with the blue and green-light sensitive enzymes. For the combination of an adenylyl and guanylyl cyclase for dual photoactivation, I utilized a blue light-sensitive adenylyl cyclase PAC and a green light-sensitive guanylyl cyclase RhGC. To avoid overlap of photoactivation by their excitation light, I tested different excitation wavelengths for PAC and RhGC and found that the combination of UV light (385 nm) for PAC and yellow light (590 nm) for RhGC could separate their photoactivation in vitro. I also found a combination of two-photon excitation wavelengths for dual photoactivation of PAC (800 nm) and RhGC (1,100 nm) in vitro.

Furthermore, I validated the expression and subcellular distribution of these photoactivatable enzymes in living neurons of cultured hippocampus brain tissue, suggesting the possibility of dual photoactivation in vivo.

In summary, I established dual photoactivation protocols to independently manipulate cAMP and cGMP by light. This will be particularly valuable for investigating cAMP and cGMP signalling interactions, which are known to regulate a variety of physiological mechanisms. In the following chapter, I will discuss the application of this technique for investigating the roles of cAMP/cGMP signalling in neurons and demonstrate its optimization for studying cAMP/cGMP signalling at both single synapses and target brain regions.
Chapter 4
Applications of multicolour optogenetics in vivo

4.1 Introduction of Chapter 4

cAMP and cGMP are critical second messengers that serve as intracellular signalling molecules in various cell types. In neurons, cAMP and cGMP play important roles in synaptic plasticity and learning and memory (Kandel, 2012; Kleppisch & Feil, 2009). However, the precise details of their dynamic interaction are not fully known. This is largely due to the lack of available tools for controlling their spatiotemporal signalling independently in distinct cellular subregions and at relevant neuronal timescales.

Optogenetics combines optical and genetic approaches to provide powerful tools for non-invasive manipulation of neuronal activity by light. For dissecting intracellular signalling, new types of optogenetic tools have been reported for controlling specific molecular functions by light. We have successfully optimized and utilized the blue light-sensitive adenylyl and guanylyl cyclases, PAC and BlgC (Ryu et al., 2010; Stierl et al., 2011), to increase intracellular cAMP or cGMP levels by light in living neurons. Our preliminary results showed their bidirectional effects in structural synaptic plasticity and short-term recognition memory, suggesting complementary interaction mechanisms between cAMP and cGMP signalling.

To directly elucidate the interactive functions of cAMP and cGMP in neurons, here, I demonstrate the application of multicolour optogenetics for cAMP and cGMP from the single synapse level (Chapter 4.2) to learning and memory function (Chapter 4.3).

4.2 Bidirectional role of postsynaptic cAMP and cGMP in structural synaptic plasticity

Synaptic plasticity, such as long-term potentiation (LTP) and depression (LTD), is believed to be a major neuronal mechanism that underlies learning and memory processes (Martin et al., 2000). During synaptic plasticity, neurons undergo both structural and functional changes that are mediated by a variety of molecular events that occur from millisecond timescales, to minutes, hours or days. This includes activity-dependent structural enlargement of dendritic spines called
structural long-term potentiation (sLTP) which is crucial for activity-dependent synaptic maturation and modification of neural circuits. Furthermore, actin is the major cytoskeletal component of dendritic spines, and its regulation by CaMKII is necessary for the induction of sLTP (Okamoto et al., 2007; Kim et al., 2015).

In addition to Ca$^{2+}$/CaMKII signalling, postsynaptic cAMP is known to be involved in the late phase of LTP in rodent hippocampal CA1 pyramidal neurons. Also, pharmacological application of the potent adenylyl cyclase activator forskolin was shown to induce sLTP of dendritic spines suggesting a role for cAMP in regulating sLTP (Otmakhov et al., 2004). In contrast, our preliminary results revealed that postsynaptic cGMP, which is involved during LTD (Zhuo et al., 1994; Feil et al., 2003), reverses forskolin-induced cAMP-dependent sLTP, suggesting that postsynaptic cAMP and cGMP have bidirectional roles in the regulation of sLTP. However, the details of their dynamic interaction during sLTP are not well known.

In this section, I applied the newly developed multicolour optogenetic technique with two-photon microscopy (Chapter 3) to manipulate cAMP and cGMP at target dendritic spines during sLTP. By co-expressing both PAC and RhGC (with GFP as volume filler) in hippocampal CA1 pyramidal neurons and photoactivating these enzymes at target dendritic spines by a custom-made two-photon microscope, I demonstrate the functions of cAMP and cGMP in the rapid and bidirectional regulation of structural synaptic plasticity.

