The Role of PAK Signaling in Synaptic Transmission and Plasticity and Social Memory in Mice

by

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

Social interactions are essential to our mental health and their deficits are hallmark characteristics in numerous brain disorders. Various subregions within the medial temporal lobe have been implicated in social memory, however, the underlying molecular and cellular mechanisms that tune these particular neural circuits in memory processing remain unclear. PAKs (p21-activated kinases) are a family of serine/threonine protein kinases that are target enzymes of the Rho family small GTPases and central regulators of both the actin cytoskeleton and neuronal morphology. PAK 1 and 3 are involved in the regulation of the structural and functional properties of the synapse, including dendritic spine morphology, synaptic transmission and plasticity, and learning and memory. Recent human and animal studies have revealed that perturbations in PAKs are implicated in several neurological and mental disorders including autism spectrum disorders (ASD) and intellectual disability (ID). These disorders are often characterized by social impairments, yet the cellular correlates within the neural circuits that subserve PAK function in social behaviour remains unknown. In this thesis, I explore five objectives which elucidates the role of PAK signaling in the entorhinal cortical perforant projections to the dentate gyrus (EC-DG) in social memory retrieval. First, I characterize the
biochemical and cellular properties of a novel transgenic mouse model where the expression of a dominant negative mutation of PAK3 can be temporally and spatially controlled. Second, I demonstrate that the reversible disruption of PAK signaling impairs social recognition memory without affecting other behavioural responses. Third, using electrophysiological recording and imaging techniques, I show that presynaptic neurotransmitter release is specifically impaired at the EC-DG synapse in the mutant mice. Fourth, I use biochemical analyses and show that cofilin and the actin cytoskeleton are significantly altered in the mutant mice. Lastly, I demonstrate that acute optogenetic manipulation of the EC-DG terminals is sufficient to rescue the social memory deficits in the PAK3 transgenic mouse model. Together, these results identify a novel signaling pathway mediated by PAKs within the EC-DG circuit that governs social memory retrieval. Furthermore, these findings suggest potential circuit and molecular mechanisms important for the treatment of social disorders.
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List of Abbreviations

ACSF........................Artificial cerebrospinal fluid

AD............................Alzheimer’s disease

ADHD........................Attention-deficit hyperactivity disorder

AID...........................Autoinhibitory domain

AMPA.........................2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid

APP............................Amyloid precursor protein

Arc.........................Activity-regulated cytoskeleton-associated protein

ArchT........................Archaerhodopsin

ASD..........................Autism spectrum disorders

BLA............................Basolatera amygdala

CaMKII.......................Calcium/calmodulin-dependent protein kinase II

CDC42........................Cell division control protein 42 homolog

CDP..........................Cut like homeobox 1 (CUX)

ChR2..........................Channelrhodopsin

CRE............................cAMP response elements

CREB..........................cAMP response element-binding protein

CRIB..........................Cdc42/Rac interaction-binding

DAG..........................Diacylglycerol

DG............................Dentate gyrus
DOCK………………………Dedicator of cytokinesis

DOX…………………………Doxycycline

dTg………………………Double transgenic

EC…………………………Entorhinal cortex

EPM………………………Elevated plus maze

EPSC………………………Excitatory postsynaptic current

EPSP………………………Excitatory postsynaptic potential

ERK…………………………Extracellular signal-related kinase

EYFP………………………Enhanced yellow fluorescent protein

FLNA……………………FIlamin

FMR1……………………Fragile X mental retardation 1

FMRP……………………Fragile-X mental retardation protein

FRET……………………Fluorescence resonance energy transfer

FXS………………………Fragile X syndrome

GAD……………………Glutamate decarboxylase isoform

GAP…………………………Guanine nucleotide activating protein

GDI…………………………Guanine dissociation inhibitor

GEF…………………………Guanine nucleotide exchange factor

GFAP……………………Glial fibrillary acidic protein

GFP…………………………Green fluorescent protein
GIT1.................................G-protein coupled receptor kinase interacting target

HD.................................Huntington’s disease

HFS.................................High-frequency stimulation

HRG.................................Heregulin

HTT.................................Huntingtin gene

ID.................................Intellectual disability

LIMK.................................LIM-domain containing kinases

LPP.................................Lateral perforant pathway

LTD.................................Long-term depression

LTP.................................Long-term potentiation

MAP-kinase........................Mitogen-activated protein kinase

mEPSC...............................Miniature excitatory postsynaptic current

MLC.................................Myosin light chain

MLCK.................................Myosin light chain kinase

MPP.................................Medial perforant pathway

NCK.................................Nck adaptor proteins

Nef.................................Neurofibromatosis 2 tumour suppressor gene

Nfl.................................Neurofibromatosis type 1

NMDA.................................N-Methyl-D-aspartic acid

OFT.................................Open field test
PAK.................................p21-activated kinases

PBD...............................PAK binding domain

PCR..................................Polymerase chain reaction

PI3K...............................Phosphatidylinositol 3-kinase

PIX..................................PAK-interacting exchange

PKA..................................Protein kinase A

PKC..................................Protein kinase C

PLD1...............................Phospholipase D1

PP.....................................Perforant pathway

PPD.................................Paired pulse depression

PPF..................................Paired pulse facilitation

PSD..................................Postsynaptic density

Rac1.................................Ras-related C3 botulinum toxin substrate 1

Rho..................................Ras homolog gene family

SNARE..............................Soluble NSF attachment receptor protein

SSH..................................Slingshot phosphatase

tetO.................................Tetracycline operator sequence

TRE.................................Tetracycline-responsive element

tTA.................................Tetracycline transactivator protein

WBS.................................Williams-Beuren syndrome
1 Introduction

Data Attribution- Certain ideas presented in this chapter were adapted from the following original publications:


1.1 Social Memory

1.1.1 Social Memory Processes

Cognition refers to the accumulation of knowledge that is acquired through our senses and experiences. It is classified into phases that involve; the integration of new information derived from our sensory modalities, the interpretation of the information into our learning, and the retention of the information into our knowledge (Maniadakis and Trahanias, 2014). One of the most complex cognitive and affective systems of human memory lies within the social domain. Social cognition is a multifaceted process that may involve working memory; to store and process the perception of faces and emotions, the interpretation of social cues, and the ability to give an appropriate response (Frith and Frith, 2007). The formation of social relationships via social interactions are essential for our physical and mental health. Thus, social memory is an extremely dynamic process that is often unpredictable, requires the ability of interpersonal inference, and constant adaptability (Lieberman, 2007). Specifically, it is a cognitive process that involves the encoding, storage, and retrieval of socially salient information. The detailed cellular and molecular mechanisms underlying each of these phases of memory will be discussed in section 1.1.3.

1.1.1.1 Memory Encoding

The first phase of memory formation involves the encoding of new, unencountered information. The social experiences derived from our sensory modalities first enters into the memory system, where the individual components from the interactions are extracted, processed, and converted in to a form that
the neural system can interpret. Memory encoding is part of the memory acquisition process that is critical for the incoming information to be processed prior to being stored into our memory. Thus, sensory information that is not encoded is not incorporated into our memories. The encoding process itself has a constructive nature, where different elements from the new experience are linked to existing knowledge in order to generate meaningful connections. Within the social domain, memory encoding involves a combination of complex social judgements and perceptions that range from face processing to interpersonal sensitivity, which guide social behaviour (Mitchell et al., 2004).

The encoding process can occur through two main mediums; automatic and effortful processing. Automatic or implicit processing occurs without conscious awareness, whereas effortful or explicit processing requires a conscious capacity in processing a particular memory (Turk-Browne et al., 2006). Examples of automatic processing include our ability to remember who we encountered yesterday whereas, effortful processing would be remembering a friend’s birthday. These types of processing allow us to gather cues from our senses, either through encoding the information visually, acoustically, or semantically, and subsequently converts them into a construct that can be stored. Visual encoding refers to the perceptions that are presented in the form of a picture, whereas acoustic encoding involves sounds, and semantic encoding alludes to information that are associated with a particular meaning.

Pioneering studies have identified the hippocampus and the medial temporal lobe to be critical in the acquisition and retention of explicit or declarative memory (Penfield and Milner, 1958; Scoville and Milner, 1957; Warrington and Weiskrantz, 1968). Whereas, implicit memory which does not require conscious awareness, relies mostly on the other brain systems including the cerebellum, striatum, and amygdala (LeDoux, 1996). Studies have shown that the processes of encoding for both explicit and implicit memory requires full attention (Mulligan, 1998). Since the hippocampus receives multimodal sensory information, the explicit encoding of the new experiences requires the integration of multiple brain structures. During the encoding process, the coordinated activation of different groups of neurons within these brain structures contribute to the firing and strengthening of the particular region. Ultimately, the patterns of activation within these neuron ensembles during memory encoding, play a
role in the association and persistence of the memories. In recent years, techniques including optogenetics have been developed to acutely control the activity of genetically defined populations of neurons. By combining optogenetics with advanced temporal gene expression control systems, certain populations of neurons can be tagged during a specific event and subsequently reactivated for further memory testing. For example, studies have used the tetracycline-controlled transcriptional activation system to selectively express various opsin, such as channelrhodopsin (a light-gated ion channel) or halorhodopsin (a light-controlled chloride pump), in neurons of the dentate gyrus of the hippocampus that were active during the encoding phase of contextual fear conditioning (Liu et al., 2012b). Since the viral opsin is expressed in dentate granule neurons that were active during the engram formation, the engram cells that contain the associated memory can be reactivated and tested in an alternative context (these experiments are discussed in detail in section 1.1.3.1). Ultimately, these findings identified a population of hippocampal dentate granule cells to be critical for the encoding of fear memory associated with a conditioned context. Thus, this approach allows for the targeting of isolated ensembles in different areas in the brain and the understanding of the mechanisms that govern the encoding of particular types of memories.

1.1.1.2 Memory Storage

Once the information is encoded into the neural system, the new memory is stored in a specific spatial and temporal manner. The memory can be classified into different forms including; sensory memory, short-term memory, or long-term memory (Cowan, 2008). Within these three types of memory stores, the information is retained for various durations of time. Sensory memory stores information from our senses for brief durations that range from a fraction of a second to a few seconds. The overall capacity for sensory memory storage is immense, but the sensory information retained is unprocessed and maintained temporarily. Short-term memory stores can selectively access the information within our sensory memory stores. It can hold the information for a period of approximately 30-45 seconds. However, short-term memory stores have a limited capacity. Lastly, short-term memory can be converted into long-term memory through functional and structural mechanisms (discussed in detail in section 1.1.3.2). Since long-term memory has an unlimited storage capacity, encoded information can be stored for a lifetime.
The research on the mechanisms underlying memory storage has been primarily focused on the synapse. A single neuron can form thousands of synapses, a highly specialized intercellular structure that permits electrical or chemical signals to be sent to another neuron. Functional and structural changes at the synapse, referred to as synaptic plasticity, are believed to be the basic mechanism of memory storage. This is corroborated by studies in *Aplysia* have shown that synapses are modified by learning experience and that these modifications within the neurons underlie memory storage (Bailey and Chen, 1983). Additionally, in rodents, the synaptic responses following learning within the hippocampus have been found to display long lasting increases in synaptic transmission and plasticity (Bliss and Lomo, 1973; Hebb, 1949). Other studies have shown that a functional enhancement in the efficacy of synaptic transmission alone is insufficient to store a complex memory. Specifically, the storage of complex information also requires morphological changes in the dendritic spine, the major postsynaptic site of the synapse (Harris et al., 1992). The correlation between the structural and functional changes at the synapse is evident in recent studies employing a two-photon glutamate uncaging technique (Pettit et al., 1997), which demonstrated that the repetitive uncaging of glutamate induces a sustained dendritic spine enlargement within the targeted spine, but not adjacent neighbouring spines (Matsuzaki et al., 2001). Thus, the size and stability of dendritic spines often correspond to the structural basis of memory storage. The details of the synapse, spine, and synaptic plasticity will be discussed in greater detail in section 1.1.5.

### 1.1.1.3 Memory Retrieval

Once memories are encoded and stored within the appropriate neural circuits, the recall process is thought to involve the subsequent re-organization of the same information from the past. The assessing of the stored knowledge or experience is a critical process involving the selection, reactivation, and reconstruction of the memories, so that they can be used in the future (Tonegawa et al., 2015). There are two methods to retrieve stored memories; recognition and recall (Rugg et al., 1998). Recognition is a process that compares the new incoming information with the existing stored information. The association is initiated as a response to the presentation of a particular sensory cue that elicits a memory from the past and triggers the retrieval process. Recall is the recollection of information without a
presence of a cue or stimulus. It involves remembering a fact, event, or other information that is not present during the recall process.

The reactivation a particular memory store upon its retrieval can result in the transient destabilization of the trace that may lead to a change in its representation. Specifically, memory retrieval elicits a pattern of neural activity that is similar to the initial response of the particular event. Therefore, studies have demonstrated the same neural cells are directly involved in the encoding and retrieval of a memory trace (Paulsen and Moser, 1998; Ramirez et al., 2013a). However, studies have demonstrated that consolidation can occur actively through a separate mechanism during the retrieval of the information at a later stage (Daumas et al., 2005; Nader et al., 2000). Specifically, during retrieval, the original memory is in a labile state that is prone to the effects of cellular and molecular processes which change the original consolidation (Sara, 2000). For example, one group found the importance of the hippocampus in the consolidation of contextual fear memory but the inhibition of this region was not required for the retrieval process (Daumas et al., 2005). Various imaging studies have implicated the medial temporal lobe in the retrieval of contextually rich information during the early stages of long-term explicit memory (Kirwan and Stark, 2004; Smith and Squire, 2009). Furthermore, others have shown that the prefrontal cortex interacts with the medial temporal lobe during the retrieval process, by allowing for the top-down selection of relevant information (Eichenbaum, 2000).

As discussed in previous sections, optogenetics combined with techniques in advanced temporal gene expression have been used to label neuronal ensembles following activity within a particular time frame using an immediate early gene marker. The labelled populations can be subsequently activated to mimic engram retrieval. Studies have employed a similar technique where neurons that were active within the hippocampus (specifically in the dentate gyrus, CA1, and CA3 regions) during a contextual fear conditioning paradigm were tagged with a long-lasting fluorescent protein, H2B-GFP, and a light-driven outward proton pump, archaerhodopsin (ArchT) (Tanaka et al., 2014). During the retrieval process, labelled neurons in either the dentate gyrus, CA1, or CA3 were selectively inactivated, which led to impairments in contextual fear memory recall. Thus, the reactivation of the entire ensemble of hippocampal neurons that were active during memory formation is critical for the retrieval of the
memory. These findings corroborate the findings that the retrieval process involves the re-accessing and reconstruction of the patterns of brain activity produced during initial learning. However, the precise brain regions and their corresponding circuits that facilitate social memory encoding, storage, and retrieval are still elusive. Section 1.1.3 will discuss the mechanisms underlying these processes in more detail.

1.1.2 The Circuitry Involved in Social Memory

1.1.2.1 Circuitry of the Hippocampus

The hippocampus is one of the most well-characterized forebrain structures, known to regulate synaptic plasticity and long-term memory. The classical trisynaptic circuit of the hippocampus consists of the perforant pathway (PP) that relays information from the layer II cells of the entorhinal cortex (EC) to the dentate gyrus (DG), the mossy fibers that connect the DG to the CA3 region, and the Schaffer collaterals that complete the circuit from the CA3 region to the CA1 region (Figure 1.1). The Schaffer collateral pathway joining the CA3 and CA1 regions is the most intensely studied synapse regulating learning and memory. They are known to mediate the rapid encoding of spatial and contextual information, novelty detection, and episodic memories through a NMDA receptor-dependent manner (Kesner, 2013; Nakazawa et al., 2003). Neurotoxic lesions and pharmacological inhibition of the CA3 and CA1 regions produce differential effects on the encoding and retrieval of spatial memories (Dumas, 2005; Ji and Maren, 2008; Lee and Kesner, 2004). Specifically, the hippocampal CA3 region mediates the rapid encoding of spatial information, novelty detection, and episodic memories through a glutamate receptor-dependent manner, whereas the CA1 region is essential for the context-dependent retrieval of memory (Lee and Kesner, 2004; Nakazawa et al., 2003). The mossy fibres that extend from the DG to the CA3 region have been extensively studied as providing the extrinsic input to the hippocampal network and serve as a model for the functional roles of presynaptic kainite receptors in synaptic transmission and plasticity (Contractor et al., 2001; Kamiya and Ozawa, 2000).
Figure 1.1 The Trisynaptic Circuitry of the Hippocampus. This simplified schematic illustrates the trisynaptic circuitry of the hippocampus. The network begins with the PP, which is made up of connections from layer II of the EC which synapse onto the granule cells in the DG. The PP comprises of the lateral and medial PP. The cells of the DG project to the CA3 region of the hippocampus via mossy fibers. Lastly, CA3 neurons synapse onto CA1 neurons in the Schaffer collateral pathway. The layer III cells of the EC can also directly project to the CA1 region of the hippocampus. Ultimately, the signals transmitted to the CA1 neurons project back to the layer V cells in the EC for further processing.
Recent studies have shown that specific regions of the hippocampus, including the CA2 and ventral CA1 regions are critical for different processes governing social memory (Hitti and Siegelbaum, 2014; Okuyama et al., 2016). The CA2 region has been shown to receive bilateral inputs from the CA3 and the EC, however, its function is not well-studied. Hitti and Siegelbam identified that the CA2 hippocampal region, which is interposed between the CA3 and CA1 regions, is a critical hub of long-term sociocognitive memory processing. Specifically, the authors used a genetically targeted method where the CA2 pyramidal neurons were injected with a Cre-dependent adeno-associated virus containing tetanus neurotoxin into Amigo2-Cre mice. This technique silenced the outputs from the CA2 pyramidal neurons, which was confirmed through immunohistochemistry and electrophysiological recordings. The mice demonstrated normal spatial memory, locomotor activity, and anxiety-like behaviour, but significant deficits in social recognition memory. Thus, the CA2 was proposed to be a sociocognitive hub for social memory or more complex memory processing. In a recent study, the ventral CA1 region of the hippocampus was demonstrated to be important for the storage of social memories (Okuyama et al., 2016). Specifically, these authors used optogenetic targeting via injections of ArchT to manipulate ventral hippocampal CA1 neurons and their projections to the nucleus accumbens shell. They show that the inhibition of the ventral CA1 cell bodies or their axon terminals in the nucleus accumbens resulted in a social discrimination deficit. Together, these two studies suggest that the dorsal CA2-ventral CA1-nucleus accumbens shell circuit may act to process, encode, and store salient social information (Hitti and Siegelbaum, 2014; Okuyama et al., 2016). In humans, hippocampal and medial temporal lobe lesions result in multiple memory deficits including impaired social memory. This is supported by initial experiments on face recognition in Henry Molaison (H.M.) and studies that demonstrate recognition memory impairments for a familiar conspecific following lesions; provided that the testing occurs immediately after the encoding phase (Corkin, 2002; Okuyama, 2018). Thus, the human and rodent data suggest that the hippocampus is required for the encoding and retrieval of social memory. However, various upstream cortical targets, including the EC, which send their projections to the hippocampus, have yet to be examined in the realm of social memory.
1.1.2.2 Circuitry of the Entorhinal Cortex

The EC is composed of six distinct layers that differentially send and receive projections to and from various areas of the brain (Figure 1.2). Layer I of the EC lacks neurons and consists of a dense band of transversely oriented fibers. The highly processed sensory inputs to the hippocampus make up layers II and III. Layer II cells project predominantly to the DG and CA3 region of the hippocampus (indirect perforant pathway), whereas the layer III cells directly project to the CA1 region and the subiculum (direct perforant pathway). A deep border next to layer III is referred to as layer IV and consists of a sparse cell layer known as the lamina dissecans. Layer V cells are stratified and is made-up of the reciprocal projections from the CA1 region that return to the EC. The outputs from this layer project to the basolateral amygdala (BLA) and diverse cortical areas, including the posterior parietal cortex, retrosplenial cortex, and the postrhinal cortex. Lastly, the multilaminated layer VI cells project to the thalamus. With respect to the hippocampal circuitry, the least studied are the axons of the PP that originate from layer II of the lateral or medial EC. The PP segregates into the lateral (LPP) and medial (MPP) respectively. Although the PP is the primary input to the hippocampus, the functional and behavioural role of the cortico-hippocampal circuitry on learning and memory remains unclear. Sensory information from the different modalities is first processed in distinct cortical association areas and then relayed through the medial and lateral pathways of the EC to the hippocampus. The medial EC (MEC) is responsible for computing and conveying spatial information, whereas the lateral EC (LEC) is responsible for object related information to the hippocampus (Eichenbaum et al., 2012). Through anatomical and tracing studies, it is known that the deep layers of the EC forms entorhinal subcortical connections to the basal forebrain, claustrum, amygdala, basal ganglia, thalamus, and brainstem (Canto et al., 2008). Figure 1.2 is a summary of the major inputs and outputs from each of the layers in the EC.
**Figure 1.2 Connections of the Entorhinal Cortex.** This schematic was adapted from Canto and Witter, 2011. The EC contains six distinct layers that receive input and send output to various regions. Cortical inputs mostly from layer II comprise of the PP. The output from the hippocampus targets layer V of the EC, which in turn sends projections to subcortical areas, the striatum, the amygdala, and the thalamus.
In humans, the EC plays a crucial role in learning and memory in concert with the hippocampus as part of the brain’s navigational system (Eichenbaum et al., 2007; Stensola et al., 2012). O’Keefe et al., found that rodents have a topographic neural map or place fields consisting of grid cells of the spatial environment in the EC (O’Keefe and Burgess, 2005). Furthermore, neurotoxic lesions to the EC in mice impair contextual fear learning though spatial learning remains unaffected (Burwell et al., 2004). In other studies, ablation of the anterior rhinal cortices which include the entorhinal and perirhinal cortices results in severe impairments in visual recognition memory (Gaffan, 1992). Therefore, as the major input and output structure of converging sensory modalities, the EC is a central signaling hub responsible for relaying diverse “where” (in the MEC) and “what” (in the LEC) memory engrams to downstream subcortical targets for further processing. Specifically, the EC within the medial temporal lobe is the main interface that relays sensory information between the neocortex and the hippocampus (Canto et al., 2008; Eichenbaum and Lipton, 2008). Previous human and primate studies have implicated the medial temporal lobe in social cognitive tasks, such as social recognition and context evaluation (Insel et al., 2015; Olson et al., 2013; Ross and Olson, 2010; Sandi and Haller, 2015), but because the temporal lobe is a large, heterogeneous region with numerous distinct cortical areas (Blaizot et al., 2010; Bonner and Price, 2013), the precise regions directly involved in these behaviours have not been identified. Human neuroimaging studies have revealed that deficits in the EC-DG PP pathway are associated with cognitive impairments related to neurological diseases, including Alzheimer’s disease, Parkinson’s disease, and schizophrenia (Iseki et al., 1998; McDonald et al., 2000; Robinson et al., 2014). Similarly, in rodents, despite extensive studies showing the critical roles of the EC-DG circuit in the discrimination, encoding, and storage of spatial and contextual memories (Basu and Siegelbaum, 2015; Eichenbaum, 2014; Kitamura et al., 2015a; Sasaki et al., 2015), few studies exist to specifically elucidate the role of this circuit in social behaviour. However, early experiments have shown that toxic lesions of the EC impair social recognition memory (Bannerman et al., 2001; Bannerman et al., 2002; Petruslis et al., 2005; Petruslis and Eichenbaum, 2003), but given the caveats of the toxin approach that may cause unintended damage to other areas and have limited control over cellular and subcellular compartments; definitive assessment of the precise cell types mediating social behaviour within the EC-DG network has not been possible. My thesis project will use recent techniques, including inducible transgenic mice and optogenetics, to examine the role of the EC-DG circuit in social learning and memory.
1.1.2.3 Other Neural Circuits

Previous studies have identified other neural targets including the BLA, striatum, anterior cingulate cortex, fusiform area, and medial prefrontal cortex, to be responsible for modulating the formation and consolidation of social recognition memory in mice (Garrido Zinn et al., 2016; Kogan et al., 2000; Molosh et al., 2014; Sanders and Shekhar, 1995). A genetic study has previously shown that the cAMP-responsive element binding protein (CREB) is necessary for the formation of social recognition memory within rodents (Silva et al., 1998). Importantly, significant changes in the targets of CREB, including the immediate early genes, such as c-fos and Arc, were observed in the hippocampus, but also in the medial prefrontal cortex, anterior cingulate cortex, and amygdala (Tanimizu et al., 2017). In the same study, additional network analyses revealed that the different regions demonstrated functional connectivity during the consolidation of social recognition memory in mice. Specifically, the hippocampus was proposed to be an important hub to integrate the information from the other brain networks and to generate social recognition memory, whereas the cingulate cortex and amygdala were responsible for coordinating brain activity during social interaction. Furthermore, various limbic connections such the ventral tegmental area and the nucleus accumbens have also been found to be important in social interactions, specifically associated with same-sex affiliation and pair bonding (Gunaydin et al., 2014). In this study, the authors used fiber photometry to optically record the neural dynamics within this circuit during real-time social behaviour. The activity from the social interactions were found to be mediated by type 1 dopamine receptor signaling. The importance of the nucleus accumbens is corroborated by the recent findings discussed above from Okuyama et al., that show ventral CA1 neurons that project to the nucleus accumbens shell comprise of the neural circuit that store familiarity aspects within social memory (Okuyama et al., 2016). In another study, mice with the deletion of a neurofibromatosis type 1 allele (NF1+/-) demonstrated social memory deficits with impaired lateral amygdala glutamate and GABAergic neurotransmission and synaptic plasticity (Molosh et al., 2014). The authors demonstrated that the genetic deletion of PAK1 or the pharmacological inhibition of PAK1 in NF1+/- mice restored all social deficits and synaptic impairments. Thus, these studies highlight the range of brain areas outside of the hippocampus that may also play a role in the regulation of the different facets of social behaviour. However, the cellular and molecular mechanisms that tune these particular regions in social cognition is not yet understood.
1.1.3 The Cellular and Molecular Mechanisms Underlying Social Memory

1.1.3.1 Memory Encoding Mechanisms

Within neuronal circuits, the exact cellular and molecular processes that encode, store, and retrieve memories within a network remain unclear. With respect to the encoding of a particular memory, one hypothesis postulates that the allocation of neurons dictates the initial conversion of a memory trace or engram into a construct that can be eventually stored (McGaugh, 1972). The activity-dependent recruitment and subsequent refinement of the synaptic connections causes competition between the neurons in becoming chosen to be allocated to the engram. The process of neuronal allocation allows for relevant memories to be stored stably and efficiently, by decreasing interference during the encoding of multiple attributes simultaneously (Won and Silva, 2008). Studies have examined that neurons are recruited to be part of the memory representation, based on the level of the transcription factor, CREB (Lonze and Ginty, 2002; Silva et al., 1998). Neurons that have a higher level of CREB expression were found to be more likely to be allocated to store the memory trace than those with lower levels of CREB (Won and Silva, 2008). In other words, the neurons that express more CREB outcompete the adjacent neurons for the memory trace. For example, studies have virally overexpressed CREB in neurons within the lateral amygdala and CA1 of the hippocampus, regions that play a key role in the regulation of associative-fear and spatial memory (Yu et al., 2017; Zhou et al., 2009). This study demonstrated that neurons within the lateral amygdala that express a relatively higher level of CREB were more likely to be recruited to be part of the fear memory engram. Other studies have used genetic or pharmacological techniques to suppress CREB expression in particular neurons. As a consequence, the infected neurons do not become allocated within the engram and their suppression erases the associated memory (Han et al., 2009; Zhou et al., 2009). The encoding processes, specifically for social memory, are not as well studied as compared to contextual fear memory. However, it is reasonable to postulate that the underlying mechanisms may be conserved between different forms of memory.
It has been proposed that CREB governs the likelihood of neuronal allocation within memory encoding via the modulation of neuronal excitability (Zhou et al., 2009). CREB is activated by learning experience, which induces neuronal depolarization and the elevation of cAMP (cyclic adenosine 3',5'-monophosphate) and activation of protein kinase A (PKA). The phosphorylation of CREB at Ser-133 by PKA activates new gene transcription (Gonzalez and Montminy, 1989; Sheng et al., 1991). Other kinases, including Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII) and mitogen-activated protein kinase, have been shown to phosphorylate and activate CREB (Lonze and Ginty, 2002; Shaywitz and Greenberg, 1999). For example, CREB has been proposed to play a role in synaptic allocation by stimulating the expression of voltage-dependent sodium channels (1ß) subunit and inhibiting the expression of voltage-dependent potassium channels (Kv1.4) (McClung and Nestler, 2003). An alternative hypothesis as to how CREB regulates synaptic allocation is through the geometry of dendritic spines. Specifically, studies have examined that the dominant-negative (dn) expression of CREB decreased the spine head size and also disrupted the coordinated regulation of spine geometry (Sargin et al., 2013). Therefore, these studies suggest that CREB regulates the density and morphology of dendritic spines and/or enhances excitability in specific cells, which may increase the probability of the particular spine of being recruited for the engram. Thus, CREB is a key transcription factor governing the neuronal and synaptic allocation during different forms of memory encoding. The mechanisms underlying the subsequent phase of memory storage will be examined next.