4.2.1 Co-expression of PAC and RhGC in hippocampal slice cultures

To confirm that the co-expression of PAC and RhGC does not affect the structural potentiation of dendritic spines (sLTP), I compared the basal sLTP between neurons that expressed GFP alone and GFP with PAC and RhGC. I first prepared organotypic hippocampal slice cultures from Sprague-Dawley rat pups. The slices were co-transfected with DNA vectors encoding PAC and RhGC (for cAMP and cGMP photoactivation, respectively) and also GFP (as volume filler) using a gene gun method. The slices were visualized through a two-photon microscope and I identified CA1 pyramidal neurons that displayed strong GFP fluorescence signal. For assaying structural potentiation, single dendritic spines from the main dendritic branch were targeted for sLTP experiments. sLTP was induced by photolysis of MNI-L-glutamate, a biologically inert form of the excitatory glutamate neurotransmitter, by application of 720 nm two-photon laser pulses (5 mW, 4 ms duration, 1 Hz frequency, 60 repetitions) approximately 1 µm from the...
target spine (Figure 10A). Using this protocol, the structural potentiation of dendritic spines was monitored over time by two-photon imaging of GFP fluorescence.

In neurons co-expressing PAC and RhGC, induction of sLTP by glutamate uncaging led to an immediate dendritic spine enlargement of 141% (Figure 10B). In addition, the enlarged spine structure persisted at least 30 minutes after uncaging, as targeted dendritic spines maintained an enlargement of 33% demonstrating structural potentiation (sLTP) (Figure 10B). The sLTP-mediated enlargement of spines from these neurons was not significantly different from neurons that were transfected with GFP (166% and 28%, respectively; p>0.05) (Figure 10C, D). This suggests that the co-expression of enzymes PAC and RhGC with GFP does not affect sLTP under these experimental conditions.

Figure 10. Comparison of structural LTP (sLTP) between neurons expressing GFP and GFP + photoactivatable enzymes (PAC + RhGC).

(A) Schematic representation of caged glutamate uncaging protocol. A caged glutamate uncaging protocol for LTP was applied 1 µm from the tip of the dendritic spine membrane. (B) Two-
photon time course imaging of dendritic spines during sLTP. Left: representative dendritic spine images before and after glutamate uncaging at target dendritic spines. Right: time course of dendritic spine size measured by GFP fluorescence intensity. The black trace represents uncaging of dendritic spines from CA1 pyramidal neurons expressing GFP (n=14 spines/8 neurons). The magenta trace represents uncaging of dendritic spines co-expressing GFP, PAC and RhGC (n=12 spines/7 neurons). (C) The size of dendritic spines expressing GFP or co-expressing GFP, PAC and RhGC was not significantly different 1 minute after uncaging (sLTP induction) or 30 minutes after uncaging (potentiation). n.s. denotes not significant, p > 0.05 by unpaired t-test. Data are mean ± SEM.

4.2.2 Effect of increased cAMP during structural synaptic plasticity

I next established a novel protocol for two-photon photoactivation of PAC for dual photoactivation during sLTP. We previously established a protocol for PAC photoactivation using 1,000 nm excitation and showed cAMP-dependent enhancement of sLTP. However, the excitation wavelength overlaps with efficient RhGC photoactivation and is not suitable for their separate activation in the same neurons. Therefore, following the in vitro two-photon dual photoactivation assay in Chapter 3, I established a new two-photon photoactivation protocol (800 nm excitation, 10 s duration) for cAMP-dependent sLTP with caged glutamate uncaging (Figure 11A). The photoactivation method led to prolonged dendritic spine enlargement in target spines, which maintained a 44% enlargement 30 minutes after glutamate uncaging (Figure 11B), demonstrating rapid enhancement of sLTP by postsynaptic cAMP production.
Figure 11. Potentiation of sLTP by two-photon photoactivation of PAC.

(A) Schematic representation of the experimental conditions. Dendritic spines of CA1 pyramidal neurons co-expressing GFP, PAC and RhGC were photostimulated for 10 seconds with 800 nm two-photon laser light for light-dependent production of cAMP by PAC. Following stimulation, sLTP was induced by caged glutamate uncaging 1 µm from the target spine membrane. (B) Time course of dendritic spine size measured by GFP fluorescence intensity with PAC activation (cAMP) and glutamate uncaging. Dendritic spines with PAC-mediated increase of cAMP show enhancement of sLTP when immediately applied prior to uncaging (n=13 spines/9 neurons). The grey dashed line represents basal sLTP of dendritic spines after uncaging alone. Data are mean ± SEM.