1.1.3.2 Memory Storage Mechanisms

The process that converts the encoded information and stores them into long-term memory is known as consolidation. As mentioned earlier, this process has been proposed to involve molecular, cellular, and structural changes. One of the first mechanisms identified for long-term memory consolidation is the requirement for de novo protein and RNA synthesis through transcription and translation (Davis and Squire, 1984). As discussed above, CREB is activated by numerous signal transduction pathways during memory encoding; such as growth factor stimulation of tyrosine kinase receptors, the activation of Ras and extracellular signal-related kinase (ERK), and increased levels of cAMP, protein kinase C (PKC), and PKA (Stern and Alberini, 2013). These pathways lead to the transcriptional regulation of immediate early genes, such as Arc, which are induced by high frequency stimulation or behavioural
training, then upregulated in subsets of neurons, and result in downstream synaptic changes underlying synaptic plasticity (Alberini, 2009). Similar to memory encoding mechanisms, specific mechanisms underlying social memory storage are unclear. Current studies have focused on the expression of activity-dependent immediate early genes following spatial or contextual memory encoding and processing (Guzowski et al., 1999). Arc has been proposed to function in long-term synaptic plasticity and long-term memory tasks. A study has shown that the intrahippocampal infusion of antisense Arc, which inhibits the protein expression, was found to disrupt the consolidation and long-term spatial memory without affecting the encoding of the memory (Guzowski et al., 1999). Thus, Arc plays a fundamental role in the stabilization of activity-dependent hippocampal plasticity and long-term memory in rodents.

Long-term memory storage is associated with synaptic and structural remodeling, including the modification of synapses, which result in changes in short and long-term synaptic plasticity. The details of the synapse in synaptic and structural plasticity will be discussed in greater detail in section 1.1.5. It has been proposed that receptor trafficking, specifically of N-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors underlie the enhancement of memory storage during synaptic plasticity (Tang et al., 1999). In particular, the overexpression of the GluN2B subunit of the NMDA receptor facilitates long-term potentiation (LTP), a type of plasticity that involves the persistent strengthening of synapses, whereas the GluN2A subunit facilitates long-term depression (LTD), an activity-dependent reduction in postsynaptic strength (Wang et al., 2009; Zhou et al., 2018). With respect to AMPA receptors, long-term memory storage has been correlated to an increase in the number of AMPA receptors, which are known to be trafficked from the endosomal compartment (Ehlers, 2000). The precise mechanisms and the auxiliary subunits by which the NMDA and AMPA receptors are driven to the synapse is not fully elucidated, however, CaMKII, PKC, and PKA have been proposed to directly facilitate the coordination of the delivery of these receptors. Furthermore, it has been proposed that the signaling through the GluN2B subunit of the NMDA receptor can in turn activate CREB, which was previously discussed as being important for memory encoding. With respect to memory storage, the activation of CREB results in the increase of RasGRF1 expression, a Ca^{2+}/calmodulin-dependent Ras-guanine nucleotide-releasing factor (Krapivinsky et al., 2003). These genetic changes can lead to morphological modifications at the synapse including the
growth of new spines and can further increase AMPA receptor insertion. Pharmacological studies have also established that NMDA receptors are crucial in activity-dependent synaptic changes in learning and memory (Staubli et al., 1989). NMDA receptor activation and ERK trafficking, has been linked to the transcriptional regulation of the protein, Arc (Chowdhury et al., 2006). Despite its role in activity-dependent hippocampal plasticity and long-term memory (as mentioned above), Arc has also been shown to be important in the reorganization of the actin cytoskeleton within dendritic spines via Rho GTPases (Diana et al., 2007). Following the induction, Arc mRNA is transported from the cell body to the dendrites, where it localizes to the actin cytoskeleton via an actin binding protein, cofilin, within dendritic spines of activated synapses (Lyford et al., 1995). Thus, these studies suggest changes in dendritic spine shape and size are also key mechanisms governing memory storage.

1.1.3.3 Memory Retrieval Mechanisms

The mechanisms underlying the various processes in memory can be categorized into different levels. Gene and molecules are the fundamental mechanisms that govern the cellular and synaptic mechanisms, which function together within circuit mechanisms. The cellular and synaptic mechanisms involved in memory encoding and storage are most extensively studied, however, to a lesser extent examined for memory retrieval. Given the lack of studies investigating the cellular and molecular mechanisms in retrieval, this section will primarily focus on the circuitry mechanisms underlying this process.

Memory retrieval refers to the process that involves the reactivation of patterns of neural activity associated with the original experience. It involves the reconstruction of patterns of brain activity that are produced during the initial learning. Thus, it is viewed as a re-consolidation process in which prior memories become labile in a state of plasticity. Previous studies have examined how the hippocampus is capable of integrating the multimodal information gained through our senses during memory encoding and storage, but its role in retrieval is unknown (Ben-Yakov et al., 2015; Kandel and Schwartz, 1982). Regardless of the type of memory, it has been proposed that during the retrieval process, a partial pattern of cues can be reactivated in multiple cortical regions that are coordinated by
the hippocampus (O'Keefe and Dostrovsky, 1971). One approach to directly test the memory retrieval of active neuron ensembles is to use transgenic mice with altered genetic modifications, such as immediate early genes, that confer activity-dependent labelling. Using the immediate early gene, c-fos, as an activity-dependent marker, studies have introduced opsins into specific neurons based on their activation in response to a particular behavioural paradigm such as fear conditioning training (Reijmers et al., 2007). A recent study has shown that the activity of DG neural ensembles are sufficient to represent a particular context and serve as a cue for memory encoding and retrieval (Ramirez et al., 2013b). The authors use a c-fos/tTA (tetracycline transactivator) transgenic mouse line that induces the expression of a particular gene of interest downstream of the tetracycline-responsive element (TRE) (Gossen and Bujard, 1992). They further injected an adeno-associated virus encoding ChR2 expression within the DG. The transgenic mice were raised with a tetracycline analog which blocks the expression of ChR2. To label the engrams, the animals were taken off the tetracycline analog and were able to explore a novel context. The animals were subjected to fear conditioning training where c-fos activity-dependent labeling of specific neuron ensembles was achieved. Subsequently, the artificial reactivation of the ChR2-labelled neural ensembles within the DG was capable of eliciting memory recall in the mice, even though they were presented to an unfamiliar context. Thus, these results show that the reactivation of the neuronal populations that are active during initial contextual learning is necessary for the subsequent retrieval of the memory.

Other studies have focused on understanding the signaling patterns shared between the cortical and hippocampal neuronal ensembles involved in memory encoding and retrieval (Wilson and McNaughton, 1994). It has been suggested that the consolidation of different types of memories occurs in different regions. Specifically, the hippocampus was proposed to be important for recent memory retrieval but the cortical areas could be useful for remote memory retrieval. These findings were based on studies that showed that hippocampal lesions impaired recent memory retrieval but spared remote retrieval (Frankland and Bontempi, 2005). Whereas, lesions in the cortical areas, including the anterior cingulate cortex, impaired remote memory retrieval but had no effect on recent memories (Kim and Fanselow, 1992). More recently, the CA1 to dorsal subiculum to the medial EC layer 5 has been proposed to play a role in updating the retrieved episodic memories, whereas the direct CA1 to medial EC layer 5 circuit is important for fear memory formation (Roy et al., 2017). These authors found that
the optogenetic inhibition or activation of the dorsal subiculum terminals during memory recall, but not during memory formation, bidirectionally regulates contextual fear conditioning, trace fear conditioning, and conditioned place preference tests. It is proposed that during recall, a formed memory is retrieved by the subiculum and relayed to the EC layer 5. Simultaneously, a stimulus modifying the existing memory is transmitted directly from the CA1 and co-delivered to the EC layer 5. This is particularly important for fear memory to ensure that memories are rapidly updated. The mechanisms underlying the role of the subiculum in recall-induced freezing were associated with the activation of the long-tail cells, which have calcium transients that last for a long approximately 10 second duration. The authors propose that only the specific re-activation of previously formed CA1 engram cells are capable of eliciting such a strong input stimulus. Thus, this study corroborates the possibility that memory encoding and recall are regulated by distinct neural but complementary circuits, which are critical for modifying existing memory engrams. Therefore, this data suggests that the hippocampus does not store all the consolidated memories but may rather function as a hub that recruits the appropriate secondary circuits during the retrieval process. To date, most studies examine the specific mechanisms involved in fear and contextual storage and retrieval. Limited studies have demonstrated the role of these mechanisms in social memory retrieval. Furthermore, to understand the implications of social memory processing, various neurodevelopmental disorders associated with social deficits will be examined.

1.1.4 Social Deficits Associated with Neurodevelopmental Disorders

1.1.4.1 Autism Spectrum Disorders

ASD is a category of pervasive developmental disorders that affects approximately 1 in 150 children within North America (Rapin and Tuchman, 2008). Recent studies have claimed that the prevalence has continued to increase over the past two decades to roughly 1 in 36 children within the United States (Sharma et al., 2018). The term ASD encompasses neurological and developmental conditions with a spectrum of severity including; autism, Asperger syndrome, and other pervasive developmental disorders that are not otherwise specified (Levy et al., 2009). ASD is diagnosed in early childhood, as symptoms usually appear within the first two years, but can manifest into adolescence and even into adulthood. The genetic basis of the disease is evident, as the prevalence is five times greater in boys
than in girls (Christensen et al., 2016). While, the genetic basis accounts for 25% of ASD cases; other causes include, environmental factors, neurotransmission deficits, abnormalities in the immune system, hormones, and gender (Gai et al., 2012). According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), ASD is characterized by severe impairments that fall within three domains including, the conceptual, social, and practical deficits. The diagnosis was created by the American Psychiatric Association. It highlights difficulties in communication and interaction with others, including non-verbal behaviours. Also, restricted interests and stereotyped repetitive behaviours associated with body posture and loss of interest in daily function. Together, these symptoms strongly affect the person’s ability to maintain social relationships in their everyday life. Compared to the other symptoms of ASD, social dysfunction is the most pronounced and debilitating. Specifically, within the social domain, the diagnosis refers to difficulties in the display of empathy, social judgement, interpersonal communication and language skills, and other social capacities. The combination of these deficits can both directly and indirectly contribute to experiences in social isolation in children diagnosed with ASD. According to the mind-blindness theory, the social withdrawal characteristics are due to impairments in conceptualizing and mentalizing the psychological dynamics with others (Baron-Cohen et al., 1985). Furthermore, the occurrence of ASD is often explained by the high co-morbidity with other disorders including, anxiety and depression, which results in social anxiety, loneliness, and decreased motivation to socialize (White and Roberson-Nay, 2009). Other studies have shown that face processing is impaired during infancy, in individuals who develop ASD later in childhood (de Klerk et al., 2014). Functional MRI investigations have reported reduced activity in cortical areas (including the frontoparietal cortex, amygdala, hippocampus, basal ganglia, and anterior cingulate cortex) related to face processing, social interactions, and with general problem-solving abilities (Amaral et al., 2008; Frith, 2003; Lombardo et al., 2011).

1.1.4.2 Intellectual Disability

ID, formerly referred to as mental retardation, is characterized by sub-average intelligence or mental ability, with a lack of practical skills and adaptive functioning necessary for everyday life. Children are usually diagnosed with ID prior to the age of 18 years (DSM-5, 2013). The diagnosis may range from a mild to a profound level. The current prevalence is approximately 1% of the population, where 85%
have the mild and high-functioning form and the remaining 15% is comprised of the moderate and profound forms of the disease (Maulik et al., 2011). The cause of ID can stem from genetic conditions, including alterations in prenatal development due to alcohol, drugs, infections, childbirth issues, or illness and injury (Reichenberg et al., 2016).

Children with ID develop their cognitive ability at a slower rate than normal children. ID is characterized by deficits in three major areas; including intellectual functioning, adaptive behaviours, and social interactions. According to the diagnostic criteria, the individual must demonstrate significantly below-average cognitive skills and concurrent impairments in adaptive functioning (First, 2013). Compared to the average intelligent quotient (IQ) score of 85-115, individuals with ID achieve scores below 70 which is two standard deviations below the normal range (Boat, 2015). Furthermore, individuals with ID often display deficits in adaptive functioning that result in the inability to adhere to socially and culturally appropriate standards. The deficits in adaptive functioning can prevent normal functioning in various daily activities including social communication and active participation with others. Regardless of the classification of severity, children with ID demonstrate significant impairments in social functioning. Though individuals with more severe ID are more likely to display limited communication skills compared to those with the mild or moderate levels that are slower in attaining proper social skills. The degree of social impairment has been demonstrated to be positively correlated with intellectual ability (Wing and Gould, 1979). Often, individuals with ID display inappropriate social behaviours due to limitations in their ability to communicate effectively in both expressive and receptive abilities, as a result of hindered speech, impaired hearing, and language delays (Matson et al., 1985). Ultimately, this exacerbates their inability to adapt and cope with unpredictable changes in their environment (Kraijer, 2000).

1.1.4.3 Other Disorders

Although ASD and ID are two of the most commonly studied neurodevelopmental disorders characterized by social deficits, several other brain disorders also exhibit social impairments. Attention-deficit hyperactivity disorder (ADHD) is a behavioural disorder that is characterized by inattention,
hyperactivity, and impulsivity that interferes with the individual’s functioning and development (Friedman et al., 2003). It includes behaviours where the individual has difficulty sustaining focus, extreme restlessness, and rash decision-making; that is not the result of a lack of comprehension. The worldwide prevalence of this disorder, which can be observed in children and adults, is approximately 5%, but the diagnosis is highly heterogeneous geographically (Polanczyk et al., 2007). It is considered to be the most common psychiatric disorder in childhood. Children diagnosed with this disorder often experience significant social difficulties, where approximately 50% experience rejection by their peers (Barkley et al., 1990). Others have shown that 2/3 of children with this disorder demonstrate a comorbid psychiatric disorder, such as anxiety or depression, which exacerbates the social impairments (Cantwell, 1996). Furthermore, the aggressive and disruptive behaviours observed in these children are often accompanied by social misconduct (Carlson and Mann, 2000). Genetic studies have classified ADHD as a highly heritable disorder, in which the phenotypic variance is accounted for by mostly genetic influences (Tian et al., 2006). Some studies have demonstrated that in children with ADHD, the dorsal anterior cingulate cortex shows stronger functional activity during the resting state, which plays a role in the regulation of emotional and executive functioning (Bush et al., 1999).

Another interesting example with social impairments is Williams-Beuren syndrome (WBS). WBS is a rare genetic neurodevelopmental disorder with a prevalence of 1 in 7500 (Stromme et al., 2002). Children diagnosed with WBS (at the age of approximately 4 years), typically display distinctive cognitive developmental delays but are characterized by hypersociability especially towards strangers (Bellugi et al., 1999a). They demonstrate a mean IQ level of 60, which is lower than the average for children diagnosed with intellectual disability (Bellugi et al., 1999b). Children with WBS develop a unique neurocognitive profile, where they display a relative superiority in language skills, strong interest in social engagements, and a strength in processing social compared to non-social stimuli (DSM-5, 2013). Though individuals with WBS exhibit strengths in language and facial recognition, they show concrete visual-spatial cognitive, intellectual, and motor deficits (Gagliardi et al., 2003). Recent studies have also demonstrated that WBS patients have difficulties establishing and maintaining friendships through social reciprocity and understanding. These difficulties range from a lack of social understanding, motivation, and awareness (van der Fluit et al., 2012). Research on adults with WBS demonstrate that the excessive friendliness predisposes these individuals to social isolation and as a
result, they rarely engage in social contact with others (Gillberg and Rasmussen, 1994). Neuroimaging studies have noted an abnormal hippocampal formation in these individuals, which corroborates the role of the hippocampus in verbal formation and memory in humans (Meyer-Lindenberg et al., 2005). The volume of the fusiform face area, a region for maintaining the accuracy in face processing, was found to be disproportionately enlarged (Golarai et al., 2010). However, the volume of the insula, a region that regulates emotional responsiveness and empathy, was reduced in individuals with WBS (Jabbi et al., 2012).

According to the DSM-5 classification, schizophrenia is a mental disorder that affects about 1.2% of the global population (2013). Symptoms for this disease begin to manifest themselves in early adulthood and continue throughout life. The symptoms for schizophrenia can be divided into three categories: positive, negative, and cognitive. The positive symptoms include delusions, hallucinations, incoherent and disorganized speech, and catatonic behaviour. Whereas, the negative symptoms include blunting of affect, poverty of speech and thought, apathy, and anhedonia. The cognitive deficits in schizophrenia include deficits in attention, working memory, and long-term memory. However, arguably the most debilitating functional outcome of this disease is the inability to participate in meaningful interpersonal interactions. The combination of the positive, negative, and cognitive symptoms further exacerbates the social interaction deficits. Specifically, these individuals demonstrate altered face processing, loss of motivation, lack of interpersonal interest, and inattention to social or cognitive input (Robertson et al., 2014). The causes of schizophrenia have been associated with a combination of genetic and environmental factors. These factors include mutations in genes leading to neurotransmitter dysfunction and signaling dysregulation and other environmental factors such as, maternal immune activation, oxidative stress, and prenatal hypoxia (Tsuang, 2000). Structural and functional imaging studies have identified various brain structures to be implicated in schizophrenia that may serve to regulate social cognition; including the prefrontal cortex, superior temporal gyrus, amygdala, and anterior cingulate cortex (Gur et al., 2002; Russell et al., 2000; Yucel et al., 2002).

In summary, social impairments are a common core symptom of many brain disorders, underscoring the importance of social cognition. As mentioned, changes at the synapse through the activation of
connected neurons within a circuit, differences in gene expression, and structural remodeling, are believed to underscore the cellular basis of learning and memory (Kandel et al., 2014). Indeed, these alterations lead to synaptic deficits, which are core to many neurological, neurodevelopmental, and mental disorders. The following sections will discuss the detailed structure and function of the synapse.

1.1.5 The Synaptic Mechanisms Underlying Memory and Neurodevelopmental Disorders

1.1.5.1 The Synapse and Synaptic Transmission

The mammalian brain is composed of billions of cells. The proper function of the brain depends on how these cells communicate with each other. Neurons are nerve cells that are specialized in transmitting electrical and chemical information. The synapse is the main site of communication between neurons either through ions or chemical messengers known as neurotransmitters. It is the specialized intercellular junction that is formed between a presynaptic nerve terminal and a postsynaptic target. The signaling processes within synapses are driven by a calcium-triggered release of neurotransmitters and results in synaptic transmission and plasticity, and ultimately, learning and memory. At the synapse, there are various proteins, channels, and receptors that facilitate the process of transmission of electrochemical signals from one neuron to another (Figure 1.3). On one side, it consists of a presynaptic axon terminal, or a bouton, that mediates neurotransmitter release at the active zone. After neurotransmitters are synthesized, they are packaged into synaptic vesicles. The active zone is the site of the docking, priming, and fusion of synaptic vesicles via a complex process called exocytosis (Sudhof, 2012).
**Figure 1.3 The Glutamatergic Synapse.** The synapse is the junction for communication between a presynaptic and postsynaptic neuron. Chemical synaptic transmission between the two neurons involves the release of synaptic vesicles that contain chemical neurotransmitters. When an action potential arrives at the axon terminal, it depolarizes the membrane, and activates voltage-gated calcium channels which result in calcium influx. The calcium causes synaptic vesicles to fuse with the presynaptic membrane at the active zone and to release the neurotransmitters into the synaptic cleft via SNARE proteins. The neurotransmitters diffuse across the synapse and bind to receptors, which can lead to depolarization or hyperpolarization of the postsynaptic cell. For example, glutamate, the most common excitatory neurotransmitter in the mammalian brain, can bind to ionotopic glutamate receptors (i.e. AMPA, NMDA, and kainate receptors) or metabotropic glutamate (mGlu) receptors. The activation of these receptors can either lead to depolarization (i.e. in the case of AMPA receptors) or activation of second messenger systems (i.e. in the case of mGlu receptors) such as protein kinases or phosphatases which lead to long lasting effects. For example, during LTP, activation of NMDA receptors and subsequent calcium influx can lead to the activation of the protein kinase, CaMKIIα, which ultimately increases AMPA receptor insertion into the postsynaptic membrane, thus resulting in the enhancement of synaptic transmission.
Specifically, following the arrival of an action potential, the depolarization of the presynaptic terminal elicits the influx of calcium ions into the terminals via voltage-dependent calcium channels. Synaptotagmin has been identified as a calcium sensor that triggers neurotransmitter release (Brose et al., 1992). The binding of calcium with synaptotagmin stimulates vesicular exocytosis via the SNARE complex that includes synaptobrevin (VAMP), syntaxin, and SNAP 25. The protein complex mediates synaptic vesicle fusion through zippering and then opens the fusion pore to release the neurotransmitters into the synaptic cleft (Sudhof and Rizo, 2011). On the postsynaptic site of the receiving neuron, there are receptors for neurotransmitters, which are activated by the released neurotransmitters and elicit interneuronal signaling.