4.2.3 Effect of increased cGMP on cAMP-mediated structural synaptic plasticity

To achieve multicolour two-photon photoactivation of PAC and RhGC for manipulating cAMP and cGMP, respectively, in target spines, I next established a novel protocol for two-photon photoactivation of RhGC. In dendritic spines of CA1 pyramidal neurons expressing PAC, RhGC and GFP, cAMP levels were first increased by 800 nm laser excitation of PAC to induce cAMP-dependent sLTP (Figure 12A). Immediately following, cGMP levels were also increased in the same dendritic spines by applying 1,100 nm two-photon laser excitation for 10 seconds to photoactivate RhGC. Finally, caged glutamate uncaging was applied to induce sLTP (Figure 12A).
Dual photoactivation of PAC and RhGC prevented cAMP-mediated structural enhancement (Figure 12B; orange), which was observed in dendritic spines stimulated with 800 nm laser excitation alone prior to uncaging (Figure 12B; blue). Rather, dual stimulated dendritic spines showed similar structural potentiation to basal sLTP, which was represented by a 22% enlargement of spine size 30 minutes after caged glutamate uncaging (Figure 12B). This was not significantly different from sLTP of dendritic spines expressing GFP alone or PAC and RhGC (with GFP) in the absence of any photoactivation (p>0.05) (Figure 12C). These results indicate the bidirectional effect of cAMP and cGMP in sLTP utilizing a multicolour two-photon optogenetic approach for the first time. This also indicates that induction of postsynaptic cAMP and cGMP is sufficient to bidirectionally regulate sLTP, suggesting a competitive interaction mechanism between cAMP and cGMP during sLTP of dendritic spines.

Figure 12. Two-photon photoactivation of RhGC reveals inhibition of cAMP-dependent sLTP.

(A) Schematic representation of the experimental conditions. Dendritic spines of CA1 pyramidal neurons co-expressing GFP, PAC and RhGC were stimulated to photoactivate PAC (cAMP) (top) or photoactivate RhGC (cGMP) following PAC (cAMP) (bottom). In the dual stimulation condition (bottom), spines were stimulated with 1,100 nm two-photon laser light for RhGC-mediated increase of cGMP. Following photostimulation in both experiments, sLTP was induced by high frequency glutamate uncaging. (B) Time course of dendritic spine size measured by GFP fluorescence intensity. The blue trace represents PAC stimulation prior to uncaging (n=13 spines/9 neurons). The orange trace represents RhGC stimulation following PAC stimulation and
prior to uncaging (n=7 spines/2 neurons). Dual stimulated spines display de-potentiation of cAMP-mediated enhancement of sLTP, indicating a role for cGMP in regulating structural plasticity. The grey dashed line represents sLTP of dendritic spines after uncaging alone. Data are mean ± SEM. (C) Comparison of sLTP at 30 minutes after uncaging. *, p < 0.05 by Dunnett’s test. All data are mean ± SEM.

4.3 The bidirectional role of cAMP/cGMP in short-term recognition memory

I next applied multicolour optogenetics for cAMP and cGMP (Chapter 3) in the mouse brain to study the roles of cAMP and cGMP in learning and memory.

Synaptic functions such as plasticity are tightly correlated with learning and memory. Specifically, the murine hippocampal dentate gyrus (DG) region was previously shown to be involved in short-term memory (Kesner, 2007). By increasing cGMP levels in the mouse hippocampal DG neurons using optical fiber-coupled LEDs for local photoactivation, our preliminary data showed cGMP-dependent suppression of short-term novel object recognition memory formation. In contrast, we found involvement of cAMP for the formation of novel object recognition memory by decreasing endogenous cAMP levels. These results suggest bidirectional regulation of short-term object recognition memory by cAMP and cGMP.

To directly determine the bidirectional effect of cAMP and cGMP in the same mice, I co-expressed PAC and RhGC in the mouse brain and also designed custom light delivery equipment for their dual photoactivation. By establishing dual photoactivation of PAC and RhGC in the mouse brain during the novel object recognition mouse behaviour assay, I examined bidirectional cAMP and cGMP effects in DG-dependent short-term object recognition memory.

4.3.1 Dual PAC and RhGC expression in freely behaving mice

To manipulate both cAMP and cGMP by light in freely behaving mice, I prepared mice which co-express RFP-PAC and RhGC-GFP fusion proteins in the DG cells of the hippocampus. I first utilized a RFP-PAC transgenic mouse line for PAC expression the hippocampal DG. The mouseline was designed to express RFP-PAC in excitatory neurons (Figure 13A). Interestingly, we found a mouseline where expression of RFP-PAC was robust in the hippocampal DG, which
was visualized by confocal RFP fluorescence imaging of acute mouse hippocampal slices (Figure 13B).