There are two major classes of neurotransmitters; the inhibitory neurotransmitters, including GABA which decrease the likelihood that the postsynaptic neuron will produce an action potential, and the excitatory neurotransmitters, including glutamate increases the chance that the postsynaptic neuron produces an action potential. The results for my thesis mainly focus on glutamatergic transmission, which will therefore be the focus of the discussion for this section. Within the mammalian brain, glutamate is the major excitatory neurotransmitter. Glutamate can act on AMPA, NMDA, and mGlu receptors in the post-synaptic membrane. AMPA receptors are hetero-tetrameric complexes composed of four subunits, GluA1-GluA4. Each subunit contains a large extracellular N-terminal ligand-binding domain, three full transmembrane domains, an intracellular-re-entrant loop, and a cytoplasmic carboxyl terminal (C-terminal) domain. Whereas GluA1 and 4 possess predominantly long C-terminals, GluA2 and GluA3 have relatively shorter cytoplasmic C-terminal tails (Sheng and Lee, 2001). Prior to birth and within the first postnatal week, the GluA4 subtype is predominantly expressed in excitatory hippocampal and cortical neurons (Akaneya, 2007; Zhu et al., 2000). However, GluA4 levels decline following birth contributing to the developmental switch to the GluA1 and GluA2 receptor subtype. Gradually with age, hippocampal Schaffer collateral synapses are largely comprised of GluA1/2 and GluA2/3 heteromers (Wenthold et al., 1996). GluA1/2 receptors are inserted into the synapse following activity and are eventually replaced by GluA2/3 receptors over time in an activity-independent manner (Shi et al., 2001; Wenthold et al., 1996). Importantly, the presence of the GluA2 subunit in AMPA receptors renders the channel impermeable to calcium and a linear current-voltage relationship. Thus, in the absence of GluA2, AMPA receptors are permeable to calcium and exhibit a strong inward
rectification at positive potentials (Jonas and Burnashev, 1995). Numerous studies have shown that AMPA receptors play an important role in mediating fast synaptic transmission in the central nervous system and are critical for the expression of synaptic plasticity, including LTP and LTD (Bredt and Nicoll, 2003; Collingridge et al., 2004; Hestrin et al., 1990; Kessels and Malinow, 2009; Malinow and Malenka, 2002), which will be further discussed in section 1.1.5.2. Another key ionotropic glutamate receptor is the NMDA receptor that is essential for the induction of synaptic plasticity. NMDA receptors are also tetramers composed of two obligatory GluN1 subunits and two GluN2 or GluN3 subunits that determine the receptor properties and kinetics. The topology of each subunit is similar to that of AMPA receptors consisting of the N-terminal domain for binding by allosteric modulators, the agonist-binding domain (for agonists such as glycine/D-serine or glutamate), the pore domain, and the C-terminal domain that binds to various intracellular protein partners. The expression of GluN2A-D subunits also varies across developmental stages (with GluN2B expression being high before birth and GluN2A expression increasing over postnatal development), which contributes to the diverse role of each subtype in synaptic plasticity. GluN2A and GluN2B are the primary subtypes found in the hippocampus and cortex (Paoletti et al., 2013; Sans et al., 2000). Synaptic NMDA receptors in mature neurons predominantly consist of GluN1/GluN2A or GluN1/GluN2A/GluN2B heteromers (Petralia et al., 2009). NMDA receptors are considered to be coincidence detectors for the induction of synaptic plasticity. This is because at resting potentials the channel is blocked by extracellular magnesium and thus inactive. It is only following presynaptic glutamate release and postsynaptic depolarization, is the magnesium block removed from NMDA receptors allowing for their activation and subsequent calcium influx. Lastly, mGlu receptors are a family of G-protein coupled receptors that bind glutamate within a large extracellular domain and transmit signals through the interaction with numerous intracellular signaling partners. There are 8 subtypes that are classified into 3 groups, based on the sequence homology, synaptic localization and ligand specificity, and downstream G-protein signaling pathways (Niswender and Conn, 2010). Group 1 includes mGluR1 and mGluR5, which are mostly anchored by PDZ proteins on the postsynaptic side and are important for increasing NMDA receptor activity through the stimulation of phospholipase C, adenylyl cyclase, and MAP-kinase phosphorylation. They also activate other biochemical cascades and ion channels, which alter excitability through presynaptic inhibition or postsynaptic induction. Group 2 includes mGluR2 and mGluR3, which are found presynaptically, and reduce the activity of postsynaptic potentials through the inhibition of adenylyl cyclase and calcium channels as well as promoting the activation of potassium channels. Lastly, group
3 includes mGluR4, mGluR6, mGluR7, and mGluR8 that are also mostly found presynaptically. Both group 2 and 3 mGlu receptors have been found to be important in the regulation of neurotransmitter release by NMDA receptors, to ultimately protect neurons from excitotoxicity (Ambrosini et al., 1995). It is specifically the binding of mGlu receptors by glutamate results in a conformation change in the heterotrimeric complex of α, β, and γ subunits, which causes the exchange of GTP for GDP within the α subunit (Tsuchiya et al., 2002). The subsequent activation of second messenger signaling pathways, allows the mGlu receptors to modulate cell excitability and synaptic transmission.

### 1.1.5.2 Synaptic Plasticity

One of the most important features of the synapse is its ability to change the strength of synaptic transmission, which is referred to as synaptic plasticity. It is believed to be the cellular mechanism underlying learning and memory. According to Hebb’s postulate, synapses are strengthened by the coincidental activation of both the presynaptic and postsynaptic neurons but are weakened by non-coincidental neuronal firing (Stent, 1973). Synaptic plasticity is also important for neural circuit remodeling during development (Kirkwood et al., 1995). Thus, alterations in synaptic transmission and plasticity have been observed in various neurological, neurodevelopmental, and neuropsychiatric disorders (Zoghbi and Bear, 2012).

Synaptic plasticity can be classified as short-term and long-term, depending on the time-scale of the changes. Short-term plasticity usually lasts for milliseconds to several minutes and has been proposed to be important for information processing, specifically the short-term adaption to sensory inputs, the transient changes in behavioural states, and the temporary, short-lasting forms of memory (Citri and Malenka, 2008). Short-term plasticity is induced by short bursts of synchronous activity that results in a transient accumulation of calcium in the presynaptic nerve terminals. One example of short-term synaptic plasticity is paired-pulse facilitation/depression, where two stimuli are presented within a certain inter-stimulus interval time period. The response to the second stimulus can be enhanced or depressed relative to the response of the first stimulus (Katz and Miledi, 1968). On the other hand, long-lasting synaptic plasticity can sustain for hours or even days. The first experimental support of the
long-lasting activity-dependent changes was reported by Bliss and Lomo, where the repetitive high frequency activation of excitatory synapses in the dentate granule neurons within the hippocampus elicited LTP in synaptic strength (Bliss and Lomo, 1973). Another long-lasting synaptic plasticity is long-term depression (LTD) of synaptic strength following low frequency stimulation (Ito and Kano, 1982). Other forms of synaptic plasticity include homeostatic plasticity and metaplasticity. Homeostatic plasticity is a neuron’s ability to adjust the strength of its synapses collectively, either up or down, to stabilize its activity. Studies have shown that synaptic scaling, one of the most extensively studied forms of homeostatic plasticity, is through the presence of calcium-dependent sensors that regulate receptor trafficking to increase or decrease the number of glutamate receptors at the synapses (Turrigiano, 2012). Metaplasticity refers to the effects that activity can have on synaptic transmission which affect the capacity of synapses to express subsequent synaptic changes, including LTP and LTD (Abraham and Bear, 1996). Metaplasticity was initially discovered through the low-level activity of NMDA receptors (low frequency stimulation in a magnesium-free medium) in the CA1 region which induced a synaptic change and inhibited the subsequent induction of LTP (Coan et al., 1989).

Although there are numerous forms of synaptic plasticity, LTP and LTD are the most extensively studied and best understood, in particular at the Schaffer collaterals connecting the CA3 and CA1 regions of the hippocampus (i.e. CA1 synapse). These forms of plasticity have been proposed to be the cellular substrate of memory formation, particularly spatial and contextual memory (Bliss and Collingridge, 1993). LTD it is typically induced by prolonged low-frequency stimulation (i.e. 0.5-3Hz), which activates NMDA receptors and causes a moderate increase in postsynaptic calcium concentration that in turn initiates the serine-threonine protein phosphatase cascade (Mulkey and Malenka, 1992). The activation of the protein phosphatases can target several substrates, including AMPA receptors, to decrease synaptic transmission. For example, LTD is associated with a dephosphorylation of Ser-845 of GluA1, which decreases the open probability of AMPA receptors and facilitates their movement away from the synaptic junction (Banke et al., 2000). LTP is typically induced by a brief high-frequency stimulation (i.e. 100Hz) of presynaptic terminals, which activate NMDA receptors and results in a brief increase of postsynaptic calcium concentration (Mayer et al., 1984). Different from LTD, this calcium rise activates protein kinase cascades, which ultimately lead to enhanced synaptic strength. For example, activation of CaMKIIα can directly phosphorylate GluA1 at Ser-845 increasing
the channel conductance of AMPA receptors. In addition, calcium influx from NMDA receptor activation stimulates the trafficking and insertion of GluA1-containing AMPA receptors from the reserve pool in recycling endosomes in dendrites to the postsynaptic density (Bredt and Nicoll, 2003).

LTP can be further divided into two temporal phases, early and late phases of LTP, which differ on the basis of their induction requirements, time of expression, and molecular mechanisms (Kandel, 2001). Early-LTP (E-LTP) relies on the modulation of activity and location of already existing proteins. For example, the phosphorylation of existing proteins and the insertion of neurotransmitter receptors into the synapse are important for E-LTP. Late-LTP (L-LTP) involves de novo protein synthesis via the activation of gene transcription factors and new gene expression (Frey et al., 1988). The signaling to the nucleus is required for L-LTP and depends on protein kinases, including PKA and ERK-MAPK, which activate transcription factors including cAMP, and immediate early genes such as c-fos (Huang et al., 1994). Thus, the time scales for E-LTP are generally shorter than three hours, whereas L-LTP can be maintained for at least three hours and usually persist for an extended period of time in the scale of days and even weeks (McGaugh, 1966). The induction of E-LTP requires a single train of high-frequency stimulation whereas, multiple spaced trains of stronger stimuli are needed for L-LTP (i.e. high frequency stimulation 4x100Hz stimulation). E-LTP and L-LTP are thought to be involved in the short-term and long-term stages of memory, respectively (Huang, 1998).

LTP and memory storage share certain properties including its rapid process of induction, long-lasting nature, and correlation with natural brain rhythms (Shors and Matzel, 1997). However, whether LTP is directly linked to the induction and storage of memory engrams remains debatable. The notion that synaptic plasticity is a substrate of memory began in 1949, when Donald Hebb proposed a mechanism where at the site of the excitatory synapse, the activation of one cell leads to the activation of a second connected cell, and reinforces the connection (Morris, 1999). LTP is an attractive mechanism to explain memory storage as it is similarly characterized by its associativity, cooperativity, and input specificity (Nicoll et al., 1988). Associativity refers to the ability to potentiate or facilitate a weak input when it is activated in conjunction with a strong input. Cooperativity refers to the coincident activation of a critical number of synapses simultaneously. It is the property that ensures a neuron must reach a threshold of depolarization before LTP can be induced. Lastly, input specificity indicates that LTP is only elicited at activated synapses and not at adjacent, inactive synapses on the same postsynaptic
neuron. Previous studies have identified direct parallels between memory and LTP through manipulations that have been found to selectively disrupt both potentiation and the encoding of new information (Bohme et al., 1993; Lynch et al., 1983; Malleret et al., 2001). Recent studies have used novel techniques such as cytoskeleton markers, important for actin polymerization, to monitor recently induced LTP within dendritic spines following learning (Fedulov et al., 2007). Other studies have used multiple recording electrodes to observe learning-induced enhancement in synaptic strength within the hippocampus and cortex following tasks including novel object recognition, trace eyeblink conditioning, and an inhibitory avoidance experimental paradigm (Clarke et al., 2010; Gruart et al., 2006; Whitlock et al., 2006).

One of the important features of LTP and LTD is that they are closely associated with the structural remodeling of the synapse (Bosch et al., 2014). In this regard, the changes in the dendritic spines are the focus of this investigation. Dendritic spines are postsynaptic dendritic protrusions where most glutamatergic synapses are formed. Specifically, spine enlargement and shrinkage are linked to LTP and LTD respectively (Bosch and Hayashi, 2012). For example, recent technical advances using live-imaging techniques, such as a two-photon microscope, to directly image single dendritic spines have shown that stimulation protocols that induce LTP, also results in the formation of new dendritic spines and the increased spine volume of existing dendritic spines (Engert and Bonhoeffer, 1999; Matsuzaki et al., 2004). In addition, the changes in the volume of the spines were found to be positively correlated to the number of AMPA receptors expressed at the synapse (Matsuzaki et al., 2001). Therefore, dendritic spine changes are considered to be an integral part of synaptic plasticity. For this reason, I will discuss spines in more detail below.

1.1.5.3 Dendritic Spines and the Actin Cytoskeleton

Many brain disorders such as ASD and ID, are characterized by an alteration in dendritic spines, underscoring the importance of postsynaptic function and morphology in normal brain function (Chapleau et al., 2009a). Spine dynamics, specifically changes in the shape and size, have been shown to modulate their functional properties including the strength of excitatory synaptic connections and the
overall activity levels (Meng et al., 2003b). As a result of their specialized morphology, dendritic spines act to compartmentalize local synaptic signaling pathways and restrict the diffusion of postsynaptic molecules. Typically, spines consist of three compartments: a delta-shaped base at the junction with the dendritic shaft, a thin neck, and a bulbous head contacting the axon (Hotulainen and Hoogenraad, 2010). There are three categories of spines based on their morphology; thin filopodia-like protrusions, short “stubby” spines without a neck, and “mushroom” spines with a large bulbous head. During neuronal development upon synaptic contact with the presynaptic axon, thin and highly motile dendritic filopodia transform into stable, mature, mushroom spines. The remodeling of the dendritic spine is dependent on its underlying actin cytoskeleton (Figure 1.4). Actin is the major cytoskeletal component in dendritic spines and it goes through continuous treadmilling to reach equilibrium between polymerization to filamentous actin (F-actin) and depolymerization to globular actin (G-actin) (Patterson and Yasuda, 2011). Actin filaments are polar structures with one end, known as the barbed end, growing more rapidly than the other pointed end (Pollard and Borisy, 2003). The actin cytoskeleton is important for numerous cellular processes involving cell shape and motility, protein translocation, and membrane dynamics. Therefore, a shift in the equilibrium has been shown to contribute to both basal spine motility and activity dependent structural plasticity.

At the synapse, the actin cytoskeleton is also critical for organizing the postsynaptic density, an electron dense area on the spine head directly in apposition with the presynaptic terminal by anchoring postsynaptic receptors and facilitating the trafficking of proteins and translation machinery (Bramham, 2008; Renner et al., 2008; Schlager and Hoogenraad, 2009; Sheng and Hoogenraad, 2007). For example, the actin network of long and short filaments in the spine neck and head stabilize postsynaptic actin-binding proteins, including CaMKII and calcineurin (Cheng et al., 2006). Studies using fluorescence resonance energy transfer (FRET) have revealed that synaptic stimulation rapidly alters the equilibrium between F-actin and G-actin (Okamoto et al., 2004). Specifically, LTP induction shifts the equilibrium towards F-actin which increases the overall volume via the greater number of actin filaments within the spine; whereas, LTD induction shifts the equilibrium towards G-actin, which results in spine shrinkage through a decrease in the number of spine filaments. Recent studies have demonstrated that during spine enlargement, the actin pool at the tip of the spine treadmills to generate an expansive force in the spine head which may be the major determinant of spine volume (Honkura et
al., 2008). Other studies also provide evidence that LTP induces growth of new spines, which also requires actin reorganization (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999).

The signaling pathways that are involved in the regulation of the actin cytoskeleton are extensively studied. These include processes activated by synaptic receptors such as NMDA and AMPA receptors (Fischer et al., 2000). The NMDA receptors regulate actin by activating calcium-dependent signaling processes such as CaMKII, which in turn stimulate the Rho family of small GTPases, including RhoA, Rac1, and Cdc42 (Bajaj et al., 2009; Lisman et al., 2002; Raveendran et al., 2009; Tada and Sheng, 2006). The Rho GTPases are the central mediators of actin reorganization in many cells, including neurons and spines. They regulate actin via activating a number of downstream effectors, including p21-activated kinases (PAKs). Since PAKs are the key molecules to be investigated in this thesis project, they will be discussed further in the following sections.
Figure 1.4 Actin Treadmilling. Actin is the major cytoskeletal component in dendritic spines and it goes through continuous treadmilling to reach an equilibrium between polymerization to filamentous actin (F-actin) and depolymerization to globular actin (G-actin). Treadmilling is a phenomenon that allows one end of the filament to grow in length (barbed + end), while the other end shrinks (pointed – end).
1.2 PAKs

1.2.1 PAK Protein Family

PAKs were first discovered in budding yeast as Ste20-type (sterile 20) kinase and were later found to exist in all eukaryotes (Leberer et al., 1992). PAKs are a family of protein kinases that are activated by the upstream small Rho GTPase Rac1 (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division cycle 42) (Field and Manser, 2012). They are among the first targets identified that interact with Rho GTPases via the Cdc42/Rac interaction-binding (CRIB) domain (Manser et al., 1994). PAKs are divided into group I (PAK1-3) and II (PAK4-6) kinases based on the presence of an autoinhibitory domain (AID), which overlaps with the CRIB domain. Since group II PAKs lack an AID, binding by upstream Rho-family GTPases does not lead to activation of the kinase, and they are often referred to as the non-conventional PAKs (Baskaran et al., 2012; Manser et al., 1995). For this reason, the detailed role of group II kinases, including their functions and activation, are much less studied. The expression pattern and key knockout phenotypes of various PAKs are summarized in Table 1.1.

1.2.1.1 Group 1 PAKs: Expression and Significance

Group I is comprised of PAK1, 2, and 3. All group I PAKs contain an N-terminal CRIB domain that overlaps with the AID, and a C-terminal serine/threonine kinase domain (Figure 1.5). In their inactivated state, the group I PAKs form trans-dimers where the AID of one PAK protein inhibits the kinase domain of a second PAK protein. All group I PAKs have a low basal kinase activity and share approximately 70% in their total sequence homology between the members (Arias-Romero and Chernoff, 2008). However, the 90% homology between the kinase domain suggests similarities in their activation via the same phosphorylation targets. The three PAKs vary in their tissue expression. PAK1, the most studied isoform, is expressed predominantly in the brain, muscle, heart, and spleen (Kelly and Chernoff, 2012). PAK2 is ubiquitously expressed and is required for embryonic development in mice (Arias-Romero and Chernoff, 2008). PAK3 is expressed predominantly in the cerebral cortex and the hippocampus within the brain (Kreis et al., 2008).
Table 1.1 *The differential expression patterns and distinct biological functions of PAKs*

<table>
<thead>
<tr>
<th>PAK</th>
<th>Tissue Expression</th>
<th>Observed Biological Defects in Knockout Models</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brain, muscle, heart, and spleen</td>
<td>Immune system, glucose homeostasis, neuronal (synaptic and behavioural), and cardiovascular</td>
</tr>
<tr>
<td>2</td>
<td>Ubiquitous</td>
<td>Embryonic lethal (Day 8)</td>
</tr>
<tr>
<td>3</td>
<td>Brain</td>
<td>Neuronal (synaptic and behavioural)</td>
</tr>
<tr>
<td>4</td>
<td>Ubiquitous (Prostate, testis, colon, heart, and brain)</td>
<td>Embryonic lethal (Day 11.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conditional KOs: Neuronal and cardiovascular</td>
</tr>
<tr>
<td>5</td>
<td>Brain</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Testis, prostate, brain, kidney, and placenta</td>
<td>Neuronal (behavioural)</td>
</tr>
</tbody>
</table>
Figure 1.5 Group I and II PAKs Structure. All PAKs contain a N-terminal domain with a CRIB domain, which confers binding to small GTPases. In group I PAKs, the N-terminal CRIB domain is overlapped by an autoinhibitory domain (AID), which keeps the PAKs as dimers and maintains the inactive conformation. Also, the NCK (Nck adaptor proteins) and PIX (PAK-interacting exchange) binding sites facilitate these adaptor proteins to interact and recruit PAK dimers to the membrane to increase their interaction with GTPases and lipids. Group II PAKs lack the AID but have an autoinhibitory pseudosubstrate motif. Thus, binding from upstream Rho GTPases, does not lead to the consistent activation of the kinase.
In vitro studies have shown that the functions of group I PAKs range from promoting cell proliferation, regulating apoptosis, accelerating mitotic abnormalities in tumor formation, and playing a role in cognitive dysfunction (Kumar et al., 2006; Zhao et al., 2006). PAK1 is localized to the cytoplasm but can migrate to the leading edge of polarized cells following activation by GTP-bound Rac and Cdc42 (Dharmawardhane et al., 1999). PAK1 knockout (KO) mice are viable, display normal gross anatomy, and are fertile, but display immune deficiencies associated with mast cell degranulation due to an inability of F-actin disassembly following the detection of allergens (Allen et al., 2009). PAK1 KO mice also display glucose homeostatic impairments in peripheral insulin secretion that may also be caused by F-actin dysregulation and signaling defects in islet cells (Wang et al., 2011). In other studies, PAK1 KO mice demonstrated pulmonary edema and cardiac hypertrophy due to increased load stress on the heart (Liu et al., 2011). In the brain, PAK1 KO mice revealed normal brain size and synaptic structures but had selective deficits in LTP at hippocampal CA1 synapses. This was accompanied by reduced levels of F-actin in dendritic spines and enhanced activity of the actin binding protein, coflin (Asrar et al., 2009). Thus, in the brain, PAK1 plays a role in the regulation of synaptic plasticity through a coflin-dependent actin reorganization (Cole et al., 2000; Krupp et al., 1999; Meng et al., 2003b). Given the ubiquitous expression pattern of PAK2, PAK2-null mice are embryonic lethal at day 8 mainly due to defective vascularization (Hofmann et al., 2004). A recent study has examined the deletion of PAK2 in T-cells specifically in mice, which resulted in severe T-cell lymphopenia accompanied by marked defects in development, maturation, and migration of thymocytes (Phee et al., 2014). Specifically, these authors found that PAK2 facilitates dynamic actin cytoskeleton reorganization in T-cell development and activation within the immune system. PAK2 has also been found to be expressed in endothelial cells. The depletion of PAK2 in endothelial cells during embryogenesis and in adult mice results in severe vascular defects, including endothelial cell apoptosis, increased vascular permeability, and overall impaired blood vessel homeostasis (Radu et al., 2015). The conditional KO of PAK2 in hematopoietic stem cells induced profound leukopenia and mild macrocytic anemia in mice, which resulted in their death within 14 days (Zeng et al., 2015). Thus, PAK2 plays an important role in cytoskeleton dynamics in diverse cell types. PAK3 expression in the brain is associated with abnormalities in synaptic plasticity and learning and memory. Rodent studies have elucidated the role of PAK3 in dendritic spine morphogenesis, synaptic transmission, and plasticity, but exactly how PAK3 achieves its structural and synaptic regulation is unclear (Allen et al., 1998; Boda et al., 2004; Dubos et al., 2012). PAK3 KO mice showed normal locomotor activities and
revealed no deficits in the actin cytoskeleton with respect to dendrite and spine morphology (Meng et al., 2005). However, these PAK3 KO mice had selective impairments in late-phase LTP and impairments in learned taste aversion (Meng et al., 2005). Hayashi et al., (2004) generated a transgenic mouse model to inhibit the catalytic activity of all group I PAKs in the postnatal forebrain. A dn PAK which consisted of an CaMKIIα promoter, amino acids 74-146 encoding the AID of PAK1-3, and SV40 intron/polyA, was microinjected into a C57BL/6 zygote. The dnPAK mice displayed decreased spine density, increased proportion of large synapses in cortical pyramidal neurons, and enhanced AMPA and NMDA receptor-mediated synaptic transmission and LTP. Behaviourally, the dnPAK mice exhibited specific impairments in the consolidation and retention of hippocampal-dependent memory in a short-term fear conditioning paradigm. Together, the data emphasizes that group I PAKs play a critical role in regulating synaptic structure and function in the cortex. In a subsequent study, Huang et al., (2011) also investigated the effects of PAK1 and 3 double KO mice. The double KO mice exhibited impaired postnatal brain growth, deficits in dendritic arbourization and spine morphology, with alterations in synaptic transmission and plasticity (Huang et al., 2011). Furthermore, these mice displayed a host of behavioural deficits consistent with ASD and ID-like symptoms including hyperactivity, increased anxiety, and learning and memory deficits. The reductions in cofilin phosphorylation were rescued by blocking cofilin activity suggesting cofilin-dependent actin regulation may mediate the effect of PAK1 and PAK3. Together, these studies illustrate the functional redundancy of the group I PAKs and their importance in synaptic regulation and brain function.

1.2.1.2 Group II PAKs: Expression and Significance

Group II PAKs are comprised of PAK4, 5, and 6. The binding by the small Rho-family GTPases, primarily by Cdc42 results in the activation of the kinase through a different mechanism compared to group I kinases. Some studies have identified that the conformational flexibility of the glycine-rich loop within the kinase domain acts as a molecular sensor for ATP binding, which governs the structural rearrangements important for subsequent autophosphorylation and activation (Eswaran et al., 2007). An alternate explanation is that group II PAKs are constitutively phosphorylated at the activation loop (Thr423) and the binding by the small GTPases acts to transport the kinase to the desired location (Molli et al., 2009). A recent study has proposed that group II PAKs are autoinhibited by a
pseudosubstrate sequence, RPKP, which is released following the binding of a second signal with an SH3 domain to the N-terminal of PAKs (Ha et al., 2012).

PAK4 is ubiquitously expressed with high levels in the prostate, testis, and colon (Kelly and Chernoff, 2012). Studies have shown that PAK4 KO mice are embryonic lethal at day 11.5 due to improperly formed vessels within the heart which result in impaired vascularization (Arias-Romero and Chernoff, 2008). Furthermore, PAK4 null mice revealed defects in neuronal differentiation and axonal outgrowth (Qu et al., 2003). In vitro studies have further identified that PAK4 plays a role in Cdc42 induced filopodia formation (Abo et al., 1998). Other studies have investigated the conditional KO of PAK4 in the nervous system and found significantly thinner outer cortical layers compared to the controls, which was attributed to a decrease in the proliferation of neural progenitor cells caused by a disruption in β-catenin signaling (Tian et al., 2011). Compared to PAK4, PAK5 and PAK6 have a restricted pattern of expression that are evident in later developmental stages. PAK5 has been found to be uniformly expressed in the brain, but to a lesser extent in other tissues. However, PAK5 KO mice are viable and exhibit normal gross anatomy of the brain, testes, prostate, and other tissues (Li and Minden, 2003). Compared to PAK4, PAK5 is expressed in later developmental stages with overall higher protein levels in the adult brain. Thus, these findings suggest that PAK4 may be required for early embryonic development in the brain and heart, whereas PAK5 may act in the brain of adults. Lastly, PAK6 is expressed in the testis, prostate, brain, kidney, and placenta (Kelly and Chernoff, 2012). The PAK6 null mouse appears to be comparable to normal wild-type (WT) mice, with no apparent gross abnormalities (Nekrasova et al., 2008). Behaviourally, the PAK6 KO mice displayed a lower locomotor activity, decreased spatial learning and memory ability, and less overall aggression in a social context. Similar to PAK5, PAK6 is highly expressed in the brain in the hippocampus and cortex. The double KO of PAK5 and PAK6 reveal cytoskeletal changes in the brain that affect synaptic plasticity, learning, and locomotion (Nekrasova et al., 2008). Furthermore, the PAK5 and PAK6 double KO mice showed impairments in the migration of motor neuron progenitor cells during development. Thus, the functional redundancy shared by PAK5 and PAK6 may act to prevent phenotypic abnormalities in mutations associated with these genes.
1.2.2 Mechanisms of PAK Activation

1.2.2.1 Overall Domain Structure of PAKs

As mentioned earlier, PAKs contain a highly conserved C-terminal kinase domain but a variable N-terminal regulatory domain that is responsible for a variety of intracellular signaling pathways (Bokoch, 2003). Both group I and II PAKs bind Cdc42 and Rac1 GTPases through their CRIB domain, but only the group I PAKs can be activated by this interaction (Hofmann et al., 2004). Group I PAKs also contain an AID (amino acids 75-149) which partially overlaps with the CRIB domain (amino acids 52-132) and interacts with the kinase domain in resting cells (Zhao et al., 1998). The interaction of active Rho GTPases with the CRIB domain triggers conformational changes between the PAK dimers resulting in the autophosphorylation and subsequent activation of the kinase. Group II PAKs have a sequence that is related to the AID, but do not contain the same conserved AID domains as the group I PAKs.

1.2.2.2 Rho-family GTPases: Key Upstream Activators of PAKs

PAKs are activated by the Rho family GTPases, which belong to the Ras superfamily of low molecular weight guanine nucleotide binding proteins. The Rho GTPases comprise of seven subfamilies including RhoA, Rac, Cdc42, Rnd, RhoD, RhoBTB, and RhoH. Each GTPase comprises of a 200 amino acid Dbl homology (DH) domain and a 120 amino acid pleckstrin homology (PH). These GTPases have been shown to be molecular switches that regulate signal transduction pathways involved in gene transcription, cell cycle progression, and cellular survival (Hill et al., 1995; Olson et al., 1995). They are particularly important in linking the extracellular cues from the surface receptors to the promote the assembly and organization of the actin and microtubule cytoskeleton (Figure 1.6). For example, in neurons, the binding of Cdc42 and Rac1 to the CRIB domain of PAKs can result in the autophosphorylation and a 100-fold activation of the kinase (Chong et al., 2001). The Rho GTPases are regulated by multiple upstream proteins (Spiering and Hodgson, 2011). Guanine nucleotide exchange factors (GEFs) are positive regulators that activate the Rho GTPases, whereas guanine nucleotide activating proteins (GAPs), which stimulate Rho GTPases to hydrolyze GTP to GDP, inactivate the Rho GTPases. In addition, guanine dissociation inhibitors (GDIs) sequester Rho GTPases in a GDP-bound state thus also acting as negative regulators. The DH domains facilitate the interactions with
GEFs by stabilizing GTP-free Rho intermediates and thus, increasing the intracellular amount of GTP. The PH domains regulates the DH domain and GEF function, including targeting the Rho GTPases to the plasma membrane, modulating exchange activity, and interactions with phospholipids and proteins (Buchsbaum, 2007).

In addition to being activated by the Rho GTPases, PAKs can also interact with various upstream proteins of the GTPases. Group I PAKs can interact with Rac-GEFs, such as PAK-interacting exchange factor (PIX), to promote the exchange of GDP and GTP of Rac1 (Obermeier et al., 1998). Specifically, studies have shown that PAKs form a complex with PIX and the G-protein coupled receptor kinase-interacting target (GIT1), which can activate Rac1 and regulate the actin scaffolding protein, paxillin. PAKs have been found to phosphorylate Ser-101 and Ser-174 on Rho GDI which selectively activates Rac1 but not RhoA or Cdc42 (Dovas and Couchman, 2005).

As mentioned earlier, the AID within the N-terminal regulatory region of PAK functions to repress the catalytic activity of the kinase. The activation process involves the structural binding of Cdc42 or Rac1 to the CRIB domain which elicits a conformational change that relieves the inhibitory AID to switch on the kinase domain. This leads to PAK being in a monomeric opened conformation. The activation of PAKs leads to the auto-phosphorylation of Ser-199/Ser-204 in the PIX-binding motif consisting of residues 186-203 (Zhao et al., 2000). PIX proteins have an SH3 domain that binds to the non-canonical binding sequence conserved in group I PAKs which can activate the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Manser et al., 1998). Phosphorylation of Ser-144 in the AID causes steric hindrance with Arg-388 within the catalytic domain. However, the binding of Cdc42 to the CRIB domain increases the autophosphorylation at Ser-141 and Ser-165 at the AID in the regulatory domain, and Thr-402 in the activation loop of the catalytic domain (Gatti et al., 1999). This has been shown to be important in the modulation of the interaction with Cdc42 (Jung and Traugh, 2005). For full activation, Thr-422 at the catalytic domain is also required to be transphosphorylated to prevent the catalytic domain interaction with the AID (Chong et al., 2001). Within group II PAKs, the CRIB domain is constitutively phosphorylated and therefore, does not require the binding of Rho-family GTPases (Zhao and Manser, 2012).
Figure 1.6 The PAK Signaling Pathway. This schematic of the Rho signaling pathway is adapted from Prudnikova et al., 2015. PAKs are activated by the Rho GTPases, which are in turn activated or inhibited by various surface receptors through GEFs and GAPs. Once activated through GEFs, PAKs can then activate various effector proteins in various signaling pathways. These pathways ultimately play a role in the regulation of cell proliferation and survival, gene transcriptional activation, and actin remodeling.
### 1.2.2.3 Other Upstream Regulators of PAKs

PAK activity can be regulated by various upstream stimuli including chemoattractants, extracellular matrix molecules, growth factors, and cytokines (Bokoch et al., 1998). G-protein-coupled receptors (GPCRs) are seven-pass-transmembrane domain proteins that transduce external stimuli into intracellular signals (second messengers) to modulate cellular function (Magalhaes et al., 2012). GPCRs function as ligand-regulated GEFs or GAPs that activate heterotrimeric GTP-binding proteins. Depending on the G protein coupled to the receptor, it can either positively or negatively regulate downstream effectors including phospholipases, adenyl cyclases, and ion channels (Neer, 1995). For example, phosphatidylinositol 3,4,5-triphosphate (PIP3)-dependent Rac exchanger 2 (PREX2) is a GEF that activates Rac1 and indirectly, increases PAK activation (Barrows et al., 2015). Other GPCR second messengers including, cAMP and diacylglycerol (DAG), also rely on signaling mechanisms through GEFs to regulate PAKs (Bos et al., 2007). To date, fewer studies examine GEF-independent mechanisms of PAK activation. In one study, the authors show that PAK1 can be activated by the Gβγ subunit of the GPCR through a PI3K and protein kinase B (Akt) pathway, independent from Rac1 and Cdc42 activity (Menard and Mattingly, 2004).

Integrins are cell surface receptors that play a role in the adhesion of the extracellular matrix to the cytoskeleton of the cell. Specifically, integrins act as cell-matrix receptors that physically bind extracellular proteins such as collagen, fibronectin, and laminins (Bokel and Brown, 2002). These cells first adhere to the extracellular matrix and then spread rapidly by extending filopodia-like projections and lamellipodia (Price et al., 1998). The linkage is required to ensure proper cytoskeletal organization, migrations, assembly, and information signaling. Integrin-dependent adhesion and cell spreading involves the coordination of dynamic rearrangements in the actin cytoskeleton. Specifically, integrin engagement activates Cdc42, which stimulates PAK kinase activity (Price et al., 1998). Recent studies show that integrins also interact with Arg, an Abl-family tyrosine kinase, which promotes the binding of cortactin to F-actin and the attenuation of Rho activity to stabilize dendritic spines (Lin et al., 2013). To date, no studies have found the direct activation of PAKs through integrins in a Cdc42 or Rac1 independent manner.
As mentioned earlier, glutamate receptors have been shown to be potent regulators of Rho GTPases and therefore, important for the activation of PAKs (Penzes et al., 2001). These findings are corroborated by biochemical studies that have identified that GEFs and GAPs, including Kalirin-7 and SynGAP, are enriched in the postsynaptic density where they are ideally positioned for regulation by glutamate receptors (Collins et al., 2006). Thus, glutamatergic synaptic activity may regulate Rho GTPase and PAK activity through modifying the function of these signaling molecules. The role of glutamate receptors in the direct activation of PAKs, independent of Rho GTPases, has not been shown.

Cadherins are also part of the family of cell adhesion molecules that regulate the actin cytoskeleton through the activation of Rho GTPases and subsequently PAKs (Watanabe et al., 2009). Similar to integrins, cadherins generate intracellular signals to small GTPases that control cytoskeletal reorganization, polarity, and vesicle trafficking (Kim et al., 2000). Various signaling pathways downstream of cadherins have been identified, in particular the role of small GTPases (Perez-Moreno et al., 2003). For example, cadherin-mediated cell to cell adhesion stimulates PI3K activity which primarily activates Rac1 (Kotani et al., 1994). Specifically, Tiam1 is a Rac1 GEF that is localized at the sites of cell-cell contacts and functions downstream of PI3K (Michiels et al., 1995). Other examples include Vav2, a RhoGEF of catenin, which directly binds to the juxtamembrane domain of cadherins and activates Rac1 and Cdc42 (Noren et al., 2000). Thus, cadherin-mediated intercellular adhesions activate GEFs which lead to the action of downstream small GTPases that control the organization of PAKs and the actin cytoskeleton. To date, few studies examine the activation of PAKs through cadherins in signaling pathways independent of GEFs and the Rho family of proteins.

PAKs can be directly activated by sphingolipids which transduce specific lipid signals (Bokoch, 2003). Furthermore, the role of lipids on PAK activation is independent of Rac and Cdc42 (Bokoch et al., 1998). Sphingosine and related long-chain sphingoid bases increase the autophosphorylation of PAK1 and the overall activity towards exogenous substrates in vitro to levels similar to that achieved with Cdc42 and Rac1 (Bokoch et al., 1998). However, the precise sites within the p21-GTPase-binding
domain or CRIB that lipids interact with PAKs is unclear. Thus, sphingolipids can independently activate PAK1 through a GTPase-independent mechanism.

Other proteins known to regulate the activation of PAKs include the neurofibromatosis 2 tumor suppressor gene (Nef), filamin (FLNA), and heregulin (HRG). Nef is a small and multifunctional protein encoded by the human immunodeficiency virus type I (HIV-1) and HIV-2 (Wei et al., 2005). Nef can bind to the regulatory subunit (p85) of PDK1 that is required to activate PAKs (Linnemann et al., 2002). Filamin (FLNA) facilitates the formation of an internal network of protein filaments that comprise of the cytoskeleton. FLNA induces PAK1 activation through binding to the CRIB domain, in a mechanism that is independent of small GTPases (Vadlamudi et al., 2002). Lastly, HRG can trigger the rapid stimulation of PAK activity, through its interaction with actin and its redistribution into the leading edges of motile cells (Adam et al., 1998). Other examples of upstream activators and signals include, JAK2 (prolactin-activated tyrosine kinase), fibroblast growth factor, and platelet-derived growth factor (He et al., 2001; Rider et al., 2007; Shin et al., 2002).

1.2.2.4 Actin Cytoskeleton: Key Downstream Effector of PAKs

As mentioned earlier, PAKs can exert multiple cellular effects, including gene expression, cell proliferation and survival, and cytoskeletal organization (Rane and Minden, 2014). However, in the brain, most studies have been focused on the actin cytoskeleton; therefore, I will mainly discuss downstream effectors of PAKs that are involved in actin regulation, with a brief description of other molecules.

Myosins are cytoskeletal regulatory proteins involved in the transport and localization of cargo within dendrites that interact with actin (Hanus and Ehlers, 2016). In muscle cells, PAKs lead to the formation of polymerized actin structures such as lamellipodia and filopodia, but also result in the dissolution of stress fibers, which are linked to focal adhesions. The regulation of smooth muscle contraction is activated by phosphorylation at Ser-19 of the regulatory light chain subunits of type II myosin light
chain (MLC) (de Lanerolle and Paul, 1991; Wilson et al., 2008). PAKs have been shown to form inputs on this pathway by inhibiting the activity of myosin light chain kinase (MLCK) (Sanders et al., 1999; Wirth et al., 2003). Studies have demonstrated that PAKs can mediate the phosphorylation of MLCK, which causes a decrease in MLCK activity by 50-60% and MLC phosphorylation by 80-90% (Sanders et al., 1999; Zeng et al., 2000). Myosins are also important in controlling cell contractility in non-muscle cells. In neurons, the phosphorylation of MLC by PAK leads to an increase in actomyosin contractility, stabilized polymerized actin, and also contributes to dendritic spine formation (Zhang et al., 2005). Furthermore, the activation of PAKs and MLC were found to rescue the spine and synaptic morphological defects in the knockdown of GIT1 in hippocampal neurons, which suggest that both PAK and MLC are downstream of GIT1. Other studies have shown that PAKs can phosphorylate the regulatory MLC of myosin VI, a nonconventional myosin involved in membrane trafficking and cell migration. Therefore, these studies suggest that PIX, GIT1, Rac, and PAKs form a complex that modulates MLC activity in the regulation of dendritic spines and synapse formation (Rane and Minden, 2014).

Another key target of PAKs is ADF/cofilin. They are actin-binding proteins that act to sever preexisting actin fibers, block barbed-end elongation, and promote depolymerization (Pavlov et al., 2007). Therefore, they are potent regulators of actin reorganization, particularly in the spines (Figure 1.4). Importantly, the activity of cofilin is regulated through the direct phosphorylation of Ser-3 by LIM-domain containing kinases (LIMK), which renders it in its inactive form and thus, unable to bind to actin (Yang et al., 1998). LIMKs are the key substrates of PAKs. Thus, PAKs can directly phosphorylate LIMKs at Thr-508 and increase the kinases activity of LIMKs to inactivate ADF/cofilin, therefore promoting F-actin stabilization (Ohashi et al., 2000) (Figure 1.4). Cofilin phosphorylation is also regulated by cofilin phosphatases such as slingshot phosphatases (SSH), which can also be phosphorylated and activated by PAKs and ultimately, promote F-actin dynamics (depolymerization) (Eiseler et al., 2009). Cofilin phosphorylation can also lead to the activation of phospholipase D1 (PLD1), an enzyme that is essential for the chemotaxis of phagocytic cells (Han et al., 2007). The increase in PLD1 increases membrane phosphatidic acid and activates DOCK (dedicator of cytokinesis) proteins, which are a family of membrane-associated GEFs for Rac1 (Nishikimi et al.,
Thus, cofilin can regulate the actin cytoskeleton either directly or through the activation of Rac1 as a feed-forward mechanism.

1.2.2.5 Other Pathways Affected by PAKs

In addition to the actin cytoskeleton, a number of signaling pathways are affected by PAKs (Figure 1.6). Mitogen-activated protein (MAP) kinases are central targets involved in intracellular signaling cascades that facilitate the transmission of signals from Ras GTPases to various cytoplasmic compartments. The vertebrate MAP kinase cascade is comprised of four cascades, including ERK, JNK (c-Jun amino-terminal kinase), p38, and BMK, which all contain the TXY MAP kinase kinase motif in the activation loop and is dually phosphorylated by members of the MAP-kinase family (Abe et al., 2002). Initial studies in Ste20 budding yeast demonstrated that PAK signals from Cdc42 to the pheromone-responsive MAP kinase and to the extracellular signal-regulated kinase (ERK) pathway (MEK), which together, is important for cell proliferation and differentiation (Zhao et al., 1995). Although PAK signaling also stimulates MAP kinase in mammalian cells, the mechanisms of PAK activation is different in that it feeds into the canonical pathway through Raf-1, a major target of mammalian Ras (Field and Manser, 2012). Raf1 and MEKs can be activated by PAKs through Ser-338 or Ser-298 phosphorylation, respectively, which suggests that the Raf-MAP-ERK cascade is important for the induction of processes involved in the signal transduction of mitogenic signals within the cell (King et al., 1998; Slack-Davis et al., 2003; Wang et al., 2013b). PAK can also indirectly phosphorylate ERK at Thr-202 and Tyr-204 which results in its recruitment to the cell membrane (Payne et al., 1991). However, a study has demonstrated that group II PAKs are regulated at the activation loop by MAP kinase kinase 6 (MKK6), which is an upstream activator of p38 MAP kinase (Kaur et al., 2005). Since group II PAKs lack the AID domain and do not require the binding of Cdc42 for activation, the authors show that group II activation can be initiated by the phosphorylation of Ser-560 in the activation loop of the catalytic kinase domain.

PAKs can also activate Akt, a serine/threonine kinase, in a kinase-independent manner by aiding the recruitment of Akt to the cellular membrane to promote cell growth and survival (Higuchi et al., 2008).
Studies have revealed that Akt is activated downstream of Rac and has an essential role in promoting cell motility without affecting actin reorganization at the leading edge in mammalian cells (Higuchi et al., 2001). For full activation, Akt requires phosphorylation at Thr-308 located in the kinase activation loop and Ser-473 located in the C-terminal hydrophobic motif (Mao et al., 2008). Akt is translocated to the membrane where it is phosphorylated at Thr-308 and subsequently activated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Alessi et al., 1997). Higuchi et al., propose that PAK activates Akt by at least two mechanisms. Firstly, PAK promotes translocation of Akt to the plasma membrane. Secondly, PAK functions as a scaffold protein to facilitate the formation of the complex between PDK1 and Akt. The growth factor-dependent association of PAK with PDK1 and Akt can be attributed to conformational changes in PAK. In this model, growth factor stimulation causes the activation of Rac, which binds to the N-terminal CRIB domain of PAK and releases the C-terminal domain, resulting in its association with Akt and PDK1. Thus, the scaffolding function of PAK is strictly regulated by upstream signals in a spatially and temporally restricted manner. Although PAK is able to activate Akt, other studies have shown that both Akt and cPDK1 can phosphorylate and activate PAK1 (Menard and Mattingly, 2004; Zhou et al., 2003). Thus, there appears to be a bi-directional feedback between PAKs and PDK1/Akt (Higuchi et al., 2008).

1.2.3 The Biological Function of PAKs Across Organisms

1.2.3.1 PAK Function in Protozoa

In protozoa, PAKs are responsible for regulating cell shape and polarity through the phosphorylation of various cytoskeletal proteins, including microtubule binding proteins, myosins, and septins. It is proposed that PAKs in protozoans are similar to group 1 PAKs but lack the key inhibitory motif within the AID and displaces the kinase activation loop (Lei et al., 2000). Relatively, protozoan PAKs are much more diverse compared to metazoans. Saccharomyces cerevisiae have three conventional PAKs, including Ste20, Cla4, and Skm1. These PAKs contain a typical CRIB/AID motif located at the N-terminal relative to the kinase domain and act downstream of Rac. Protozoa express forms of PAKs that contain lipid binding PH domains including Cla4p and Skm1p (Park and Bi, 2007). Ste20 is an essential protein involved in the mating pathway upstream of the MAP kinase cascades (Dan et al., 2001). Cla4 is involved in septin ring assembly, actin polymerization, and mitotic entry and exit. Cla4
participates in a negative feedback loop to end the polarized growth phase to ensure proper budding and cytokinesis (Benton et al., 1997; Keniry et al., 2004). The loss of Ste20 results in sterility, while the deletion of Cla4 leads to aberrant cytokinesis, and the deletion of both Ste20 and Cla4 kinases is lethal (Cvrckova et al., 1995). Fission yeast such as *Schizosaccharomyces pombe* contain two PAK kinases, PAK1 (Shk1 or Orb2) and PAK2, which are effectors of Cdc42 (Marcus et al., 1995). The loss of PAK1 in fission yeast leads to actin defects which affect the microtubule cytoskeleton, mating, and results in a loss of polarity (Kim et al., 2003). The loss in Skb15, a highly conserved repeat protein which negatively regulates Shk1 in fission yeast, results in deficits in the actin cytoskeleton organization, chromosome segregation, and cytokinesis. Studies have demonstrated that the microtubule regulator, Tea1, is a critical downstream effector of PAK1 (Kim et al., 2003). Thus, the deficits associated with the knockdown of Skb15 are suppressed by Tea1 loss of function (Kim et al., 2003). The amoebae *Dictyostelium discoideum* contain DdPAKa, DdPAKb, and DdPAKc. DdPAKa is regulated by PDK1 and Akt at Thr-579 (Chung et al., 2001). DdPAKa-null cells display defects in myosin II assembly and directional motility (Kumar et al., 2009). DdPAKb is activated by Rac isoforms and acidic lipids (Brzeska et al., 2001). The loss of DdPAKb results in mild chemotaxis defects (Lee et al., 2004). DdPAKc belongs to the Cla4 class of kinases with a N-terminal PH domain. DdPAKc-null cells exhibit defects in cell polarization and pseudopodia formation but normal cell motility. Strains that exhibit loss of both DdPAKb and DdPAKc demonstrate a severe loss of cell movement within the chemotaxis pathway (Lee et al., 2004).

## 1.2.3.2 PAK Function in Invertebrates

Within *Drosophila melanogaster*, three PAKs exist which include dPAK1, the mushroom bodies tiny (Mbt)/dPAK2, and dPAK3 (Zhao and Manser, 2012). Signaling in *Drosophila* are initiated by Trio, a GEF, that activates dRac, dPAK1, and Dock (Nck) (Newsome et al., 2000). This cascade is important for relaying external signals from the receptors to the actin cytoskeleton. The Nck/Dock-dPAK1 signaling complex regulates the movement of photoreceptor axons under the guidance of a growth cone at the leading edge of the sensorimotor axon. Other studies have shown that dPAK1 plays a role in defining the size and shape of the *Drosophila* embryonic salivary gland lumen by regulating the size and elongation of the cells via the regulation of E-cadherin levels at the adherens junctions and
basolateral membrane (Pirraglia et al., 2010). Furthermore, mutations in dPAK1 prevents the elongation of the follicular epithelium, which is composed of F-actin bundles, and covers the developing egg chambers during oogenesis. In all these examples, the activation of Nck/Dock signaling initiates the translocation of the complex to the plasma membrane, where it increases the local activity of intracellular proteins such as Rac and the subsequent activation of dPAK1. Compared to dPAK1, Mbt/dPAK2 are involved in photoreceptor cell morphogenesis; specifically the proliferation, differentiation, and survival of neuronal cells (Melzig et al., 1998). Mbt-null mutant flies have fewer neurons overall and demonstrate defects in the connectivity between brain regions. It has been shown that the actin depolymerization factor, Twinstar/Cofilin, within the Mbt signaling cascade plays an important role in the organization of the actin cytoskeleton and adherens junctions (Menzel et al., 2007). Lastly, dPAK3 function is the least characterized but its loss affects bouton morphology, microtubule distribution, and synaptic transmission (Ozdowski et al., 2011).

There are three PAK-related protein isoforms within Caenorhabditis elegans, including CePAK1, CePAK2, and CePAK3/MAX-2 (Zhao and Manser, 2012). CePAK1, which is similar to group I PAKs, is expressed in the hypodermal cell boundaries during embryonic body elongation and colocalizes with CeRac1 and CeCdc42 (Chen et al., 1996). Studies have demonstrated that CePAK1 and CePAK3 function with the Rac GTPases in axon guidance for cell migration (Lucanic et al., 2006). It has been found that the morphogenesis of the C. elegans gonad requires a CePAK1/CePIX/CeGIT1 complex but not the small G-proteins (Lucanic and Cheng, 2008). Furthermore, CePAK1 can be used to promote the maturation of a hemidesmosome, which is an integrin-linked desmosome that can resist mechanical stress, through the coordination of the morphogenesis of epidermal and muscle tissues (Zhang et al., 2011). Specifically, the tension exerted through changes in mechanical pressure maintains GIT1 at hemidesmosomes and stimulates PAK1 and Rac-bound to PIX. CePAK3 has been found to be important for motor neuron axon guidance in a Rac-independent manner. It is particularly important for the guidance of ventral cord commissural motoneuron axons (Lucanic et al., 2006). CePAK2 shares extensive homology with MBT in that both PAK proteins lack a N-terminal proline-rich Src homology 3 (SH3) domain binding motif, which mediates the binding to the Nck adaptor protein (Bokoch et al., 1996).
1.2.3.3 PAK Function in Vertebrates

Within zebrafish, there are six forms of PAKs termed PAK1, PAK2a, PAK2b, PAK4, PAK5, and PAK6. Additionally, zebrafish have three PIX genes that are ubiquitously expressed and abundant within the nervous system (Tay et al., 2010). The role of each of the isoforms is unclear, but recent studies have shown that PAK2a and PAK2b genes are important for regulating autonomous endothelial cells within brain vessels during vascular development (Liu et al., 2007). Specifically, a loss-of-function of PAK2a is associated with cerebral hemorrhage without other obvious phenotypes (Buchner et al., 2007). PAK2a is activated via the activation of Rho GTPases, βPIX, and GIT1; and required for cerebrovascular stabilization (Liu et al., 2012a). Other studies have shown that the deletion of PAK4 inhibited cardiomyocyte proliferation and angiogenesis (Peng et al., 2016). Furthermore, these studies demonstrate that Rac1 and Cdc42 are not upstream effectors of PAK4 in the heart.