To co-express RFP-PAC with RhGC-GFP, I designed and tested a recombinant adeno-associated vector in which the CaMKIIα promoter drives expression of RhGC-GFP (Figure 14A). Virus particles (serotype 9) were microinjected into the bilateral DG of RFP-PAC transgenic mice using the stereotaxic coordinates A/P: -2.0 mm, M/L: ±1.3 or 1.6 mm and D/V: -2.0 mm (Figure 14B). After 3 weeks, confocal imaging of the virus-injected mouse brain slices revealed the fused RhGC-GFP in the target hippocampal regions (Figure 15).

Figure 13. Validation of the RFP-PAC transgenic mouseline.

(A) Schematic representation of the transgene construct (top) and genotyping of PAC transgenic mice on agarose gel (bottom). 859 bp band represents the transgene construct. TG (RFP-PAC transgenic mouse), WT (wild type mouse). (B) Distribution of the transgenic protein RFP-PAC in the transverse hippocampal slice. RFP fluorescence was imaged by a confocal microscope and merged with the transparent light image. Top: WT, bottom: TG. Figure adapted from Luyben et al. (unpublished).
Figure 14. Dual enzyme expression strategy for photoactivation of PAC and RhGC in vivo.

(A) Schematic representation of the recombinant AAV vector (serotype 9) encoding RhGC-GFP following the CaMKIIα promoter. (B) Schematic representation of the combined transgenic and viral strategy for dual expression of RhGC and PAC in the hippocampal DG. RFP-PAC transgenic mice exhibit strong RFP fluorescence signal in the DG granule cells (left). rAAV encoding RhGC-GFP following the CaMKIIα promoter (2 µL) was microinjected into the mouse brain using stereotaxic coordinates to target the bilateral DG neurons. Three weeks following surgery, GFP fluorescence signal can be detected in the DG (right).
4.3.2 Design of an implantable LED system for dual colour optogenetics in vivo

To enable dual photoactivation of RhGC and PAC at the target hippocampal DG region in freely behaving mice, I custom designed a small bilateral LED device with dual optic fibers (Figure 16A). Each bilateral optic fiber can emit either UV (385 nm) or yellow (590 nm) LED light. The LED is controlled by a system remote that communicates with a small (1 g) wireless receiver that physically attaches to the LED.

Using the dual expression mice described above, the optic fiber-LED system was implanted into the brain using stereotaxic coordinates to target photoactivation to the DG (A/P: -2.0 mm, M/L: ±1.3 or 1.6 mm and D/V: -2.0 mm). The resulting mice co-express PAC and RhGC in the DG cells of the hippocampus and bear a fixed LED system to control their local photoactivation (Figure 16B,C). This strategy provides both temporal and spatial control of optogenetic enzyme activity for manipulating cAMP and cGMP in freely behaving mice.

**Figure 15. Viral RhGC-GFP expression in the RFP-PAC transgenic mouse hippocampus.**

Distribution of rAAV RhGC-GFP in hippocampal slices following virus microinjection in transgenic (RFP-PAC) mouse. The GFP and RFP fluorescence was imaged by a confocal microscope and merged with the transparent image. Images were taken 4.5 weeks after virus microinjection.
Figure 16. Schematic design of custom LED system for optogenetic stimulation of freely behaving mice.

(A) RFP-PAC+ mice with viral RhGC-GFP expression were equipped with a dual fiber optic and bilateral LED system. The optical fibers were inserted into the mouse brain and targeted to the DG cells of the hippocampus. The LED can attach to a small receiver for wireless control through a remote stimulator. The optical fibers can emit either UV (385 nm) or yellow (590 nm) light for optogenetic stimulation of PAC and RhGC, respectively. (B) Photograph of a subject mouse with the wireless receiver attached to the surgically implanted LED assembly. (C) Photographs of implantable LED attached to wireless receiver with UV (top) and yellow (bottom) light emission.

4.3.3 Role of cAMP and cGMP in short-term recognition memory

To determine the effect of cAMP and cGMP on short-term memory formation, I used an object recognition memory test to assay the dual expression mice described previously. The novel object recognition test (NORT) is a memory test, which unlike other mouse behaviour tests, requires no external (e.g. electric shock) or internal (e.g. hunger/thirst) forces (Antunes & Biala, 2012). Rather, it is based upon the animal’s innate curiosity of its surrounding environment. Specifically, the NORT and its variations evaluate the animal’s preference for novelty. Preference for a novel object indicates that a memory exists of the familiar object. Interruption of memory (e.g. brain lesion, pharmacological treatment) can result in reduced novelty preference while enhancement of memory (e.g. genetic manipulation, pharmacological treatment) can lead to increased novelty preference.
To test the roles of cAMP and cGMP in short-term memory formation, we designed a NORT paradigm to assay short-term object recognition memory (Figure 17). Our NORT paradigm consists of two phases, a habituation phase and a test phase. During the habituation phase, mice were given 5 minutes to familiarize with two identical objects placed in an open field arena. At the end of habituation, mice were returned to their home cages for a test delay period of two minutes. During the test delay, one of the identical objects in each arena was switched with a novel object. During the test phase, mice were given another 5 minutes to explore the familiar object and the novel object. The total trial time did not exceed 15 minutes. This was to ensure that short-term memory formation, which does not last after 24 hours, would be evaluated.