Within *Xenopus laevis*, the definitive number of PAKs remains unknown due to the incomplete classification of the genome. Some studies have proposed that there are four genes in the genome, including xPAK1, xPAK2, xPAK3, and xPAK5, which are important in oocyte maturation and embryonic development. The activation of Rho GTPases such as Cdc42 aid in oocyte maturation through the transition into phases of meiosis. It has been found that xPAK1 regulates oocyte maturation during prophase of the cell cycle, as well as overall survival and death. The dn expression of xPAK1 in *Xenopus* oocytes has resulted in heightened caspase activity and promoted apoptosis during embryogenesis. Compared to xPAK1, xPAK2 is inactivated following Cdc42 phosphorylation and the stimulation of the MAP kinase pathways during maturation (Cau et al., 2000). Studies on xPAK3 have demonstrated its importance during the differentiation of neuronally programmed cells through a transcription factor, neurogenin (Souopgui et al., 2002). Thus, together xPAK1/2/3 all support cell survival. Lastly, xPAK5 is activated by extracellular calcium and binds with actin and the microtubule network in the regulation of cell adhesion and movement (Luo et al., 2007).
Within humans, the six subtypes of PAKs are similarly classified as two major groups. Human group I PAK isoforms contain a N-terminal regulatory domain which consists of a GTPase binding domain (CRIB) and an AID. Group I PAKs can be activated in a Rho GTPase dependent or independent manner. All human PAKs contain a highly conserved C-terminal kinase domain. Group II PAKs exist as monomers and the kinase domain was found to be constitutively active (Eswaran et al., 2007). The binding by Rho-family GTPases induces translocation of the group II PAKs to different cellular compartments. Human PAKs play an important role in actin organization, cell cycle motility, apoptosis, and cell survival (Kumar et al., 2009).

I have briefly discussed the functions of PAK in mice that are relevant to its pattern of expression and significance in sections 1.2.1 and 1.2.2. Therefore, I will expand on those discussions in this specific section. The PAK1 KO mice have normal behaviour, fertility, and viability (Asrar et al., 2009). The PAK1 null mice demonstrate an inability to disassemble F-actin following allergen stimulation, suggesting the importance of PAK1 in mast cell degranulation, through the regulation of F-actin disassembly in the immune system (Allen et al., 2009). The PAK1 KO mice also have defects in glucose homeostasis, specifically higher fasting glucose levels, that are attributed to the signaling defects in islet cells during insulin secretion (Wang et al., 2011). Therefore, these null mice suggest that PAK1 may play a role in glucose metabolism and may represent a therapeutic target in certain forms of diabetes. Other studies have examined the effects of PAK1 KO specifically in the cardiomyocytes of mice. The mice were found to be healthy but demonstrated a progression from ventricular hypertrophy to heart failure that was associated with pulmonary edema (Vinciguerra et al., 2009). Specifically, the pressure overload within the heart is associated with the JNK cascade, linking PAK1 with the MAP kinase pathway in ventricular hypertrophy (Liu et al., 2011). Within the brain, PAK1 KO mice demonstrate deficits in LTP in hippocampal CA1 synapses, despite normal basal and presynaptic transmission (Asrar et al., 2009). The PAK1 KO mice exhibit deficits in the actin cytoskeleton that are accompanied by the mis-regulation of phosphorylated cofilin in the dendritic spines. PAK2-null mice have resulted in embryonic lethality at E. day 8 due to defective vascularization (Arias-Romero and Chernoff, 2008). PAK3-null mice display normal dendrite and spine morphology, and basal synaptic strength, but reduced L-LTP (Meng et al., 2005). Interestingly, phosphorylated CREB is reduced in the PAK3 KO mice suggesting a role for PAK3 in CREB regulation. In PAK1/3 double KO mice, the
deficits are much more pronounced that the single KO mice. These include reduction in brain volume, alterations in neuronal morphology, impaired synaptic transmission and LTP, and deficits in spatial learning and memory (Huang et al., 2011). In the PAK1/3 KO mice, cofilin-dependent actin regulation is also impaired more severely than in the single KO mice. Together, these results suggest that PAK1/3 regulate brain structure, neuronal morphology, synaptic function, and behaviour.

Various mouse models have also been created for the group II PAKs to explore their in vivo function and behavioural outcomes. PAK4 KO mice display embryonic lethality at embryonic day 11.5. PAK4 KO embryos demonstrate a thinning of the myocardial walls of the heart, this is accompanied by the pooling of blood, which result in impaired ventricular functioning (Liu et al., 2008). Within the nervous system, the PAK4 KO mice display neuroepithelia or transparency in the hindbrain and forebrain (Zou et al., 2011). The conditional KO of PAK4 in the brain result in altered brain anatomy that may be due to a decrease in the differentiation and proliferation of neural progenitor cells (Qu et al., 2003). Whereas, PAK5 KO mice are viable, healthy, and fertile (Nekrasova et al., 2008). Similarly, PAK6 KO mice do not exhibit any overt abnormalities in their development and behaviour. However, PAK5/6 double KO mice showed specific learning and locomotor deficits although they were healthy and viable without obvious structural and morphological abnormalities in the brain (Nekrasova et al., 2008). Furthermore, the PAK5/6 double KO mice had a reduced number of neural progenitor cells, likely through their effects on the cytoskeletal changes in the brain.

In summary, PAKs are key signaling protein kinases that regulate multiple cellular processes, particularly the actin cytoskeleton and actin-based processes. PAKs can be activated by various signals, including synaptic neurotransmitter receptors and adhesion molecules mainly through the Rho GTPases. PAKs affect actin by targeting multiple effector proteins such as LIMKs, cofilin, and MAP kinase. Both in vitro and in vivo studies in various model systems all have indicated that PAKs are critical for neuronal development and function, synaptic plasticity, and memory. Therefore, it is not surprising that PAKs and associated signaling proteins have also been implicated in various neurological and mental disorders, which will be discussed in the following section.
1.2.4 PAKs in Neurodevelopmental Disorders

1.2.4.1 PAK Signaling in ASD

Various candidate genes have been identified via genetic screening and post-mortem studies to be associated with ASD. A network-based functional analysis of genetic associations has identified a large biological network of rare genetic variants on several chromosomal loci (2q, 5, 7q, 15q, and 16p) in autism (Gilman et al., 2011; Li et al., 2012). Specifically, the genes and their signaling proteins were found to be important for synapse development, axon targeting, and neuron motility. PAKs and LIMKs, which regulate the dynamics of the actin network were found to be part of the highest scoring cluster using the network-based analysis of genetic associations of rare de novo copy number variation regions observed in autistic individuals (Gilman et al., 2011). Other mutated versions of genes involved in the upstream or downstream regulation of Rho-family GTPases, including GAPs and GEFs, are also candidates of genome-wide association studies (Ba et al., 2013). Hence, PAKs may influence autistic phenotypes through their role in either dendrite and/or axon signaling processes.

1.2.4.2 PAKs in Fragile X Syndrome

Fragile X syndrome (FXS) is the most commonly inherited form of ID and autism (Hayashi et al., 2007; O'Donnell and Warren, 2002; Pietropaolo, 2014). FXS is a genetic diagnosis affecting 1 in 1250 males and 1 in 2500 females (Crawford et al., 2001). It is a multi-organ disease with widespread effects leading to secondary effects including; macroorchidism in males and premature ovarian insufficiency in females (Ascano et al., 2012; Brennan et al., 2006). FXS patients can also exhibit an increased incidence of seizures, anxiety, depression, and subtle motor impairments (Basuta et al., 2011; Berry-Kravis et al., 2007; Chonchaiya et al., 2012). This neurodevelopmental disorder is caused by the loss of the fragile X mental retardation protein (FMRP) encoded by the fragile X mental retardation 1 (FMR1) gene (Verkerk et al., 1991). Clinical studies reveal that patients with FXS have increased expansion and hypermethylation of trinucleotide (CGG) repeats within the promoter of FMR1 (Fu et al., 1991; Kremer et al., 1991a; Kremer et al., 1991b). FMRP is predominantly expressed within the cytoplasm of cells and have been implicated in translational processes including RNA interference and RNA subcellular localizations by facilitating nucleo-cytoplasmic shuttling (Devys et al., 1993; Eberhard and
Grummt, 1996; Fridell et al., 1996). Human studies have revealed that FXS patients demonstrate abnormal dendritic spine development which result in morphological changes including an abundance of long, thin, and immature spines (Hinton et al., 1991; Irwin et al., 2001). Recent studies have demonstrated that the inhibition of PAK1, either genetically or pharmacologically, can ameliorate the cognitive and social deficits in several animal models of fragile X syndrome (Dolan et al., 2013; Hayashi et al., 2007). Specifically, in these studies, the antagonism by the dn form of PAK postnatally on FMR1 KO mice was able to reverse the FXS-related abnormalities related to synaptic plasticity and morphology (Dolen et al., 2007). Behaviourally, FMR1 KO mice also display phenotypes including hyperactivity, repetitive behaviours, and seizures (Spencer et al., 2011). Other studies reveal that FMR1 KO mice also show defective Rac and PAK signaling (Chen et al., 2010). Thus, this study proposes that PAK may repress FMRP’s activity by phosphorylating FMRP itself or phosphorylating an upstream regulator or a downstream effector of FMRP. This is further corroborated by FRAX486, a potent PAK inhibitor, that has been proposed to be a potential therapy for adults with FXS. The treatment with FRAX486 reversed the seizures and behavioural abnormalities and restored the density of dendritic spines within the cortical neurons in the FMR1 KO mice (Dolan et al., 2013). This suggests that PAK inhibition could be used as a potential post-diagnostic therapeutic target. Furthermore, FMR1 KO mice exhibit enhanced basal ERK phosphorylation, which is important for the regulation of spine morphology, synaptic plasticity, and behaviours (Dolen et al., 2007). Since ERK is phosphorylated and activated by PAK, the inhibition of PAK normalizes the levels of phospho-ERK in FMR1 KO mice to basal levels (Eble et al., 2002). Alternatively, FMRP may antagonize PAK-mediated signaling by binding and repressing the translation of mRNAs encoding Rac1 (Hayashi et al., 2007).

1.2.4.3 PAKs in ID

This is supported by human studies that have demonstrated that 25-35% of patients with ID have genetic associations with nonsense or missense mutations on the X chromosome resulting in loss of function in the PAK3 protein. These individuals often express mutated versions of genes involved in Rho signaling or PAKs, especially PAK3 (Allen et al., 1998). Mutation screening of the whole coding region within the PAK3 gene has revealed a missense mutation in exon 2 of the N-terminal end of PAK3 (R67C) which plays a major role in the Rho GTPase binding and stimulation of PAK activity (Bienvenu et al., 2000). Other kinase mutations including R419X and A365E have been shown to
completely inactivate the catalytic function of the kinase (Kreis et al., 2007). A number of mouse models have thus been generated based on the PAK genes to investigate the in vivo role of these genes and how their mutations contribute to ID (Allen et al., 1998; Boda et al., 2004; Dubos et al., 2012). Hayashi et al., (2004) generated a transgenic mouse model to inhibit the catalytic activity of group 1 PAKs in the postnatal forebrain. The dnPAK transgene, which consisted of an CaMKIIα promoter, amino acids 74-146 encoding the AID of PAK3, and SV40 intron/polyA, was used to create the mouse model (Hayashi et al., 2004). The dnPAK transgenic mice displayed decreased spine density and an increased proportion of large synapses in cortical pyramidal neurons. Surprisingly, these mice showed enhanced AMPA and NMDA receptor-mediated synaptic transmission and LTP (Hayashi et al., 2004). Behaviourally, the dnPAK mice exhibit specific impairments in the consolidation and retention of hippocampal-dependent memory in a fear conditioning paradigm. Thus, the data suggests that PAKs in general play a role in synaptic structure and plasticity and cognitive processes. PAK1 and PAK3 single KO mice were previously created by replacing a part of the coding and adjacent upstream/downstream intronic sequence by a pgk-neomycin resistant cassette to completely eliminate the entire kinase domain of PAK1 and 3 respectively (Asrar et al., 2009; Meng et al., 2005). PAK1 KO mice revealed normal brain anatomy but selective deficits in LTP at hippocampal CA1 synapses with changes in levels of the actin binding protein, cofilin (Asrar et al., 2009). PAK3 KO mice resulted in no deficits in either cofilin activity or the actin cytoskeleton with mild impairments in late-phase LTP and learned taste aversion (Meng et al., 2005). In a recent study, Huang et al., (2011) investigated the role of PAKs by analyzing double KO mice lacking both PAK1 and PAK3 to eliminate functional redundancy. The double KO mice were generated by crossing previously characterized single PAK1 and PAK3 KO mice (Meng et al., 2005; Asrar et al., 2009). The double KO mice exhibited impaired postnatal brain growth, deficits in dendritic arbourization and spine morphology, with alterations in synaptic transmission and plasticity (Huang et al., 2011). Furthermore, the double PAK1/3 KO mice displayed a host of behavioural deficits consistent with ID-like symptoms including hyperactivity, increased anxiety, and learning and memory deficits. The alterations in spines were rescued by blocking cofilin activity suggesting that cofilin-dependent actin regulation may mediate the effect of PAK1 and PAK3. Actin, the major cytoskeletal component in dendritic spines, is known to regulate a host of synaptic properties particularly postsynaptic receptor trafficking and spine morphology (Meng et al., 2004). Since the genetic deletions of either PAK1 or PAK3 in mice have only produced modest effects, PAK1 and
PAK3 appear to have at least some overlapping functions in synaptic and cognitive regulation (Asrar et al., 2009; Huang et al., 2011; Meng et al., 2005).

1.2.4.4 PAKs in Other Brain Disorders

Genetic studies have revealed that WBS is caused by the deletion of a 1.5-million-bp-segment on chromosome 7 (7q11.23) (Pober, 2010). This microdeletion is caused by misalignment of chromosome 7, a region spanning 28 genes flanked by highly homologous clusters of genes during meiosis that results in unequal homologous recombination and consequently deletion (Pober, 2010). Genetic association studies have implicated a variety of the deleted genes to contribute to the cognitive and behavioural profile of WS. One of the most well-studied genes is LIMK1 (Frangiskakis et al., 1996), which as I discussed earlier, is a key target of PAKs and a potent regulator of actin dynamics via phosphorylating and thus inactivating ADF/cofilin (Arber et al., 1998; Sumi et al., 1999; Yang et al., 1998). Animal studies showed that LIMK1 KO displayed deficits in locomotion, fear responses, and visuospatial cognition (Meng et al., 2002; Todorovski et al., 2015). In addition, LIMK1 KO mice were altered in ADF/cofilin activity, reduced F-actin, impaired L-LTP and spine deficits. Taken together, these studies suggest a critical role for PAK-LIMK signaling in pathogenesis of WBS.

Abnormalities in PAKs and associated proteins have also been found to be linked to schizophrenia. Missense mutations in PAK3 have been associated with schizophrenia involving premorbid non-syndromic ID (Morrow et al., 2008). Other studies have found that perturbations of the Cdc42/PAK1 and Rac1/PAK1 pathways in the anterior cingulate cortex may enhance MLC phosphorylation, which are associated with actin cytoskeletal dysfunction and spine loss in schizophrenia (Rubio et al., 2012). In a recent study, Ayalew et al., (2012) used a translational convergent functional genomics approach to identify and prioritize the possible genes involved in schizophrenia. Through gene-level integration of genome-wide association data combined with gene expression studies in humans and animal models, these authors identified top candidate genes, such as Disrupted-in-Schizophrenia 1 (DISC1) (Ayalew et al., 2012). DISC1 is a scaffolding protein with numerous binding partners that facilitate the formation of protein complexes (Camargo et al., 2007; Morris et al., 2003). DISC1 is expressed systemically in
the heart, liver, kidney, and thymus, but highly enriched in the cerebral cortex, hippocampus, hypothalamus, amygdala, cerebellum, and olfactory bulbs in the brain (Austin et al., 2004; Brandon et al., 2009; Ma et al., 2002). The DISC1 gene was first identified as a risk factor for mental disorders including schizophrenia, schizoaffective disorder, recurrent major depression, and adolescent conduct and emotional disorder, in a Scottish pedigree that displayed a translocation between chromosomes 1 and 11 (q42;q14.3) (St Clair et al., 1990). The first DISC1 mouse model was engineered based on a spontaneous 25-bp deletion in exon 6 of the DISC1 gene in a 129S6 strain that abolished the production of the full-length DISC1 protein (Koike et al., 2006). The DISC1-deficient mice exhibit cognitive dysfunction specifically in spatial working memory (Koike et al., 2006). In recent studies on the same DISC1-deficient mice display impaired dendritic growth that are accompanied by changes in short-term plasticity at mossy fiber/CA3 synapses (Kvajo et al., 2011). Furthermore, Juan et al., (2014) reveal decreased neuronal excitability and morphological alterations in layer II/III pyramidal neurons in the medial prefrontal cortex in the same DISC1-deficient mice. Interestingly, DISC1 has a regulatory role in the postsynaptic density in association with the NMDA-type glutamate receptor and Kalirin-7, a Rac1-specific GEF expressed in the postsynaptic spines (Ma et al., 2001). In addition, the modulation of PAKs using various chemical inhibitors have been shown to ameliorate dendritic spine deterioration in DISC1-deficient mice within the prefrontal cortex both in vivo and in vitro (Hayashi-Takagi et al., 2014). Therefore, PAKs may also play a key role in the pathogenic process of DISC1-related schizophrenia.

Alzheimer’s disease (AD) is a debilitating age-associated neurodegenerative disorder with a prevalence of 4.4% worldwide (Hall and Roberson, 2012; Qiu et al., 2009). The disorder results in a decline of intellectual function begins with impaired communication and ultimately, progresses to interfere with one’s ability to function independently (Janus, 2014). Therefore, as one of the most devastating neurodegenerative disorders, potential biomarkers have been identified via genetic and biochemical techniques. AD has a well-defined neuropathological profile including the presence of extracellular amyloid plaques, accretion of intracellular neurofibrillary tangles, neuronal damage, and death in selected brain regions (Arnold et al., 1991; Braak and Braak, 1994; Naslund et al., 2000; Petersen et al., 1999; Vigo-Pelfrey et al., 1993). Cognitive decline of AD has been directly linked to synaptic dysfunction, neuronal loss, and dendritic spine defects (Ma et al., 2008). With respect to PAKs, studies
have demonstrated a significant loss of PAK1 and PAK3 expression in the cytoplasm of AD brains during the late-stage diagnosis (Zhao et al., 2006). The phosphorylated PAK1 at Ser-141, which is an index of the kinase activity, was reduced by 73% in the AD temporal cortex (Zhao et al., 2006). In amyloid precursor protein (APP) transgenic mice, the protein level of PAK1-3 at the early stages was increased but the total and cytoplasmic levels of active phosphorylated-PAK1 in the old mice was reduced (Nguyen et al., 2008). Furthermore, the AD transgenic mice demonstrated decreased levels of phosphorylated-PAK1 which are accompanied by plaque and tangle formation (Arsenault et al., 2011). This suggests that PAKs may play an important role in AD. To examine how PAKs work in AD, cultured hippocampal neurons were treated with β-amyloid oligomers and it was found that abnormal PAK activation and translocation was followed by the subsequent loss of cytoplasmic phosphorylated-PAK (Ma et al., 2008). The exposure to Aβ oligomers also resulted in a rapid loss of F-actin and dendritic spines. In other in vitro rat hippocampal studies, intracellular inclusion bodies that contain coflin and actin rods were prominent features in the hippocampus and cortex of AD brains (Cichon et al., 2012). The changes in the level of translocated cytosolic phosphorylated-PAK1 and coflin in rat hippocampal cultures, were associated with the reduction in the actin-regulating protein, debrin (Takahashi et al., 2003). Changes in phosphorylated-PAK, coflin and debrin were observed in APP transgenic mice (Zhao et al., 2006). These results suggest that PAK/LIMK/cofilin signaling may play a significant role in the regulation of synaptic defects and memory deficits in AD through affecting actin in the spines. It was also found that the protein subunits of NMDA receptors, specifically GluN2B, were decreased in AD transgenic mice (Bi and Sze, 2002). The receptors allow calcium entry, which can activate GIT1 and Rac GEFs, and subsequently downstream PAK activation in synaptogenesis (Saneyoshi et al., 2008). Therefore, PAK signaling via NMDA receptors may be impaired which cause deficits in synaptic plasticity and learning and memory associated with AD.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder that predominantly affects the basal ganglia. It is characterized by unwanted choreatic movements, behavioural and psychiatric dysfunction, and dementia (Hooghwinkel et al., 1968). The prevalence is roughly 1/15,000 within the Caucasian population, with a mean onset of symptoms beginning at 25 years (Roos, 2010). HD is caused by elongated CAG (cytosine, adenine, and guanine) repeats (36 repeats or greater) encoding a polyglutamine (polyQ) tract in exon 1 of the HD gene (on the short arm of chromosome
4p16.3), HTT (Rubinsztein et al., 1996). The longer the CAG repeat, the earlier the onset of the disease was observed. Specifically, the loss-of-function of the HTT gene might contribute to neuronal toxicity resulting from the polyQ expansion (Rubinsztein et al., 1996). Other studies show that the primary toxicity caused by the mutation of the HTT gene is via a gain-of-function mutation through the intracellular aggregates of the mutant HTT protein (Nagai et al., 2007). Thus, HD shares similar clinical manifestations including behavioural and psychiatric disturbances to AD. It has been shown that the HTT gene prevents PAK2 cleavage by caspase-3 and caspase-8 which normally mediates cell death through the constitutively active C-terminal kinase domain (Luo and Rubinsztein, 2009). Other studies have shown that PAK1 interacts with the HTT protein which can modify the overall toxicity of the HTT protein (Luo et al., 2008). In this study, PAK1 overexpression enhances the toxicity within the neurons and causes the aggregation of the mutant HTT protein. In another study, PAK-interacting exchange factor (αPIX) was identified as a novel mutant HTT interacting protein, which accumulates in the mutant HTT protein aggregates (Eriguchi et al., 2010). Thus, PAKs may play an important role in mutant HTT aggregation within the pathogenesis of HD.

Therefore, changes in the PAK genes themselves or PAK signaling are associated with a wide range of brain disorders, underscoring the significance of PAKs in brain function.

1.2.4.5 Synaptic Mechanisms Underlying PAK Function

1.2.4.5.1 Postsynaptic Mechanisms

The exact mechanisms by which PAKs regulate synaptic function and memory are unclear. However, they may involve presynaptic neurotransmitter release and/or postsynaptic spines. Most studies regarding PAKs at the synapse have been centered on dendritic spines. Specifically, genetic deletions of PAK1 and PAK3 in mice have been shown to be important in postsynaptic dendritic spine morphogenesis through the regulation of cofilin (Asrar et al. 2009; Huang et al. 2011). Other studies have shown that the inhibition of the catalytic activity of PAKs through the expression of a dnPAK3 transgene in the transgenic mice, result in a decreased number of spines and a shift in the synaptic distribution towards larger sizes (Hayashi et al., 2004). These in vivo findings corroborate previously
observed spine anomalies in rat hippocampal organotypic slice cultures transfected with small interfering RNA that mediated the suppression of PAK3 or a dnPAK3 carrying the MRX30 mutation (Boda et al., 2004). These in vitro studies demonstrate an increased number of elongated and tortuous dendritic spines that resemble filopodia. The aberrant structures failed to express postsynaptic densities and did not make synaptic contact with the presynaptic terminals. In another study that explored the role of PAKs in developing cortical networks, the suppression (using a truncated protein containing the AID of PAK3) of the kinase activity of PAK3 in rat hippocampal slice cultures interfered with activity-mediated spine dynamics (Dubos et al. 2012). Moreover, various PAK3 ID-associated mutations (i.e. R419X and A365E) in the catalytic domain altered spine morphology and decreased spine density in the hippocampus, whereas mutations in the regulatory domain (i.e. R67C) decreased spine density alone (Kreis et al., 2007). Thus, the different PAK domains regulate distinct roles in the regulation of the structure and function of the hippocampal synapse. Other studies have found that KO mice of the downstream effectors of PAKs such as LIMK1/2 are severely affected in coflin phosphorylation, spine morphology and LTP (Meng et al., 2002). Specifically, the authors observed thin necks and small heads, which is characteristic of sessile spines in the KO animals. Together, these studies have demonstrated that PAKs regulate spine morphology and synaptic plasticity through the activation of LIMK and subsequent coflin-dependent actin regulation within the spines.