To study the effect of cAMP and cGMP in short-term memory formation, photoactivation of PAC or RhGC lasted for 5 minutes during the habituation phase (Figure 17). While the mice were habituating with two identical objects (i.e. forming a memory), they were stimulated either with UV light to increase cAMP or yellow light to increase cGMP. In the control condition, mice completed the habituation phase without any light stimulation. To examine the effect of cAMP and cGMP on object recognition memory formation, the animals’ exploration was recorded and novelty preference was calculated.

First, I tested novel object recognition without photoactivation to test if the enzyme expression, virus injection or implant surgery had any effect on the animals. In the absence of photoactivation (Light OFF control), mice displayed a strong recognition memory as evidenced by an increase in time spent exploring the novel object compared to the familiar object (71% novel object preference) (Figure 18A). This was similar to the performance of intact wildtype mice from our preliminary study (66% novel object preference).

In a different trial, the same mice were photostimulated with 385 nm UV light (to increase cAMP) during the habituation phase and also spent more time exploring the novel object compared to the familiar object during the test phase (67% novel object preference) (Figure 18B). This was not significantly different from the control (Light OFF) conditions (p > 0.05) and is similar to our previous result using blue light to photoactivate PAC in the RFP-PAC transgenic mouse.

In contrast, when mice were stimulated with 590 nm yellow light (to increase cGMP) during habituation, they no longer showed significant preference for the novel object (49% novel object preference).
preference) indicating a strong suppression effect of cGMP (Figure 18C). This result was significantly different from when the same mice were stimulated with UV light to increase cAMP in the DG (Figure 18D) and demonstrates the effect of RhGC photoactivation in the mouse brain.

**The Novel Object Recognition Test**

![Diagram of the Novel Object Recognition Test](image)

Figure 17. The Novel Object Recognition Test for short-term memory.

RFP-PAC+ transgenic mice with viral RhGC-GFP expression were equipped with the bilateral LED system and placed in an arena with two identical objects. In Light ON conditions, the LED was continuously turned on to photoactive either PAC with UV light (cAMP) or RhGC with yellow light (cGMP). In the Light OFF condition, no light was applied. Mice were given five minutes to explore the objects after which they were returned to their home cage. One of the identical objects was replaced with a novel object and mice were placed back into the arena for another five minutes. For all conditions, no light was applied throughout the novel object test phase. The time each mouse spent exploring the objects was recorded during the experiment using a video camera.
Figure 18. Behavioural assay of short-term memory by the Novel Object Recognition Test.

(A) Object preference during the novel object test phase in the absence of enzyme photoactivation during the habituation phase (n=10 trials/5 mice). Mice in the Light OFF condition show significant preference for the novel object compared to the familiar object indicating normal short-term memory formation. (B) Object preference during novel object test phase after UV light stimulation during habituation phase (cAMP) (n=10 trials/5 mice). Mice show significant preference for the novel object compared to the familiar object. Novel object preference in the UV Light ON condition is not significantly different from the Light OFF condition. (C) Object preference during novel object test phase after yellow light stimulation during habituation phase (cGMP) (n=11 trials/5 mice). Mice show no significant preference for
the novel object indicating a decrease in memory formation. (D) Schematic summary of novel object test results (left) and normalized novel object preference (relative to Light OFF) in the UV Light ON (cAMP) and yellow Light ON (cGMP) conditions (right). *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s. denotes not significant, p > 0.05 by unpaired t-test. All data are mean ± SEM.

4.4 Summary of Chapter 4

By utilizing the multicolour optogenetic approaches for controlling cAMP and cGMP established in Chapter 3, here, I demonstrate two in vivo applications: 1) dual two-photon photoactivation at target single synapses for studying the role of cAMP/cGMP in structural synaptic plasticity and 2) dual photoactivation in mouse hippocampal DG for elucidating the involvement of cAMP/cGMP in short-term object recognition memory.