1.2.4.5.2 Presynaptic Mechanisms

In presynaptic terminals, studies suggest that actin dynamics can affect the efficacy of presynaptic neurotransmitter release at the active zone (Morales et al., 2000), and it is proposed that F-actin regulates the dynamic translocation of synaptic vesicles from the reserve pool to the readily releasable pool, as well as in the vesicular fusion to the plasma membrane at the active zone (Cingolani and Goda, 2008). The regulation of actin is likely mediated by Rho GTPases. For example, monomeric GTPases of the Rho family regulate the exocytosis of synaptic vesicles (Brown et al., 1998). Studies in chromaffin cells show that Cdc42 is involved in the calcium-induced rearrangement of cortical actin and secretion of catecholamine vesicles following stimulation (Gasman et al., 1999). In rat brains, Rho, Rac1, and Cdc42 were found to be present in synaptosomes and associated with highly purified fractions of synaptic vesicles (Doussau et al., 2000). In Aplysia neurons, various toxins were used to
inhibit Rho family GTPases that led to reduced neurotransmitter release at synapses. In these studies, Rac1 was found to be important for presynaptic function, specifically for vesicle trafficking, docking, or priming (Doussau et al., 2000). The downstream effector cofilin was also found in presynaptic terminals (Wang et al., 2000) and shown to regulate neurotransmitter release (Wolf et al., 2015). These results suggest that PAKs may play an important role in neurotransmitter release at excitatory synapses through actin, but no direct evidence is available. Interestingly, a recent study has shown that the disruption of PAK1 can also lead to significant impairments in inhibitory postsynaptic transmission possibly through affecting GABA release through an actin-independent mechanism (Xia et al., 2016). Thus, compared to postsynaptic spines, the role of PAKs in presynaptic terminals is less clear. Thus, in this thesis, I will focus on investigating the role of PAKs in presynaptic function at glutamatergic synapses and how it affects social memory.
2 Rationale, Hypotheses, and Objectives

2.1 Summary of Current State of Knowledge

Social cognition is critical for the formation of social relationships and is essential to our everyday physical and mental health. Social impairments are found in wide range of neurological, neurodevelopmental, and neuropsychiatric disorders, including ASD, ID, and schizophrenia. Thus, great effort has been made to identify and understand the cellular, molecular, and circuit mechanisms that govern social behaviour. Both animal and human studies have demonstrated the involvement of the medial temporal lobe in social cognition. In particular, rodent studies have identified several brain regions, including the BLA, striatum, hippocampus, fusiform area, and frontal cortex, to be responsible for modulating the various aspects of social behaviour. However, how these various regions interact is unknown. In addition, the molecular processes involved remain elusive. The EC-DG circuit within the medial temporal lobe has been extensively studied and shown to be critical for spatial learning and memory. However, its involvement in social behavior is unclear. Given that the EC serves as a main interface for information relay between various cortical regions and the hippocampus, it is possible that the EC-DG circuit may play a key role in social behavior, but direct evidence is lacking. The PAK family protein kinases, as major targets of Rho GTPases, have been intensively investigated and shown to be important for synaptic plasticity, spine morphology, and spatial and contextual learning and memory. To date, the studies on PAKs are mainly concerned with the postsynaptic site. Importantly, changes in the PAK genes or related proteins (i.e. Rho GTPases and LIMKs) have been found in many brain disorders characterized by social impairments. However, direct evidence to support the involvement of PAKs in social cognition is still lacking.

2.2 Rationale and Approaches of the Project

Based on the current understanding of different brain regions involved in social memory and the various PAK functions, as discussed in the previous Introduction sections, my thesis project will directly examine the involvement of PAK signaling and the EC-DG circuit in social recognition memory. As illustrated above, many previous studies, including those in our own lab, have used KO models. However, because these chronic KO models can lead to developmental compensation by other proteins and do not have spatiotemporal regulation of gene expression, it would be impossible to
determine whether the behavioral deficits in the KO mice are caused by PAKs by using these mouse models. Neither would it be possible to isolate the specific circuits and time window involved. Therefore, for my thesis project I used two new approaches to overcome these problems: inducible gene expression and optogenetics, which will be briefly discussed below.

First, I used a tetracycline inducible system, which consists of two components: a tetracycline transactivator (tTA) and a tetracycline operator sequence (tetO) (Figure 2.1). A gene of interest (or transgene) is linked to tetO and upon binding by tTA, tetO activates the transcription of the gene. The binding of tTA to tetO can be regulated by tetracycline or its analogs (i.e. doxycycline (DOX)). Therefore, the expression of the gene can be inducibly regulated through the administration of tetracycline or DOX. The expression of the gene can be further regulated by restricting the expression of tTA using cell/region-specific promoters, thus achieving a precise temporal and spatial control of transgene expression. As discussed earlier, I chose to focus on PAK3 because it is predominantly expressed in the brain and its mutations are implicated in both ASD and ID. In previous studies carried out in our lab, we show that PAK1/3 KO mice display impaired spatial learning and memory, anxiety-like behaviour, and hyperactivity, suggesting a role of PAKs in these processes. However, we have yet to examine the role of PAKs in the social behavior. I expressed a mutant transgene of PAK3 (R67C) under the control of the tetO operator in the transgenic mice, so that the expression of the mutant PAK3 can be inducibly controlled by DOX. The R67C mutant is chosen because it is linked to ID and previously shown to have decreased binding to the upstream Rho GTPases (Allen et al., 1998), thus acting in a dominant negative fashion to impair the activation of PAK signaling. To further control the transgene expression, I used tTA transgenic mice where its expression is under the control of the CaMKIIα promoter. This restricts the expression of tTA in excitatory neurons in certain forebrain areas. As described below, this combination of the tTA/tetO tet off system allowed the inducible expression of the mutant PAK3 transgene within the EC-DG circuit. Thus, definitive analysis of PAK signaling in this circuit within the context of social memory was explored.

Second, I used an optogenetics approach (Figure 2.2) to achieve temporal manipulations of the EC-DG circuit to determine the precise time window where the circuit and PAK signaling were involved in
social memory. Optogenetics is a technique that combines genetic and optical methods to manipulate synaptic transmission in specific circuits. In particular, I chose to express ChR2 or ArchT since spatial, temporal, and neurochemical precision can be easily achieved to selectively control the projections from layer II cells of the EC to the DG in a bidirectional manner. Compared to the tetracycline inducible system, the optogenetics approach is more precise in examining the specific temporal and spatial circuitry involved in social memory. ChR2 is a light-gated cation channel that can lead to depolarization and the generation of light-evoked action potentials (Wang et al., 2007). Whereas, ArchT is a light-driven outward proton pump that hyperpolarizes and inhibits action potentials from being fired (Han et al., 2011). Viral expression systems such as adeno-associated viral vectors (AAVs) have fast and versatile implementation with high infectivity rates resulting in robust expression in axons with a high number of projection fibers such as the PP. These AAV-based expression vectors also allow for the integration of promoter elements to limit the expression of the opsins in certain populations of neurons. Therefore, to test if the EC is indispensable in social memory, I silenced the PP through AAV-mediated expression of ArchT under the CaMKIIα promoter. Furthermore, to determine if PAK activity in the EC was sufficient for inducing social memory, I activated the PP to enhance social memory through AAV-mediated expression of ChR2 under the CaMKIIα promoter.

Thus, the use of the tTA/tetO and optogenetics, combined with traditional electrophysiological recordings, biochemical assays and behavior tests, will provide a superior and comprehensive approach and allow me to conclusively address the role of PAK signaling in social memory and its underlying synaptic, molecular, and circuit mechanisms.
Figure 2.1 Tet-off vs. Tet-on Systems. The tetracycline inducible system consists of a Tet response element (TRE) which is made up of the Tet operator (tetO) sequences fused to a minimal promoter. In the tet-off system, which is used in this project, the tetracycline-controlled transactivator (tTA) will bind to the tetO sequences to drive the expression of the gene of interest (i.e. transgene). However, in the presence of a tetracycline or its analog, DOX, tTA will no longer be able to bind to the tetO sequences and this leads to turn off of the transgene expression. In the tet-on system, the tTA is replaced by a new transactivator reverse tetracycline-controlled transactivator, rtTA, and the presence of DOX induces the binding of rtTA with the tetO sequences to drive the expression of the transgene.
Figure 2.2 Optogenetics Approaches. Optogenetics is a technique that can be used to spatially and temporally control the activity of genetically-defined neurons using light. In this schematic, ChR2 is an ion channel that opens in response to 473nm blue-light which allows the influx of cations and depolarizes the EC terminals, resulting in enhanced synaptic transmission along the EC-DG synapse. In response to 532nm green-light, ArchT is an outward proton pump that hyperpolarizes the EC terminals, resulting in the silencing of synaptic transmission along the EC-DG synapse.
2.3 Overall Hypothesis and Specific Aims

The overall hypothesis of the project is that the PAK signaling within the EC-DG circuit regulates social recognition memory through affecting cofillin mediated actin reorganization and synaptic transmission at the PP terminals (Figure 2.3). To test this hypothesis, I will employ a combination of techniques, including the use of transgenic mice, biochemical analysis, electrophysiology, optogenetics, and behavioral tests to achieve five specific aims:

- **Aim 1**: To characterize and validate the transgenic mouse model where the expression of the mutant PAK3 (mPAK3-GFP) transgene is region/cell-specific and reversible, using molecular techniques, including Western blot analysis and immunostaining.

- **Aim 2**: To assess the behavioural consequences of mPAK3-GFP transgenic expression with a focus on social behavior using various behavior testing paradigms including the three-chamber social interaction and social habituation/dishabituation tests.

- **Aim 3**: To examine the synaptic properties of mPAK3-GFP transgenic mice using electrophysiological recording and imaging techniques.

- **Aim 4**: To identify molecular signaling processes by which PAK signaling regulates social memory and synaptic function with a focus on LIMK/cofilin mediated actin changes in mPAK3-GFP transgenic mice using immunostaining and Western blot analysis.

- **Aim 5**: To determine the time window where the EC-DG neural circuit is involved in social memory by optogenetic manipulations of the circuit.

I will discuss each of the five aims with a brief introduction, relevant materials and methods, results, and discussion in the following Chapter.
**Figure 2.3 Overall Hypothesis.** PAK signaling through its interaction with Rac and Cdc42 is important in the regulation of the actin cytoskeleton through the Rho signaling pathway within the EC-DG synapse. As mentioned previously, Rho GTPases including Rac1 and Cdc42 lead to the autophosphorylation of PAKs and subsequently, the activation of the downstream effector, LIMK. The phosphorylation of LIMK inactivates cofilin activity and facilitates actin polymerization. The actin cytoskeleton regulates neurotransmitter release, which may be responsible for social memory processes.
3 Experimental Procedures

Data Attribution- Materials and Methods presented in this chapter were adapted from the following original publication:


3.1 Creation and Housing of Animals

Subject mice were group housed on a 12-hour light/dark cycle with food and water ad libitum. All experimental procedures were conducted during the light cycle (7am-7pm) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and approved by both the Animal Care Committees at the Hospital for Sick Children and the University of Toronto (Toronto, Ontario, Canada). In all experiments, the mice were first coded by an independent investigator prior to experimentation and decoded following the completion of the experiments for data grouping and subsequent analyses.

3.1.1 Plasmid Construction

The PAK3 construct was derived from the pHA-PAK3a plasmid and confirmed with sequencing. The mice were generated based on a R67C mutation from oligonucleotide set: 5’-ccaataagaagaagagagagagatcctctcag-3’ and 5’-ggaagagagatcctcgtggactctttctttctttattgg-3’ leading to the HA-PAK3a-R67C plasmid (Kreis et al., 2007). The R67C mutation is located on the regulatory domain (N-terminal end) of the CRIB. This mutation impairs PAK3 binding to Rho GTPases including Cdc42 and prevents the subsequent activation of PAK3. The BamHI/XbaI fragments of the different HA-tagged PAK3 constructs described above were subcloned into the BamHI/XbaI-linearized pEGFP vector (Clontech, Ozyme, St. Quentin en Yvelines, France), to obtain a GFP-tagged PAK3 plasmid GFP-PAK3-R67C (mPAK3-GFP).
3.1.2 Generation of Transgenic Mice

Similar to previous recombinant cloning techniques, a transgene DNA construct containing the PAK3-R67C tag with GFP (mPAK3-GFP) at its C-terminus under the control of the tetracycline operator sequence (tetO) was made (Meng et al., 2003a; Meng et al., 2002). The transgenic vector was linearized and subsequently, pronuclear microinjected into FVB/N zygotes. From 37 independent founder lines, ten of these founders were crossed with previously characterized CaMKIIα-tTA mice (Jackson Lab) to produce independent double transgenic (dTg) mouse lines for the evaluation of transgene expression. Only one line (#67) showing restricted mPAK3-GFP expression in the EC-DG circuit was further analyzed in this thesis project. The dTg mice were backcrossed to C57BL/6 for 6 generations. To further minimize any potential effect of the genetic background, all the mice used in this thesis project are the littermates derived from hemizygous breeders, which include: WT (tTA- /mPAK3-GFP), single positive transgenic (Tg1: tTA+/mPAK3-GFP and Tg2: tTA/mPAK3-GFP+) and double positive transgenic mice (dTg: tTA+/mPAK3-GFP+). Since no differences were found in any of the measures carried out in the present study between WT and single positive transgenic mice, the data from these genotypes are pooled together and combined as a single WT control group.

3.1.3 Genotyping: Polymerase Chain Reaction

For tail digestion, each tail sample (~1mm) cut from a transgenic mouse (~2-3 weeks old) was digested overnight at 60°C in 500mL of tail lysis buffer (50mM Tris-HCl pH 8, 100nM EDTA, 100mM EDTA, 100mM NaCl, and 1% SDS) and 10uL of 10mg/mL proteinase K (PRK403, Bioshop) in an eppendorf tube. The following day, 190µL of 5M NaCl and 600µL chloroform were added to each tube and centrifuged at 10,000rpm for 10 minutes. The use of chloroform removes proteins from the DNA sample allowing proteins to be denatured and partition into the phenol while the DNA remains in the water phase. Next, 400µL of the supernatant (water phase containing the DNA) was transferred into a new eppendorf tube and an equal volume of 100% ethanol was added and mixed to precipitate the DNA. The sample was centrifuged at 14,000rpm for 5 minutes to spin down the DNA which pelleted at the bottom of the tube. To ensure that all the chloroform was washed out, the supernatant was removed and 300µL of 70% ethanol was added to the pelleted DNA and centrifuged at 14,000rpm for 5 minutes. The remaining ethanol was removed leaving the pelleted DNA to dry for ~1 hour. The pelleted DNA was then dissolved in 100µL of sterile water to a final concentration of roughly 40-50ng/µL.
The following primers (ACGT, Toronto, Ontario, Canada) were acquired: tTA-1 sense: CGCTGTGGGGCATTTTACTTTAG, tTA-2 antisense: CATGTCCAGATCGAAATCGTC, mPAK3-GFP sense: GAACAGTAACAACCGAGACTC and mPAK3-GFP antisense: GGTGACTGCATCAAAACCCAC. The primers were then diluted to a concentration of 150µg/mL with sterile water. For the mPAK3-GFP reaction, a PCR buffer (690µL) comprised of 2mM dNTP (DD0056, BioBasic Inc), 25mM MgCl₂, 10x PCR buffer (500nM KCl, 100mM Tris-HCl pH 8, 1% Triton X-100) and sterile double-distilled water was used, while for the tTA reaction, a PCR buffer (1011µL) comprised of 2.5mM dNTP, 10x Thermo Reaction Buffer (B9004S, New England BioLabs) and sterile double-distilled water was used. To each PCR buffer, 2µL of Taq polymerase (MO267L, New England BioLabs) was added. Each PCR reaction contained 13.5µL of PCR buffer and 1.5µL of template DNA.

The PCR cycling protocols for mPAK3-GFP and tTA were used for all tails to confirm the genotype of each animal. For the mPAK3-GFP PCR protocol, an initialization step at 94°C for 3 minutes was followed by a denaturation step at 94°C for 30 seconds. This was followed by an annealing step of 58°C for 1 minute and an extension/elongation step at 72°C for 1 minute. The denaturation to extension steps were repeated for 33 cycles and subsequently kept at 72°C for 10 minutes and terminated at 4°C. For the tTA PCR protocol, an initialization step at 94°C for 3 minutes was followed by a denaturation step at 94°C for 30 seconds. This was followed by an annealing step of 57°C for 1 minute and an extension/elongation step at 72°C for 1 minute. The denaturation to extension steps were repeated for 35 cycles and subsequently kept at 72°C for 2 minutes and terminated at 10°C.

Following the PCR, to each reaction, 3µL of 6X DNA loading buffer (0.25% bromophenol blue, 0.5M EDTA, 30% glycerol, and sterile water) was added and loaded into a 1% TAE agarose gel (1xTAE 40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8) with 0.003% RedSafe (21141, iNtRON Biotechnology). The DNA bands on the agarose gel were visualized using a UV transilluminator (UVP BioDoc-It Imaging System, California, USA). In addition to polymerase chain analysis, all the mice
used for these quantifications were also confirmed by the presence or absence of the transgene (mPAK3-GFP) expression following all experimentation, using Western blot analysis and/or immunohistochemistry using anti-GFP antibodies (see below sections for detailed explanations for each technique).

3.2 Behavioural Tests

For the following behavioural studies, all mice were bred and subsequently housed in groups of two to five animals in cages (15cm wide x 33cm long x 12cm high) located in a room with an ambient temperature set at 20 ± 1°C with lights on starting at 7am for 12 hours. Animals were tested between 9am and 6pm. Prior to behavioural testing, mice were habituated to the experimenter by handling 2 times each day for 3 consecutive days. For social interaction tests, mice were single housed 6 hours or 24 hours prior to testing. For all tests, C57BL/6J, dTg mutants, or WT groups were sex-balanced. For experiments involving the systemic treatment of DOX, animals were treated continuously with 0.5mg/ml doxycycline hyclate ≥98% (HPLC) (24390-14-5, Sigma-Aldrich) in acidified water, replaced every other day for aminimum of four weeks. For the optogenetic experiments, the mice were between 8-10 weeks old at the time of surgery for the viral injections and optic fiber implantation, and all experiments were completed by the age of six months.

3.2.1 Open Field Test

The Open Field (OFT) apparatus was purchased from ANY Maze (Illinois, USA). The square apparatus is made from clear plexiglass (40cm wide x 40cm long x 40cm high). It was placed 80cm above the floor on a table in the center of a 3.05m x 3.05m room. Each subject mouse was individually tested in one 10-minute session as previously described (Huang et al., 2011). The subject was introduced to the apparatus at the same corner of the arena facing the wall. For the analysis, a square (20cm x 20cm) was drawn within the arena and denoted as center, while the remaining area (surrounding the center) was denoted as periphery. The following behaviours were recorded automatically through the ANY Maze software: locomotion which was measured as the time (in seconds) a subject moved when 70% of its area was within either the center or periphery zone, immobility when the animal does not change location and at least 75% of its body remains static for at
least 2 seconds, and freezing when the system calculated a score below 30% for at least 250ms. Other behaviours were recorded manually via the ANY Maze software such as rearing, where the subject’s body is raised supported only by its hind legs and grooming, which included fur cleaning and/or combing and face or body washing. All behaviours were recorded separately for the central and peripheral fields of the arena.

3.2.2 Elevated Plus Maze Test

The Elevated Plus Maze (EPM) apparatus was purchased from ANY Maze. The subject mice were tested as described previously with slight modifications (Huang et al., 2011). The apparatus is made of four arms; two grey plastic open arms (39.5cm long x 5cm wide) perpendicularly conjoined at a center with two plastic enclosed arms (39.5cm long x 5cm wide x 10cm high walls). Each arm was secured by a table leg that elevated the maze 80cm from the floor. To initiate the test, a mouse was placed in the center of the maze. The ANY maze tracking software was used to record the movement of the animal’s body throughout the maze via an overhead camera for 5 minutes (or 6 minutes in the optogenetic experiments). For the optogenetic experiments, habituated ArchT or control EYFP expressing mice with the optical fibers attached (on the day of testing) received a 3-minute home cage habituation prior to being placed on the center of the apparatus. The mouse was allowed to explore the apparatus without light (from the start of the test to the 2-minute mark). Continuous 532nm green-light (15mW) was delivered bilaterally for 2 minutes (from the 2-minute to 4-minute mark) and the procedure was repeated with light off (4-minute mark to 6-minute mark). For all tests, the time the mouse spent in the open or closed arms was expressed as a percentage of the total time spent in both the open and closed arms.

3.2.3 Three-Chamber Social Interaction Test

Social interaction was assessed using the three-chamber apparatus consisting of three (45cm wide x 20cm long x 30cm high) plexiglass chambers, which was purchased from ANY maze (Kaidanovich-Beilin et al., 2011; Moy et al., 2004). The connected chambers were separated by two removable partitions in the plexiglass walls that allowed animals to freely travel between the three chambers. A
cylindrical wire cage with a diameter of 8cm and height of 17cm with bars spaced 1cm apart was used to house the stranger mice during the test. These cages were placed on the outer two chambers, while the middle chamber was left empty. Prior to the day of testing (~24 hours), the handled subject mice were each habituated to the empty three-chamber apparatus for ten minutes. On the day of testing, each testing session involved three stages; a 10-minute habituation stage (stage 1), followed by a 5-minute sociability or encoding phase (stage 2), and lastly, a 5-minute social memory or discrimination stage (stage 3). Each stage was separated by a 45 second to 1-minute interval where the subject was kept in the middle chamber using the two partitions. Prior to the start of each testing session, the subject mouse was placed in the center chamber for 3 minutes, before the removal of both door partitions in the beginning of the test. In the habituation phase, animals were allowed to acclimate to the entire test area with partitions removed and both wire cages empty. In the sociability phase, a juvenile male/female C57BL/6 mouse (sex matched to the subject) was added to the wired cage on one side of the arena (counterbalanced between subjects), while the other cage was left empty. In the recognition memory phase, a second unfamiliar juvenile C57BL/6 mouse (sex matched to the subject) was added to the empty wired cage. All the stages were recorded using an overhead camera and saved within the ANY Maze software. The amount of interaction was measured by manually scoring the sniff time/direct contact when the animal oriented its nose within 2cm of the mouse contained in the wired cage (a 2cm zone surrounding the wired cage was drawn and defined as an “interaction zone” using the ANY Maze software). The amount of time spent in each of the defined “interaction” zones was also automatically generated via the ANY Maze software and used to confirm the manual result. Climbing on the wire cage was excluded from the scoring. All data presented was expressed as a percentage time spent investigating the target cage over the entire time spent engaged in investigation of either cage. To test the effect of ArchT activation in the EC or CA1 terminals, ArchT or control EYFP expressing mice were assessed with or without continuous bilateral 532nm green-light (15mW), counterbalanced over two days of testing (i.e. half of the mice were tested with the light-on on first day and the other half with light-on on second day). This stimulation paradigm was modified based on previous studies (Okuyama et al., 2016). For the CA1 experiments, ArchT mice were presented with 473nm blue-light (15mW) as control. For the optogenetics experiments, social interaction was assessed using a self-constructed three-chamber apparatus consisting of three (40cm wide x 20cm long x 40cm high) chambers (Aqrabawi et al., 2016). The same wired cages for the strangers were used in these experiments as described above. The green-light was presented during either the 5-minute sociability
stage (stage 2) or the 5-minute discrimination/memory stage (stage 3). To test the effect of ChR2 activation on social discrimination/memory recognition, ChR2 expressing subject mice were given bilateral 473nm blue-light (DG: 20Hz, 5ms pulse width, 6.5mW or CA1: 4Hz, 15ms pulse width, 10mW) in a 30 second light-on followed by 30 second light-off pattern presented during the 5-minute discrimination/memory stage. This ChR2 stimulation protocol was slightly modified based on previous studies (Felix-Ortiz and Tye, 2014; Kitamura et al., 2014; Matthews et al., 2016). To further test the effect of ChR2 in facilitating sociability/memory, the three-chamber testing protocol was slightly modified in which after the 10-minute habituation to the two empty cages, the 5-minute sociability period was reduced to 2 minutes, which was followed by a 5-minute social memory or discrimination stage. In this paradigm, subject mice were given bilateral 473nm blue-light (20 Hz, 5ms pulse width, 6.5 mW) delivered during sociability (stage 2) or discrimination stage (stage 3) in a 30 second on/off pattern only when the subject mice were interacting with the previously encountered stranger (stranger 1). For ChR2 experiments, 589nm yellow-light (DG: 20 Hz, 5 ms pulse width, 6.5mW or CA1: 4Hz, 15ms pulse width, 10mW) in a 30 second light-on followed by 30 second light-off pattern was used as control.

3.2.4 Five-trial Social Interaction Assay

Social memory was assessed using a previously described procedure (Hitti and Siegelbaum, 2014; Kogan et al., 2000). All subject mice were individually housed in covered cages for 24 hours prior to the day of testing, to ensure that other mice in the housing room were out of view. On the day of the testing, the subject mouse was placed in a chamber (45cm wide x 20cm long) and presented with a caged juvenile (3 weeks or younger) mouse matched by sex for four (or six in the optogenetics and cannula infusion experiments) consecutive 1-minute trials with an inter-trial interval of 30-45 seconds. The same wired cage was used as described in the three-chamber social interaction test. On the last trial (either fifth or seventh), a novel caged juvenile stimulus mouse (matched by sex) was presented. In the optogenetics experiments, half of the ArchT or EYFP expressing mice were assessed under 532nm green-light (15mW) off conditions while the other half under green-light on conditions in the fifth trial (familiar mouse) and seventh trial (novel mouse). Conditions were counterbalanced over two separate days. The amount of interaction was manually scored based on the sniff time, which is defined as when
the animal oriented its nose within 2cm of the wired cage. Sniff time was recorded only when the subject’s head entered the 2cm interaction zone surrounding the wired cage. Each subject’s percentage baseline calculation for trials 2-4 or 2-6 was relative to its individual interaction time from trial 1.

3.2.5 Novel Object Test

A square apparatus (same as the one used for the open field test) made from clear Plexiglass (40cm wide x 40cm long x 40cm high) was placed 50cm above the floor. Subject mice were placed facing the wall within a corner of the apparatus, looking away from the objects at the start of the test. Mice were then allowed to explore two objects (designated as object 1 and 2) for 10 minutes, which were placed in the two diagonal corners of the open field apparatus (10cm from the wall) in stage 1. Following an inter-trial interval of 5 minutes, mice were then presented with a familiar object 2 and a novel object 3 for a period of 5 minutes (stage 2). This cycle was repeated with object 3 and another novel object (object 4) for dTg mice. For optogenetic experiments, ArchT or EYFP expressing mice with optical fibers attached received a 3-minute home cage habituation prior to the object recognition test. Continuous 532 nm green-light (15 mW) was delivered bilaterally for 5 minutes during stage 2. The time spent interacting with a particular object (either 2 or 3) was expressed as a percentage of the total time spent interacting with both objects. Object locations and the delivery of light were counterbalanced between test subjects.