In the first study, I established a two-photon optogenetic excitation protocol for dual stimulation of wavelength-separated enzymes PAC and RhGC at the level of single dendritic spines. Our preliminary results showed pharmacological cAMP-dependent enhancement of sLTP and its inhibition by optogenetic activation of cGMP synthesis, suggesting structural regulation of dendritic spines by intracellular cAMP and cGMP. By photoactivating PAC and RhGC using different two-photon excitation wavelengths at the same target dendritic spines, I directly demonstrate the rapid and bidirectional effects of postsynaptic cAMP and cGMP in sLTP for the first time. Since the dual two-photon optogenetic approach enables us to control the location and timing of cAMP and cGMP manipulation at single synapses in deep brain tissues, this will advance the study of how dynamic cAMP/cGMP signalling regulates synapse structure and function.

In the second study, I optimized my multicolour optogenetic strategy for investigating the functions of cAMP and cGMP in memory formation in freely behaving mice. Using intact animals that exhibit dual expression of PAC and RhGC in the hippocampal DG, I established the photoactivation of cGMP production by yellow light, via an implantable wireless LED device, and confirmed the suppression of short-term object recognition memory formation. Photoactivation of cAMP production however, had no effect on object recognition memory when tested in the same mice. This is similar to our previous result using blue light photoactivation of PAC, suggesting that endogenous cAMP levels are sufficient to saturate novel object memory.
Although PAC photoactivation by UV light still remains to be validated using other recognition memory tests where optogenetic cAMP synthesis has been shown to have an effect (i.e. the displaced object recognition test), this dual photoactivation approach will be a valuable tool for studies of spatiotemporal cAMP/cGMP signalling in freely moving animals.
Chapter 5 Discussion

5.1 Summary of the project

Through this study, I demonstrated novel approaches for studying intracellular cAMP/cGMP signalling in the nervous system by establishing a multicolour optogenetic method for investigating cAMP/cGMP at both the synaptic and mouse behavioural levels.

The application of multicolour optogenetics has only been described using variants of the light-dependent channelrhodopsin and halorhodopsin (Zhang et al., 2007; Klapoetke et al., 2014; Schild & Glauser, 2015). Here, I established a combination of photoactivatable cyclases, the blue light-sensitive PAC (Ryu et al., 2010; Stierl et al., 2011) and green light-sensitive RhGC (Scheib et al., 2015; Gao et al., 2015), and excitatory wavelengths of one- and two-photon light that can separate their enzymatic photoactivities. This enabled independent and spatiotemporally precise manipulation of cAMP and cGMP signalling in the same neurons for the first time.

At the level of the synapse, dual two-photon PAC and RhGC photoactivation revealed rapid modulation of structural potentiation of postsynaptic dendritic spines, indicating cAMP/cGMP-mediated bidirectional regulation of synaptic structure. Furthermore, in the DG neurons of hippocampus, local photoactivation of PAC and RhGC demonstrated different effects on object recognition memory in freely behaving mice. These findings highlight the significance of cAMP and cGMP signalling interactions in the nervous system, and demonstrate the utility of multicolour optogenetics with photoactivatable cyclases for investigating cAMP/cGMP functions at single synapses and freely behaving animals.

5.2 Significance

5.2.1 Technological advantages

The multicolour optogenetics technique I established using excitation wavelength-shifted enzymes PAC and RhGC provides independent control of cAMP and cGMP signalling within the same neuronal samples (Chapter 3). With the protocols I established for both one- and two-photon photoactivation of PAC and RhGC, this technique can be applied to study cAMP and
cGMP signalling interactions at multiple levels of the nervous system with the advantages of high regional and temporal precision.

In comparison to the approaches using pharmacological agents (e.g. PDE inhibitors) or genetic manipulations (e.g. AC or GC knockout animals) for perturbing cAMP/cGMP levels in the nervous system, optogenetics for controlling cAMP/cGMP can be targeted to specific cellular or tissue regions by local application of light. By establishing both one- and two-photon photoactivation of PAC and RhGC, I show here that optogenetic control of cAMP and cGMP synthesis can be applied at the level of the single synapse (>1 µm diameter) and also at the mouse dentate gyrus (~1 mm diameter) enabling investigation of structural synaptic plasticity and memory, respectively. As well, expression of PAC and RhGC by cell-specific promoters can also target photoactivation to cell types of interest and diminish unwanted side effects under broad illumination conditions.

This method enabled us to directly examine and control the effects of cAMP and cGMP in the same biological targets, such as synapses, neurons and animals, providing a precise assay for their functions without multiple different groups for comparison (e.g. wildtype vs. drug treatment or knockout). This also allowed for spatiotemporal and interactive studies of cAMP and cGMP function through selection of the location and timing of PAC and RhGC photoactivation. Therefore, these improvements will help advance our understanding of dynamic cAMP/cGMP interactions in synaptic and brain level functions.

5.2.2 Neurobiological relevance

Using multicolour optogenetics to target PAC and RhGC photoactivation at the synapse, our first study demonstrated bidirectional regulation of synapse structure during structural long term-potentiation (sLTP) by cAMP and cGMP. The role of cAMP and its downstream signalling pathway has been primarily studied during the late phase of LTP (> 30 min) and the related long-term, protein synthesis-dependent memory formation. However, our results demonstrate and validate cAMP (PAC)-mediated enhancement of structural LTP (sLTP) (< 30 min) and its inhibition by cGMP (RhGC) in the same dendritic spines. This indicates a distinct interactive mechanism between postsynaptic cAMP and cGMP signalling for the regulation of synapse structure during the early, protein synthesis-independent phase of LTP (< 30 min). Since there exist many feedback systems for cAMP and cGMP signalling in neurons, their interaction may
be formed by a variety of mechanisms including cGMP-mediated upregulation of cAMP hydrolysis via phosphodiesterase 2.

Our second study demonstrated the contrasting effects of local PAC (cAMP) and RhGC (cGMP) photoactivation in the hippocampal DG during the formation of novel object recognition memory. Similar to our preliminary study, photoactivation of cAMP synthesis had no detectable effect on short-term novel object memory while photoactivation of cGMP synthesis strongly suppressed it. This indicates that rapid cAMP and cGMP signalling are involved in different mechanisms of object memory formation and further photoactivation paradigms can inform us on details of their location- and timing-specific signalling actions.

In the nervous system, the molecular and cellular consequences of cAMP/cGMP signalling interactions remain largely uncharacterized during structural plasticity and learning and memory. However, pharmacological inhibitors of phosphodiesterases, which degrade cAMP and/or cGMP, have shown clinical potential for attenuating symptoms of many learning and memory disorders, such as Alzheimer’s disease, highlighting the importance of intracellular cAMP and cGMP in the mammalian brain (Reneerkens et al., 2009; Heckman et al., 2015). Since multicolour optogenetics using PAC and RhGC can manipulate cAMP/cGMP levels in targeted brain regions, the technique can serve as a valuable tool for elucidating cAMP/cGMP signalling pathways for strategic development of new therapeutics and methods for brain disease research.

5.2.3 Non-neuronal applications

cAMP/cGMP signalling crosstalk has also been described in non-neuronal mechanisms such as cardiac myocyte contractility (Zaccolo & Movsesian, 2007; Stangherlin & Zaccolo, 2012) and intestinal epithelia homeostasis (Arshad et al., 2013). Thus, multicolour optogenetics using PAC and RhGC will serve as a useful tool for further investigation of cAMP and cGMP in non-neuronal research fields. For expression of PAC and RhGC in these different cell types, minimally invasive viral delivery can be used and has been successfully demonstrated in the mouse heart using AAV9 vectors carrying ChR2 (Vogt et al. 2015). Similarly, this can be combined with the development of transgenic animals, which have also been developed for ChR2 expression in the mouse heart and skeletal muscle (Bruegmann et al., 2010; Bruegmann et al., 2015). The combination of transgenic and viral transduction techniques can likely be adapted for dual expression of photoactivatable cyclases and investigation of cAMP and cGMP in these
areas. In fact, viral delivery of PAC has already been demonstrated using adenovirus in mouse pancreatic β-cells to study cAMP-mediated insulin secretion in vitro (Zhang & Tzanakakis, 2017).

5.3 Limitations

A limitation of our study using PAC and RhGC for dual activation of cAMP and cGMP synthesis is that the wavelengths of excitation light used to separate their activity are not in the enzymes’ peak excitation range. For one-photon excitation, UV light was used to activate blue light-sensitive PAC and yellow light to activate green light-sensitive RhGC. For RhGC, excitation with yellow light decreased cGMP synthesis by 4-fold. However for PAC, cAMP synthesis by UV light was similar to that by blue light. In spite of these differences, the cGMP produced by RhGC upon yellow light excitation was sufficient to produce a strong inhibitory effect on mouse learning and memory in vivo. This result was similar to our previous study using blue light excitation to photoactivate the guanylyl cyclase BlgC, thus validating the effect of cGMP on short-term object recognition memory. Meanwhile, for PAC, the UV light used for photoactivation in vivo revealed the same result as our previous study, which used blue light excitation instead. Although high power UV light can cause damage to brain tissues, the novel object memory assayed in animals stimulated with UV light was similar to that of mice assayed with lower frequency blue light and also no light in the control conditions.