3.2.6 Morris Water Maze

Handled mice were tested in the visible and hidden platform versions of the test, as described previously (Huang et al., 2011). The training and the test were conducted in a 130cm diameter pool. The visible platform was marked by a 15cm high flag above the surface of the water in the pool that was attached to a 10cm diameter circular platform submerged 2cm below the surface of the water. The visible test lasted 2 days with 3 trials conducted each day. The acquisition phase of hidden platform, where the flag was removed from the submerged platform, consisted of 3 training days with 3 trials conducted each day, and the probe trial was given 2 hours and 24 hours following the last training session to assess short-term and long-term memory, respectively. For analysis, the area of the pool was
divided into 4 equal quadrants (with a 20cm diameter circle drawn in the center of each) for the analysis and denoted as platform, right, left, and diagonal zones via the EthoVision software (Noldus Information Technology, Amsterdam, Netherlands). The spatial memory for the platform location was evaluated by the dwelling time in the zone defined within the platform quadrant compared to adjacent quadrants. The swim path was recorded by a camera connected to a video tracking system (Noldus Information Technology, Amsterdam, Netherlands) and analyzed with EthoVision software. Each trial began by placing the mouse in the water, facing the wall of the pool. The starting points for each subject were chosen randomly from any of the three quadrants other than the one containing the platform. Each subject was allowed 60 seconds to find the hidden platform. If the subject mouse failed to reach the platform within the designated time, it was guided by the experimenter to the platform. Subjects were allowed to remain on the platform for 10 seconds prior to being dried and returned to their home cage. For each subject, the latency to reach the platform as well as the distance traveled to the platform were recorded and only the latter was presented in the results. In the visible platform version of the water maze, the location of the submerged platform with the flag was varied randomly between four possible quadrants of the pool for each subject and between trials per session. In the hidden platform test, the platform location was always in the same position (northwest quadrant).

3.2.7 Fear Conditioning

3.2.7.1 Contextual Fear Conditioning

To evaluate contextual and associative fear memory, mice were tested as described previously (Aqrabawi et al., 2016; Meng et al., 2002). The conditioning chamber consisted of one closed side (21cm wide × 20cm long × 19cm high) of a shuttle box (Shuttle-Scan SC II, Omnitech Electronics Inc., Columbus, Ohio, USA) with a speaker on the box lid and a light on the side wall. The floor consisted of stainless steel bars that were connected to a computer, which controlled the duration of a test session, timing, intensity, and duration of shock or sound. Background noise was set at 52dB. Each subject was allowed to explore the chamber for 3 minutes before the onset of a discrete conditioned stimulus (CS), which consisted of a continuous sound (3600Hz, 95dB) lasting 30 seconds. During the last 2 seconds of this CS period, a subject was exposed to an unconditioned stimulus (US), a continuous foot shock (0.75mA) for 2 seconds. Three such training sessions were delivered in succession, with 30 second
intertrain intervals. 2 hours and 24 hours after the training, subjects were given a 4-minute contextual conditioning test in the same chamber in which they were trained. Fear responses were assessed by recording the subjects' freezing response using FreezeView2 (Coulbourn Instrument, Whitehall, Pennsylvania, USA) and confirmed by offline manual analyses. Freezing was defined as absence of any locomotor activity, except for respiratory movements, slight head movements, and occasional tail rattling (all within a motion index of 10 or less). The chamber was cleaned with 70% alcohol after each subject was tested. For optogenetic experiments, ArchT or EYFP expressing mice with optical fibers attached received a 3-minute home cage habituation prior to starting the test. For these experiments, 2 and 24 hours after the training, contextual memory was tested with continuous 532nm green-light (15mW) delivered bilaterally.

3.2.7.2 Trace Fear Conditioning

To evaluate contextual trace fear memory, mice were tested as described previously (Kitamura et al., 2014). The same conditioning chamber and speakers were used as discussed above. For the optogenetic experiments in EC-CA1, ArchT, and ChR2 mice with optical fibers attached received a 3-minute home cage habituation prior to training. The mice were placed in the conditioning chamber denoted as “Context A” for 3 minutes, before the onset of a discrete CS, which consisted of a continuous sound (2000Hz, 75dB) lasting 20 seconds. This was followed by a 20-second trace period and a 2-second US foot shock (0.75mA). An additional two training sessions were delivered in succession, with a 2-minute intertrain interval. Mice received continued 532nm green-light (15mW) during each of the 22-second training periods. 24 hours after the training sessions, subjects were placed in a modified conditioning chamber denoted as “Context B” for 3 minutes, followed by the same tone (2000Hz, 75dB) as presented during training for 60 seconds and a post-tone period of 3 minutes. This was repeated two additional times. Fear responses were assessed by recording the subject mouse’s freezing response using FreezeView2 and confirmed by offline manual analyses. Similar to above, freezing was defined as absence of any locomotor activity, except for respiratory movements, slight head movements, and occasional tail rattling (all within a motion index of 10 or less). The chamber was cleaned with 70% alcohol (Context A) or 4% acetic acid (Context B) after each subject was tested.
3.2.8 Olfaction Habituation/ Dishabituation Test

To test olfaction habituation and dishabituation, mice were sequentially presented with different odors as described previously (Yang and Crawley, 2009). On the day of testing, subject mice were individually housed (with cages covered) and deprived of food for 6 hours. Mice were then habituated in a clear standard cage for 30 minutes with a clean applicator (Q-tip) inserted through a hole in the top roof of the cage. Three odors (two non-social odors and one social odor) were tested. Each odor was presented in three consecutive trials for a duration of 90 seconds for each trial with an inter-trial interval of ~1 minute. The two non-social odors included a Q-tip soaked with pure vanilla extract (simple non-social cue) (McCormick, Hunt Valley, Maryland, USA; 1:100 dilution) or 2mL of diluted vanilla extract with 2g of mixed Italian herbs (complex non-social cue) (Heavenly Spices, Montreal, Canada). The social odor was obtained using a Q-tip rubbed along the bottom of a cage that housed unfamiliar mice of the same sex as the subject. The time that mice spent sniffing the Q-tip, designated as when the animal oriented towards the tip with its nose within 2 cm, was recorded manually. Time that mice spent grabbing the Q-tip was not scored.

3.2.9 Food Burying Test

Mice were fasted and evaluated on how quickly they could find a hidden food pellet, as previously described (Yang and Crawley, 2009). Similar to above, on the day of testing, subject mice were individually housed (with cages covered) and deprived of food for 6 hours. A food pellet was hidden 3cm under standard cage bedding in one corner of a clean, previously un-used cage. The subject mouse was placed facing the wall in a corner of the cage away from the pellet. The latency to uncover the food pellet was recorded manually.

3.3 Optogenetics

3.3.1 Design and Fabrication of Optic Fibers

Optic fibers were custom made using a 200µm diameter, 0.39NA fiber cable (M72L02, Thor Labs) that were cut with an electronic caliper into 7.5mm (to target the dorsal DG) or 11.4mm (to target the
ventral CA1) lengths. They were secured to ceramic ferrules (230 µm bore size, 1.25 mm outer diameter) (MM-FER2007C-2300, Precision Fiber Products) using Speed Set Epoxy glue (LePage) and allowed to dry overnight. All optic fibers were autoclaved and subsequently each optic fiber was tested to ensure at least 70% light transmission efficiency.

### 3.3.2 Stereotaxic Surgery

The rAAV5-CaMKIIα: eArchT3.0-EYFP (with a titer of 3.3*10^{12}), rAAV5-CaMKIIα-EYFP (with a titer of 3.3*10^{12}) and rAAV5-CaMKIIα-hChR2 (H134R)-mCherry-WFRE-PA (with a titer of 4.5*10^{12}) viruses were generated and with permission from Dr. Karl Deisseroth, acquired from the University of North Carolina GTC Vector Core (Chapel Hill, North Carolina, USA). The viruses were aliquoted and stored at -80°C before use. The procedure for optogenetic surgery was described previously (Aqrabawi et al., 2016; Nguyen et al., 2014). Prior to surgery, mice were single housed for at least 2 days. Mice were anaesthetized with isoflurane (1.5-2% in 1 L/minute oxygen) and placed onto a stereotaxic frame. Body temperature was maintained at 37°C using a temperature controller. A midline scalp incision was made followed by craniotomies using a 0.6mm drill bit. The AAV viral particles were injected into the EC at AP: -3.80mm, DV: -4.70mm, ML: ±3.70mm relative to bregma for the ECII-DG terminals and AP: -3.80mm, DV: -4.70mm, ML: ±3.50mm relative to bregma for the ECIII-CA1 terminals (coordinates derived from Paxinos and Franklin, 2007); by infusing 0.5µL of the virus bilaterally at a rate of 0.1µL/minute via an internal cannula connected by Tygon tubing to a 10µL Hamilton needle syringe. Following infusion, the internal cannula was left in place for 10 minutes to allow for complete diffusion of the viruses. The custom made optical fibres were then bilaterally implanted above the DG or ventral CA1 (AP: -1.70mm, DV: -1.60mm, ML: ±1.00mm relative to bregma for the DG or AP: -3.16mm, DV: -4.55mm, ML: ±3.10mm relative to bregma for the CA1; Coordinates derived from Paxinos and Franklin, 2007) and fixed to the skull with dental cement. The surgically operated mice were recovered for 4 and 6 weeks to allow for ChR2 and ArchT viral transduction, before behaviour tests were performed. The expression pattern of ChR2/ArchT and the placement of the optical fibers were confirmed by immunohistochemical staining of fixed brain sections after behaviour tests. Mice with the optical fibers misplaced or viruses that were mistargeted were excluded from the analysis.
3.3.3 Apparatus for \textit{in vivo} Optical Stimulation

The apparatus used for the \textit{in vivo} optogenetic experiments for the behavioural tests was described previously (Aqrabawi et al., 2016; Nguyen et al., 2014). Optical stimulation of PP terminals onto the DG granule cells (EC-DG circuit) or onto the CA1 pyramidal cells (EC-CA1 circuit) that originate from EC neurons were achieved by illumination with 532nm green-light to activate ArchT or 473nm blue-light to activate ChR2. The light was generated by a diode-pumped solid state laser (Laserglow, Toronto, Ontario, Canada). The output of the laser was controlled by either the ANY Maze tracking software or a waveform generator (Keysight Technologies, Santa Rosa, California, USA). For all optogenetic behavioural experiments, the laser was connected to a 1x1 fiberoptic rotary joint and a Doric mini cube (Doric Lenses, Quebec City, Canada) which split the beam into two separate mono fiberoptic arena patch cables (Doric Lenses, Quebec City, Canada) that were each attached to a ceramic split sleeve (SM-CS125S, Precision Fiber Products) and connected to the implanted optical fiber on a mouse. Prior to all behavioural tests, the output of each laser was measured using a power meter (Thor Labs, Michigan, USA).

3.4 Local Cannula Infusions

3.4.1 Design and Fabrication of Cannulas

Guide cannulas were custom-made using a 19G x 1 1/2TW needle with a diameter of 1.1mm with an average length 12mm (Becton Dickinson, Franklin Lakes, New Jersey, USA). A rubber stopper was soldered 1mm from the base of the guide cannula to secure the cannula upright so that once implanted, 1mm of the cannula rested below the dura. A cannula stopper (diameter 0.5 mm, average length 5 mm) was made with the tip of a 25G x 1 1/2 TW needle (Becton Dickinson, Franklin Lakes, New Jersey, USA) to ensure that the cannulas were unobstructed with bedding/dust following surgeries.

3.4.2 Stereotaxic Surgery

Mice were anaesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg) and placed into a stereotaxic frame. The skull was surgically exposed and two holes were drilled bilaterally to target the
ECII (AP: -3.80mm, ML: ±3.70mm; Coordinates derived from Paxinos and Franklin, 2007) or the BLA (AP: -1.5mm, ML: ±2.80mm Coordinates derived from Paxinos and Franklin, 2007). In each hole, a single guide cannula was slowly lowered 1mm in the hole (up until the rubber stopper). The bilateral cannulas were secured with dental cement and allowed to dry for 5 minutes. Following surgery, the animals were monitored until complete recovery from anesthesia and were given tear gel on each eye and 1mL 0.9% saline and 5 mg/kg Anafen for analgesia intraperitoneally. All animals had a monitored postoperative recovery period of at least 7 days before behavioural experiments were commenced.

3.4.3 Apparatus for Local Infusions

For the local administration of DOX (0.5mg/mL) (24390-14-5, Sigma-Aldrich), the drug was dissolved in saline and infused through a 25G x 1 1/2 TW needle (Becton Dickinson, Franklin Lakes, New Jersey, USA) 3.70mm below the cannula for the EC and 3.45mm below the cannula for the BLA. A volume of 0.1µL at a rate of 0.2µL/minute was infused once per day over a period of 7 days using a Dual Syringe Infusion Pump (KD Scientific, Holliston, Massachusetts, USA). For local administration of the PAK inhibitors, IPA3 (50µM) (Tocris Biosciences, Avonmouth, Bristol, United Kingdom) and PIR3.5 (50µM) (Tocris Biosciences, Avonmouth, Bristol, United Kingdom), the drugs were dissolved in Dimethyl Sulfoxide ReagentPlus > 99.5% (D5879, Sigma Aldrich) and infused through a 25G x 1 1/2 TW needle (Becton Dickinson, Franklin Lakes, New Jersey, USA) 3.70mm below the cannula for the EC and 3.45mm below the cannula for the BLA. A volume of 0.1µL at a rate of 0.2µL/minute was infused 30 minutes prior to the start of the behavioural test.

3.5 Electrophysiology

3.5.1 Preparation of In vitro Slices

The detailed procedures and analysis for the hippocampal and cortical slice recordings were completed in reference to previous studies conducted in our lab (Meng et al., 2005; Meng et al., 2002). For the extracellular field recordings, the subject mice ranged from 4-12 weeks of age, whereas the intracellular field recordings, the mice ranged from 4-6 weeks of age. The mice used for the
optogenetic recordings were the same subjects from the behavioural tests at ~4-6 months of age. For slice preparation, the mouse was sacrificed via cervical dislocation in accordance with the standard operating procedures approved by the Animal Care Committee at the Hospital for Sick Children. The mouse brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 120 NaCl, 3.0 KCl, 1.2 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, 2.0 CaCl₂, and 11 D-glucose saturated with 95% O₂/5% CO₂ for 1 minute. The brain was bisected along the longitudinal fissure and then mounted with tissue adhesive (1469SB, Vetbond) on the chuck of a VT1200S vibratome (Leica, Concord, Ontario, Canada). Horizontal 350µm hippocampal slices were cut and recovered at room temperature in continually oxygenated ACSF for at least 2 hours before recording.

3.5.2 Data Acquisition

A single slice was transferred to a submersion chamber perfused with 95% O₂/5% CO₂ saturated ACSF with (for whole-cell recordings) or without (for field recordings) 100µM picrotoxin (1128, Tocris). A perfusion flow rate was set at 2mL/minute for both the intracellular and extracellular recordings. Hippocampal and cortical neurons were visualized using an infrared differential interference contrast microscope (Axioskop 2, Zeiss). For hippocampal slice recordings, synaptic transmission was evoked by external stimulation (at 0.05Hz for field recordings or 0.1Hz for whole-cell recordings) of the MPP onto the DG granule cells or Schaffer collaterals onto the CA1 pyramidal neurons and recorded with glass pipettes (3-4MΩ) filled with either ACSF (for field responses) or with intracellular solution (for whole-cell responses). The intracellular solution comprised of (in mM) 130 CsMeSO₄, 5 NaCl, 1 MgCl₂, 0.05 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 Na₃GTP, and 5 QX-314 (at a pH of 7.25 and osmolarity of 280-300mOsm). For input/output field recordings, the stimulus intensities were measured from 2, 3, 5, 7, 10, 20, 40, 60, 80, 100, 120, and 140mV. For the paired-pulse field recordings, the inter-stimulus intervals were measured at 25, 50, 100, 200, 300, 400, 500, and 1000ms. LTP at the CA1 synapse was induced by four trains of 100 Hz lasting 1 second each, with a 20-second intertrain interval. The baseline period was recorded for 30 minutes prior to the induction of LTP followed by 50 minutes following the stimulation protocol. The response for field EPSPs were calculated from measuring the slope between 5% and 60% of the peak response, which was normalized to the mean of the baseline response. For whole cell experiments, cells were clamped at -65mV throughout the
experiment. For EPSC\textsubscript{AMPA}/EPSC\textsubscript{NMDA} ratio experiments, EPSC\textsubscript{AMPA} was the peak value obtained at -70mV, whereas EPSC\textsubscript{NMDA} was the response amplitude at +40mV taken 40ms after the onset of the response. For current/voltage relationship, EPSC\textsubscript{AMPA} (6 responses averaged) were recorded at various holding potentials (-80, -60, -40, -20, 0, +20, +40, and +60 mV) with 100µM D-APV (0106, Tocris) added to the ACSF and the EPSC amplitudes were normalized to those recorded at -60mV. For miniature EPSCs (mEPSC), the responses were recorded at -70mV with 100µM picrotoxin and 1µM TTX (1069, Tocris). To confirm ArchT expression in the EC cortical neurons, the EC layer II or adjacent somatosensory cortical neurons (as control) were identified by EYFP fluorescence and action potentials were recorded under a current-clamp mode with or without continuous 532 nm green-light illumination (1-4mW, StabArc XBO-75 HBO 109, Carl Zeiss, North York, Ontario, Canada) delivered through a 40x objective lens. The light power emitting from the objective was measured with a power meter (PM100D, Thor Labs). To confirm the effect of ArchT activation on synaptic transmission at the EC-DG projection, EPSCs evoked by stimulating the MPP were recorded for 5 minutes before the green-light was applied, 5 minutes during the green-light illumination, and another 5 minutes after the green-light was turned off. To confirm ChR2 expression, whole-cell recordings were obtained from the EC layer II or adjacent somatosensory cortical neurons and action potentials were triggered by blue-light pulses (5ms, 473nm, 1-4mW) using a Dual OptoLED (Cairn Research, Faversham, Kent, United Kingdom) delivered through a 40x objective lens. To confirm the effect of ChR2 activation on synaptic transmission at the EC-DG synapses, 1ms pulses of blue-light (473 nm, 1-4mW) was delivered to the DG molecular layer area and the light-evoked EPSPs or EPSCs were recorded from the DG granule cells. To confirm that DG granule cells do not express ChR2, NBQX (10µM) and picrotoxin (100µM) were added to the ACSF to block EPSP, action potentials or EPSC recorded from the DG granule cells. In all of the whole-cell recordings, cell series resistance was monitored throughout experiments by applying a -3mV step at the end of each response sweep. Any experiment in which the resistance changed by more than 20% was excluded from the analysis. All data acquisition and analysis were done using pCLAMP 10.6 (Axon Instruments, Foster City, California, USA) and MiniAnalysis program (Synaptosoft, Fort Lee, New Jersey, USA). In all electrophysiological experiments, n represents the number of neurons or slices used for the recording where one or two neurons per slice from a single animal were used.
3.6 Electron Microscopy

The mice were anaesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg) in accordance with the standard operating procedures approved by the Animal Care Committee at the Hospital for Sick Children. Mice were then transcardially perfused using 2.5% glutaraldehyde (111-30-8, Sigma-Aldrich) made in 0.1M sodium cacodylate buffer (97068, Sigma-Aldrich). The fixed brains were removed and post-fixed in 2.5% glutaraldehyde for 6 hours. Brains were then sliced (500μm) on a vibratome (VT1000S, Leica) and 1mm x 1mm blocks covering the molecular layer area of the DG were isolated from comparable slices taken from WT and dTg mice. The blocks were then post-fixed in 2% glutaraldehyde fixative solution for an additional 3 hours and processed according to standard methods by the Department of Pediatric Lab Medicine at the Hospital for Sick Children (Meng et al., 2003a; Meng et al., 2002). For each block, 1μm thick sections were cut and stained with 1% toluidine blue to guide further trimming to isolate equivalent DG molecular layer regions. From the chosen blocks, thin sections (60nm) were cut and stained with uranyl acetate and Reynolds lead citrate and subsequently mounted on hydrophilic carbon coated grids. The number of synapses (determined by the presence of the postsynaptic density), postsynaptic density length, and the number and distribution of synaptic vesicles (at axon terminals) were determined on electron micrographs at a final magnification of 25,000x, using a NIH program available within the Nanoscale Biomedical Imaging Facility at the Hospital for Sick Children. For all EM analyses, 10 different grids prepared from 2 mice of each genotype were used. A total of 80-100 images were acquired covering the DG PP region totaling 5800-7000μm². The quantification for the dendritic spines, synaptic vesicles, and PSD, were analyzed using ImageJ.

3.7 Western Blotting

3.7.1 Tissue Dissection and Brain Lysate Preparation

The detailed procedures for Western blotting were conducted as described previously (Huang et al., 2011). To isolate protein samples, the subject mouse was sacrificed via cervical dislocation in accordance with the standard operating procedures approved by the Animal Care Committee at the Hospital for Sick Children. The mouse brains were quickly removed from the skull and bisected along
the longitudinal fissure. For each half brain, structures were microdissected to isolate the cerebellum, cortex, and hippocampus and digested using 300µL, 450µL, and 200µL of lysis buffer respectively, based on the size of the tissue. For whole brain lysates, 600µL of lysis buffer was used. The lysis buffer is composed of 50mM Tris HCl pH 7.5, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1% Triton X-100, and 20mM NaF, and 15mM NaVO₄ (phosphatase inhibitors). For 10mL of lysis buffer, 1 cOmplete Mini, EDTA-free protease inhibitor cocktail tablet (11836170001, Roche Diagnostics GmBH) was added. The tissue was manually homogenized using polypropylene pestles and allowed to be digested on a rotator for 1 hour at 4°C. The samples were centrifuged at 12,500rpm for 20 minutes and the supernatant containing the protein was aliquoted to new eppendorf tubes. The concentration of DNA of each tube was measured using a NanoDrop 2000 Spectrophotometer (ND-2000, ThermoFisher Scientific). All samples were diluted using the same lysis buffer to a concentration of 10µg of total proteins.

3.7.2 Western Blotting and Exposing

For Western blotting, 70µL of the diluted protein sample was combined with 20µL of 6X loading buffer (1M Tris pH 8, Sodium doedecyl sulfate (SDS), Glycerol, Bromophenol blue and Beta mercaptoethanol) and 10µL of 1M dithiothreitol (DTT). Prior to loading, the samples were heated at ~70°C for 4 minutes to fully denature and reduce the samples from DNA and cellular debris. The samples were loaded on a 10% polyacrylamide gel (1.5M Tris pH 8.8 (resolving), 1.0M Tris pH 6.8 (stacking), 30% Acrylamide, 10% SDS, 10% APS, TEMED) and allowed to separate (~1.5 hours at 130V) and transferred to nitrocellulose membranes (1 hour at 120V; LC2006, Pall Corporation). Running buffer was composed of 30mM Tris, 144mM Glycine, and 10mM SDS and the transfer buffer was composed of 30mM Tris, 145mM Glycine, and 3mM SDS. The membranes were washed with 1x Tris Buffered Saline with Tween 20 (TBST) (24mM Tris, 90mM NaCl, 10% Tween-20, pH 7.4) and subsequently blocked for 1 hour with 5% non-fat dry milk (SKI400.500, BioShop) in TBST. The membrane was incubated in the primary antibody overnight at 4°C while gently shaking. The following day, the membrane was washed 3 times, for 10 minutes each with TBST, and the appropriate secondary antibody (linked with horseradish peroxidase) in 15mL of reconstituted in 5% non-fat dry milk in TBST was added to the membrane for 1 hour. The membrane was washed with TBST (3 times, 10
minutes each) and the amount of each protein was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, New Jersey, USA) using a chemiluminescent HRP antibody detection reagent (E2500, Denville Scientific).

### 3.7.3 Primary and Secondary Antibodies

Primary antibodies used included: anti-GFP (2555, Cell Signaling Technology), anti-PAK1 (Cat# 2602, Cell Signaling Technology), anti-PAK2 (2608, Cell Signaling Technology), anti-PAK3 (2609, Cell Signaling Technology), anti-Cofilin (ACFL02, Cytoskeleton), pCofilin1 (sc-12912-R, Santa Cruz Biotechnology), anti-B-actin (3700S, Cell Signaling Technology), and anti-GAPDH (2118, Cell Signaling Technology). Secondary antibodies used included: anti-mouse IgG, HRP-linked Antibody (7076, Cell Signaling Technology), anti-rabbit IgG, HRP-linked Antibody (7074, Cell Signaling Technology), and Donkey anti-Goat IgG (H+L) HRP (A16005, Invitrogen).

### 3.7.4 Analysis

The amount of protein for each blot was analyzed using the FluorChem Q software (Protein Simple, San Jose, California, USA). The integrated density value (IDV) for each individual band was normalized to the background of each lane. The density value was then expressed as a ratio between the protein of interest to a housekeeper (such as GAPDH or B-actin). These density values were subsequently normalized against the density values obtained from the WT controls.

### 3.8 Immunohistochemistry

#### 3.8.1 Preparation of Brain Slices

The mice were anaesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg) in accordance with the standard operating procedures approved by the Animal Care Committee at the Hospital for Sick Children. Mice were then transcardially perfused using 1 x PBS (Phosphate buffered saline; diluted from PBX10×; 311-012-CL, Wisent Inc) and subsequently with 4% paraformaldehyde (PFA; PAR070,
Bioshop) made in 1 x PBS. The brain was then dissected and further fixed in 4% PFA for 6-8 hours and then transferred to 30% sucrose in PBS solution for 24 hours until it was fully saturated. Prior to sectioning, the brain was embedded in Tissue-Tek® O.C.T. compound and was frozen at -80°C for 30 minutes.