A similar limitation is evident in our two-photon study, which utilized two-photon excitation wavelengths 800 nm and 1,100 nm to photoactive PAC and RhGC, respectively, in dendritic spines. Although 800 nm was shown to strongly activate PAC in vitro, prolonged activation of PAC using 800 nm in vivo was damaging to the dendrites and dendritic spines. This limited photostimulation of PAC to 1 mW of power and 10 seconds in duration. However, this protocol was sufficient for yielding cAMP-mediated enlargement of dendritic spines during LTP, and also suppression by cGMP, which we have previously observed.

Finally, co-expression of PAC and RhGC in DG neurons was achieved by a combination of virus and transgenic strategies. Fluorescence imaging of the hippocampus of transgenic RFP-PAC mice revealed strong expression of RFP-PAC in the DG. In transgenic mice injected with AAV RhGC-GFP, fluorescence imaging revealed expression of RhGC-GFP not only in the DG, but other hippocampal regions such as the CA1 and CA3. As the CA1 and CA3 are dorsal to the DG,
this expression pattern is likely due to a step in the microinjection protocol where virus particles flow dorsally through the brain tissue as the needle is raised from the skull. Although photoactivation is targeted only to the hippocampal DG by LED-coupled optical fibers, to optimize expression of RhGC, we are currently working to develop a knock-in mouseline where RhGC-GFP expression is localized to the DG by a Cre-lox recombination system.

5.4 Future directions

It is well established that cAMP/cGMP signaling crosstalk is important for mediating diverse functions in a variety of mammalian systems (Zaccolo & Movsesian 2007; Kobayashi et al., 2013). Dual colour optogenetics using PAC and RhGC will enable us to further investigate how cAMP/cGMP functions and interactions play a role in different neuronal processes, such as synaptic plasticity and memory.

For example, we previously showed that postsynaptic cAMP synthesis facilitates structural potentiation at single spines and also nearby spines during LTP. Furthermore, we showed here that cGMP, which is produced in dendritic pines during LTD, prevents cAMP-mediated structural potentiation during LTP. These results suggest a novel role for cAMP/cGMP signalling interactions in regulating the plasticity and interactions between multiple synapses, adding a neural circuit level function to their crosstalk. To test this hypothesis, we plan to use dual colour optogenetics to synthesize cAMP and cGMP in neighbouring dendritic spines of the same neuron. This would allow us to assess how synthesis of cAMP/cGMP in one spine can affect the sLTP of a neighbouring spine. This study would reveal how individual synapses interpret spatiotemporal patterns of information arriving from neighbouring synapses.

Multicolour optogenetics for cAMP/cGMP can also be applied to understand how cAMP/cGMP interactions mediate learning memory. Our findings from this study reveal that cAMP and cGMP have different effects on short-term object recognition memory. Photoactivation of PAC and RhGC in the DG of freely behaving mice showed cGMP-, but not cAMP-, mediated suppression of object memory formation in the novel object recognition test. Our previous results however, showed that increasing cAMP enhanced memory performance in the displaced object recognition test, and decreasing endogenous cAMP, by PhPDE4, impaired memory performance in the novel object recognition test. These results suggest that cAMP is a positive modulator of DG-specific object recognition memory. An interesting experiment would then be to use PAC and RhGC to
determine if cAMP can rescue cGMP-mediated suppression of short-term memory in the novel object recognition test. Using a similar approach, we could also investigate whether spatiotemporal cAMP/cGMP has any effect on animal models of learning and memory disorders. As PDEs are common drug targets in the treatment of cognitive disorders, such as Alzheimer’s disease, characterizing the spatiotemporal roles of cAMP and cGMP in the brain could help design more targeted pharmacological therapies.

5.5 Conclusion

To elucidate the dynamic and complementary cAMP and cGMP signalling functions in synaptic plasticity and memory, I developed a novel multicolour optogenetic method with optimized photoactivatable cyclases (PAC and RhGC) and designed custom light delivery equipment for their photoactivation in vivo. In pyramidal CA1 neurons of the rat hippocampus, I demonstrated dual two-photon photoactivation at target synapses and revealed cAMP/cGMP-mediated bidirectional regulation of dendritic spine structure. Furthermore, in mice that co-express PAC and RhGC, local activation of cAMP and cGMP synthesis in the hippocampal DG revealed different effects on short-term object recognition memory formation. Taken together, these one- and two-photon photoactivation methods represent novel approaches for studying interactive cAMP and cGMP functions from the single synapse to animal behavior levels. Further studies can expand and adapt this technique to investigate the diverse functions of cAMP and cGMP signalling in both neuronal and non-neuronal research fields.
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