3.8.2 Immunostaining and Imaging

The brain was embedded on a cryostat chuck using O.C.T. and sliced to 25-30μm coronal or horizontal cryostat sections at -22°C (Leica CM1950, Concord, Ontario, Canada). The brain sections were directly transferred to a Superfrost glass slide (22-037-246, Thermo Scientific). Sections were washed with 1 x PBS, permeabilized by 0.1% Triton X-100 for 15 minutes, blocked with 5% bovine serum albumin (BSA) in 0.1% Triton X-100 for 1 hour, and incubated with primary antibodies (diluted to their specified amounts as indicated in the next section) in 5% BSA in 1 x PBS, overnight at 4°C. The sections were thoroughly washed using 1 x PBS (3 washes, 10 minutes/ wash) and the appropriate secondary antibodies (diluted to their specified amounts as indicated in the next section) in 1 x PBS at 37°C for 2 hours. Following washing with 1 x PBS, the stained glass slides were cover slipped using the VectaShield hard-set anti-fade mounting medium with DAPI (Vector, Burlingame, California, USA) and allowed to dry overnight. The slides were sealed using clear nail polish. Images were collected using a 4x or 20x objective on a Nikon C2 confocal (Nikon Canada, Mississauga, Ontario, Canada), Nikon TE2000 (Nikon Canada, Mississauga, Ontario, Canada), or Zeiss confocal (Carl Zeiss Canada Ltd., North York, Ontario, Canada) microscopes.

3.8.3 Primary and Secondary Antibodies

Primary antibodies (diluted to 1:500-1:1000) used for immunohistochemical analyses included: anti-GFP (2555, Cell Signaling Technology), anti-CDP (sc-13024, Santa Cruz), anti-GAD67 (sc-28376, Santa Cruz), anti-GFAP (ab7260, Abcam), anti-Reelin (D223-3, MBL), anti-Calbindin (ab11426, Abcam), and anti-PAK3 (2609, Cell Signaling Technology). Secondary antibodies (diluted 1:1000) used included: Goat anti-Mouse IgG, Alexa Fluor 546 (A11003, Invitrogen), Donkey anti-Rabbit IgG, Alexa Fluor 568 (A10042, Invitrogen), Goat anti-Rabbit IgG, Alexa Fluor 488 (A11034, Invitrogen),
and Donkey anti-Goat IgG, Alexa Fluor 546 (A11056, Invitrogen). Alexa Fluor 568 Phalloidin (A12380, ThermoFisher Scientific) was added in conjunction with corresponding secondary antibodies for 2 hours at room temperature.

3.8.4 Analysis

The images obtained were analyzed using the NIS-Elements Viewer 4.20 (Nikon Instruments Inc., Melville, New York, USA) and ImageJ software (NIH, Bethesda, Maryland, USA). For the phalloidin experiments, to calculate the corrected total region of interest (ROI) fluorescence in the PP, the integrated density of the ROI was subtracted from the product of the area of the selected ROI in the perforant pathway and the mean fluorescence for the control ROI in the CA3 region of the hippocampus. The integrated density, area, and mean gray values were derived from the ImageJ software.

3.9 Statistical Analyses

All the data within this thesis is presented as mean ± SEM (standard error of the mean). For each data set, values were first tested using the Shapiro-Wilk test of normality. Subsequent tests (whether parametric or nonparametric) were chosen based on whether the data assumed a Gaussian distribution. All data were statistically evaluated by independent-samples t-tests (Unpaired t test or Mann-Whitney test), paired-samples t-tests (Paired t test or Wilcoxon matched-pairs signed rank test), or ANOVA (one-way, two-way or repeated measures (RM), wherever appropriate) followed by Post-hoc Holm-Sidak’s multiple comparisons. $p<0.05$ was considered as significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Independent sample size (n) represented number of animals in the behavioural tests, number of slices or neurons in the electrophysiological recordings (typically one slice/neuron from one animal), or the number of grids or synapses analyzed in the electron micrographs. All analyses were conducted using GraphPad PRISM version 6.00 for Windows (GraphPad Software, La Jolla, California, USA) and SigmaPlot 12 (Systat Software Inc., San Jose, California, USA)
4 Results

Data Attribution- Figures and Results presented in this chapter were adapted from the following original publication:


The results from this thesis project will be discussed in five sections that correspond to the five specific aims outlined earlier. In section 4.1, I will describe the biochemical and cellular characterization of a novel transgenic mouse model where the mutant form of PAK3, mPAK3-GFP, is inducibly expressed. In sections 4.2 and 4.3, I will discuss the behavioral and synaptic phenotypes of this mouse model, respectively. In section 4.4, I will summarize the changes in molecular signaling pathways, specifically cofilin and the actin cytoskeleton, in the mouse model. Finally, in section 4.5, I will discuss the optogenetic findings in the control and transgenic mice. In each of these sections, I will discuss the introduction and rationale, the materials and methods that are directly relevant to that section, followed by the results, and then a brief discussion. I performed the majority of the experiments included in this thesis, however, those that were done in collaboration will be indicated in the specific sections.

4.1 The Biochemical and Cellular Characterization of CaMKIIα-tTA/tetO-mPAK3-GFP Transgenic Mice

4.1.1 Introduction and Rationale

The advent of genetic manipulations in mice began in the early 1980s with the microinjection of exogenous DNA (often called a transgene) into the pronuclei of fertilized eggs that randomly integrate into the mouse genome. These embryos were surgically transferred into pseudopregnant recipient females, resulting in the production of transgenic mice harbouring the transgene. Over the last 30 years, the use of transgenic mice has dramatically increased our understanding of certain disease-related genes and the consequences of ablating specific cell types or introducing genetic mutations associated with particular human diseases. Recent studies have also employed inducible and cell-type restricted
expression systems, thus enhancing the utility of transgenic mice by conferring spatial and temporal specificity of the transgene expression. In this case, the use of appropriate promoters to drive the transgene expression is essential (Matsuda and Aiba, 2004).

As discussed earlier, many of the current studies in our lab focus on the role of PAKs in the brain using KO mice. For example, KO mice have been generated for all members of the PAK family and analyses of these mice have provided important insight regarding their role in brain function. However, a number of problems and concerns exist with the use of KO mice. Firstly, the phenotypes of global KO are difficult to attribute to a particular tissue or cell-type since the gene is disrupted from a zygote stage, and therefore it is impossible to ascertain whether the phenotypes observed are due to the primary effects of the loss of the gene itself or due to the accumulation of secondary effects during development that may arise due to compensation (Matsuda and Aiba, 2004). Secondly, many global KOs of the CNS genes such as PAK2 are embryonic lethal and it is therefore impossible to analyze their in vivo function at an adult stage. Lastly, another key issue with the KO strategy is that this process is not reversible. This is particularly a problem for behavioral tests where reversible manipulations of gene expression is desirable. The use of the bacteriophage Cre/loxP system has overcome some of these issues such as achieving cell-type specificity, but the lack of inducibility and reversibility is still a major challenge with the KO approach.

As mentioned, PAK 1 and PAK3 are the key members of Group I PAKs highly expressed in the brain (Dubos et al., 2012; Zhao and Manser, 2012). Human genetic studies have implicated both PAK1 and PAK3 in various brain disorders, including ASD and ID. However, the in vivo function of these PAKs in the brain remains poorly understood. To investigate this question, our lab previously generated PAK1 and PAK3 single and double KO mice (Asrar et al., 2009; Huang et al., 2011; Meng et al., 2005). However, there are number of key questions that cannot be addressed by using these KO mice. Mostly importantly, the brain circuit and the temporal and spatial specificity by which PAK1 and PAK3 are required cannot be investigated using KO mice. To overcome these problems associated with PAK KO mice, our lab generated a novel transgenic mouse model where the expression of a mutant form of PAK3 can be reversibly controlled in a spatial and temporal-dependent manner. The tTA/tetO
system (Gossen and Bujard, 1992) was chosen because it easily allows control of the transgene expression through the administration of DOX (Figure 4.1.1). The CaMKIIα promoter was used to drive tTA so that the expression of the transgene can be restricted to excitatory neurons in selected areas of the brain (Mayford et al., 1996). The R67C point mutation in the PAK3 gene (Figure 4.1.2) was chosen because this mutation occurs in the Rho GTPase-binding domain, thus acting in a dn manner by inhibiting the activation of PAK signaling (Kreis et al., 2007). GFP was fused downstream of the mutant PAK3 for convenient and easy detection of the transgene expression. The mPAK3-GFP transgene was injected into the zygotes and the founder lines were crossed with CaMKIIα-tTA transgenic mice (which were obtained from Jackson Lab) to generate double transgenic mice for analysis of the transgene expression (Figure 4.1.1B). In the double transgenic mice, tTA binds to tetO and promotes the transcription of mPAK3-GFP transgene.

In this section, I will discuss the characterization of the expression pattern and reversibility of the mPAK3-GFP transgene to validate the tetracycline-inducible mouse model. I expect that the transgene is expressed only in excitatory neurons of selected forebrain regions and can be reversibly controlled by the addition or removal of DOX.
Figure 4.1.1. The generation of mPAK3-GFP dTg mice using the tTA/tetO inducible system. (A) tetO-mPAK3-GFP transgenic lines (Tg-1) were bred to the CaMKIIα-tTA mouse line (Tg-2) to produce double transgenic mice (dTg). (B) The addition of the tetracycline analog, DOX, prevents tTA from binding to tetO and blocks the transcription of the transgene. The removal of DOX restores the expression of mPAK3-GFP transgene.
Figure 4.1.2 The dominant negative effects of the R67C mutation of PAK3 on PAK signaling.

PAKs are dimerized and inactive, but the binding of the Rho GTPases leads to the activation of the catalytic domain. The right side of the schematic illustrates the R67C mutation present on the regulatory domain at the N-terminal end of the CRIB. This mutation impairs PAK3 binding to Rho GTPase (i.e. Cdc42) and prevents the activation of the kinase catalytic domain.
4.1.2 Materials and Methods

4.1.2.1 CaMKIIα-tTA and tetO-mPAK3-GFP Transgenic Mice
The CaMKIIα-tTA mice, with the expression of the tTA under the control of the forebrain excitatory neuron-specific promoter of the CaMKIIα gene (003010), were obtained from Jackson Lab (Tg-2). The tetO-mPAK3-GFP transgenic mice (Tg-1) were generated at The Center for Phenogenomics (TCP, Toronto, Canada) using standard pronuclear DNA injection techniques (Cho et al., 2009). Briefly, the transgene construct was linearized and a DNA fragment containing tetO-mPAK3-GFP was injected into the zygotes. The founder lines were then crossed with the Tg-2 line to create a double positive transgenic line (dTg). To minimize any potential effects of the genetic background, all the mice used for the present study were the littermates derived from hemizygous breeders and they include: WT (tTA+/mPAK3-GFP), single positive transgenic (Tg1: tTA+/mPAK3-GFP and Tg2: tTA+/mPAK3- GFP+), and double positive transgenic mice (dTg: tTA+/mPAK3-GFP+).

4.1.2.2 Molecular and Biochemical Analysis
All mice were genotyped using standard PCR methods. In addition to PCR analysis, all the mice used for experiments were confirmed by the presence or absence of the transgene (mPAK3-GFP) expression using Western blotting and/or immunostaining with anti-GFP or PAK3 antibodies. Whole-brain lysates were used to confirm the presence/absence of the transgene, whereas protein lysates microdissected from the cerebellum, midbrain, and hippocampal tissues were used to determine the regional expression of the transgene. The detailed procedures for PCR, Western blotting, and immunohistochemistry are described in the Experimental Procedures chapter.

4.1.3 Results

4.1.3.1 Biochemical analysis of CamKIIα-tTA/tetO-mPAK3-GFP mice
Through Western blot analysis of whole brain lysate, I showed that the mPAK3-GFP was expressed in dTg, but not single transgenic mice beginning at 3 weeks (Figure 4.1.3A and 4.1.3B), thus avoiding potential effects of the transgene on brain development. Western blot analysis from isolated structures
including the cortex, hippocampus, and cerebellum showed that transgene expression was found in the cortex and hippocampus, which is expected based on the expression pattern of the CaMKIIα promoter (Figure 4.1.3C). The expression of endogenous PAK1, PAK2, and PAK3 was not altered (Figure 4.1.3C and 4.1.3D), suggesting that transgene expression does not interfere with the levels of endogenous PAK proteins (18.30% of mPAK3-GFP transgene relative to the endogenous level of PAK3 in the hippocampus). Therefore, mPAK3-GFP is successfully expressed using the tTA/tetO system in transgenic mice.

4.1.3.2 Subcellular localization of mPAK3-GFP expression
To establish the regional specificity of the transgene expression, I performed immunostaining of serial brain sections and showed that mPAK3-GFP was mainly detected in the EC and hippocampus (Figure 4.1.4A and 4.1.4B). The transgene was also mildly expressed in the BLA, albeit much weaker than in the EC (Figure 4.1.4C). The transgene was predominantly expressed in the superficial layer II of the medial and lateral EC, along the axon fibers in the MPP and LPP projecting to the molecular layer, but not the granule cell layer of the DG (Figure 4.1.4C). Also, no transgene expression was detected in the CA1, CA2, or CA3 sub-regions (Figure 4.1.4D). To confirm that the transgene expression is restricted to excitatory pyramidal neurons in the EC, I performed co-immunostaining of mPAK3-GFP with a layer specific marker, CDP (CUX1; Cut like homeobox 1) for the superficial cortical layers II/III (Figure 4.1.5A). I found that the transgene was expressed in 88.03% ± 2.96% of CDP-positive cells. Furthermore, I co-stained GFP with GAD67 (Glutamate decarboxylase isoform 67, an inhibitory neuron marker) (Figure 4.1.5B) and GFAP (Gliarial fibrillary acidic protein, a marker for astrocytes) (Figure 4.1.5C). I observed no significant localization between GFP and GAD67 (0%) and GFAP (1.60% ± 0.66), respectively. In addition, a significant portion (37.24% ± 4.20) of mPAK3-GFP positive neurons were reelin-positive, those that project from ECII to the DG (Figure 4.1.5D) and only a minor fraction (1.02% ± 0.49) of mPAK3-GFP positive neurons were calbindin-positive, those that project from ECII to the CA1 (Figure 4.1.5E).
**Figure 4.1.3 The biochemical characterization of dTg mice.** (A) Expression of mPAK3-GFP transgene (90kDa) in dTg, but not in Tg-1 or Tg-2 mice as probed with anti-GFP and anti-PAK3 antibodies. Expression of mPAK3-GFP transgene in dTg mice was turned off with the administration of DOX. Endogenous PAK3 (65kDa) was present in all genotypes. (B) Western blots of whole brain protein lysates showing that mPAK3-GFP transgene (90kDa) was not expressed until postnatal 21 days in dTg mice. The level of endogenous PAK3 (65kDa) was not altered in the dTg mice. (C) Restricted expression of mPAK3-GFP transgene (90kDa) in the cortex (CX) and hippocampus (HP), but not in the cerebellum (CR) or midbrain (MD). With no changes in the total amount of endogenous PAK3 protein (65kDa). (D) Western blots of total protein lysates prepared from the CX, HP, and CR, showing that the expression of endogenous PAK1 and PAK2 was not altered in dTg mice.
Figure 4.1.4 The expression of mPAK3-GFP transgene in dTg mice. (A) Coronal dorsal to ventral brain sections of dTg mice stained with anti-GFP and DAPI. Scale bar: 2000µm. (B) Horizontal brain section of dTg mice stained with anti-GFP. Scale bar: 1000 µm. (C) Coronal brain section of dTg mice stained with anti-GFP. Expression in the DG and mildly in the BLA. A 20x zoomed image of the mPAK3-GFP expression in the BLA. Scale bar: 1000µm (Coronal); 100µm (BLA). (D) Hippocampal section of dTg mice stained with anti-GFP and DAPI showing mPAK3-GFP expression in the DG-ML, but not in the DG-GCL or CA1/CA3 areas. Scale bar: 500µm.
Figure 4.1.5 Colocalization of the mPAK3-GFP transgene with various excitatory, inhibitory, and cell-specific markers. (A) EC cortical sections of dTg mice stained with DAPI, anti-GFP (to detect mPAK3-GFP cells), anti-CDP (to identify layer II/III of the EC cells), anti-GAD67 (to identify inhibitory cells), anti-GFAP (to identify astrocytes), anti-Reelin (to identify EC layer II cells that project to the DG), or anti-Calbindin (to identify EC layer II cells that project to the CA1). Scale bars: 100µm. (B) Summary graph showing colocalization of mPAK3-GFP with CDP/reelin-positive pyramidal neurons, but not with calbindin-positive excitatory neurons, GABAergic neurons, or glial cells.
Therefore, through the immunohistochemistry experiments, the expression of the mPAK3-GFP transgene was confirmed to be predominantly in excitatory pyramidal EC II neurons and their perforant axonal projections innervating the molecular layer of the DG.

### 4.1.3.3 Reversibility of the tetracycline inducible system in CamKııII-tTA/ tetO-mPak3-GFP mice

To confirm that the transgene expression is reversibly regulated, I supplemented the drinking water with DOX (0.5mg/ml). Using Western blot techniques, I found that the protein level of mPAK3-GFP transgene was significantly reduced within one week of the drug treatment and was completely turned off by four weeks (Figure 4.1.3A and 4.1.6A). Furthermore, immunohistochemistry techniques revealed that the transgene was completely turned off in the EC layer II neurons and their PP projections to the DG (Figure 4.1.6B). Similarly, other regions including the BLA displayed no transgene expression (Figure 4.1.6C).

### 4.1.4 Discussion

In this section, I demonstrated that, compared to the traditional KO approach, the tetracycline system confers several advantages, including temporal and spatial control of transgene expression, thus overcoming any possible developmental compensations. I showed that transgene expression is limited to the excitatory reelin-positive layer II EC neurons and their corresponding PP outputs to the DG. Moreover, the administration of DOX in the drinking water provided a convenient technique to achieve the reversibility of transgene expression.

The Western blot results showed that the transgenic mice expressed the mPAK3-GFP mutation in the forebrain at 3 weeks. This expression profile was consistent with that of the CamKıııα promoter used in tTA transgenic mice (Hasegawa et al., 2009). The immunostaining results further showed that transgene is expressed in excitatory neurons, again consistent with the property of the CamKıııα promoter. Interestingly, the expression of mPAK3-GFP is largely restricted to layer II EC neurons and
their corresponding outputs to the DG. Previous studies using the same CaMKIIα-tTA mice have shown broader patterns of transgene expression that span multiple areas within the forebrain. These studies have identified transgene expression in various regions of the hippocampus, that range from the DG granule cells to the CA1 or CA3, amygdala, striatum, and/or cerebral cortex (Han et al., 2012; Mayford et al., 1996; Michalon et al., 2005). The reason for the restricted expression in the EC in our transgenic mice is not clear, but it is likely due to interactions between the CaMKIIα promoter and the specific integration site of the transgene in the genome. For example, in forebrain regions such as the CA1 or CA3, tTA may bind to tetO but may not be effective for transgene expression resulting in insufficient activation of the mPAK3-GFP transgene. Although the CamKIIα mice were purchased from Jackson Lab and were fully characterized, the tetO founder line may have genomic positional effects that would result in the unique EC expression that is observed compared to the other studies. There may also be poor tetO regulation in certain areas since the expression pattern of the tetO promoter can be highly variable in different cell types and tissues, leading to differences in the relative level of tTA induction. Nevertheless, the highly localized expression of mPAK3-GFP in the EC-DG circuit has provided us a unique opportunity to investigate this circuit in synaptic and behavior regulation.

As expected, I showed that the expression of the PAK3 transgene did not alter the expression of the total protein levels of endogenous PAKs 1-3, indicating that the insertion of the mPAK3-GFP transgene does not affect the endogenous PAK genes and their expression. It is important to note that, based on the Western blot results of whole brain protein lysates, the total amount of mPAK3-GFP transgene is much lower than that of endogenous PAK3 (18.3%). This could be explained by the fact that mPAK3-GFP is only expressed in excitatory neurons of the EC, a small population of cells that excludes astrocytes and glial cells in the brain.

The immunostaining experiments further indicate that mPAK3-GFP is specific to reelin-positive neurons in the EC. Previous studies have shown that the EC layer II cells are divided into two populations: the reelin-positive “Ocean” cells that project to the DG and the calbindin-positive “Island” cells that innervate the CA1 region of the hippocampus (Varga et al., 2010). These studies have also
revealed the role of the Ocean cells in the rapid encoding and formation of context-specific representations of fear memory, and the Island cells in temporal association learning (Kitamura et al., 2015b). However, whether these two populations of excitatory neurons differentially regulate social memory is unknown. The fact that our transgene mPAK3-GFP is specifically expressed in the reelin-positive cells would allow us to selectively dissect out the function of PAK signaling in this pathway alone.

It is important to note that I observed a low expression of the mPAK3-GFP transgene in the BLA. Given that the EC sends reciprocal connections between the various layers, it is possible that layer V cells of the EC also express the mutant transgene (though much weaker), which in turn project to the BLA. Given that the level of the transgene expression in the BLA is much lower than that in the EC-DG PP circuit, it is not expected to have a significant behavioural and functional effect; however, control experiments targeting the CA1 and BLA will be performed to rule out any effects of the transgene expression in these areas.

Lastly, the biochemical and immunohistochemistry findings also demonstrate several advantages of the tetracycline inducible system. First, the system allowed for the ability to overcome any developmental compensations that are associated with KO models. Secondly, these results indicated that the administration of DOX allowed for the reversibility of the transgene expression which is akin to performing rescue experiments. Previous studies have demonstrated that the transgene can be switched off by DOX treatment at concentrations which range from 2 to 2000µg/ml in the drinking water for three weeks (Redelsperger et al., 2016; Zhu et al., 2007). I chose to use a concentration of 0.5mg/ml (500µg/ml), as prolonged administration at high doses result in adverse biological effects including, abdominal aortic aneurysms (Prall et al., 2002). Furthermore, a dose of 467µg/ml has been shown to elicit comparable results comparable to the maximal dose of 2000µg/ml (Redelsperger et al., 2016). Overall, these findings validated the spatiotemporal expression of the mPAK3-GFP transgene in a tetracycline dependent manner. Specifically, the administration of DOX in the drinking water allowed for the reversibility of the transgene expression. Thus, the “tet-off” system is an effective approach to evaluate the role of PAK in behaviour.
Figure 4.1.6 The inducible effect of DOX on mPAK3-GFP transgene expression in dTg mice. (A) Expression of mPAK3-GFP (probed with anti-PAK3) in dTg mice was turned off with the systemic administration of DOX for 4 weeks. (B) Expression of mPAK3-GFP in dTg mice was turned off in the cortex and hippocampus following DOX administration for 4 weeks. (C) Coronal sections of WT and dTg mice with DOX systemic administration (0.5 mg/ml). Scale bars: 1000µm.
4.2 The Behavioural Significance of CaMKIIα-tTA/tetO-mPAK3-GFP Transgenic Mice

4.2.1 Introduction and Rationale

Children diagnosed with ASD and ID have conceptual, practical, and social deficits. PAKs were identified through genetic screening and post-mortem studies to be involved in ASD and ID (Gilman et al., 2011). As discussed previously, animal studies have demonstrated that members of the PAK family are critically involved in the regulation of both the structural and functional properties of the synapse, including dendritic spine structure and synaptic plasticity (Asrar et al., 2009; Hayashi et al., 2004; Huang et al., 2011; Meng et al., 2005; Meng et al., 2002; Zhao and Manser, 2012). Specifically, PAK1 KO mice revealed normal brain anatomy but selective deficits in LTP at hippocampal CA1 synapses with changes in levels of actin binding protein, coflin (Asrar et al., 2009). PAK3 KO mice demonstrated no deficits in either coflin activity or the actin cytoskeleton with mild impairments in late-phase LTP and learned taste aversion (Meng et al., 2005). Interestingly, Huang et al., (2011) analyzed double KO mice lacking both PAK1 and PAK3 and showed the double KO mice exhibited impaired postnatal brain growth, deficits in dendritic arbourization and spine morphology, and alterations in synaptic transmission and plasticity (Huang et al., 2011). Furthermore, the double KO mice displayed a host of behavioural deficits consistent with ID-like symptoms including hyperactivity, increased anxiety, and learning and memory deficits, which are consistent with the practical and conceptual impairments revealed in patients with ASD and ID (Huang et al., 2011). These deficits were much more severe than each individual KO model, suggesting function redundancy between PAK1 and PAK3. Some of the double KO deficits such as abnormalities in dendritic spines were rescued by blocking coflin activity suggesting that coflin-dependent actin regulation may mediate the effect of PAK1 and PAK3. Therefore, these studies highlight the importance of PAK1 and PAK3 in synaptic regulation and learning and memory. However, whether PAKs are directly involved in social behavior processing remains unknown.

To investigate the behavioural significance of PAKs on social memory processing, CaMKIIα-tTA/tetO-mPAK3-GFP transgenic mice were evaluated on their performance using two types of social interaction tests. Unlike the previous KO models, the transgenic mouse model allowed for the
behavioural examination of PAKs at mature synapses, without developmental compensations. Furthermore, the inducibility of the transgene expression using DOX permitted the re-testing of each subject as an internal control. I examined short-term social memory using Crawley’s three-chamber social interaction test, to measure sociability and social recognition memory, and a five-trial social interaction assay, to compare social habituation and dishabituation. These paradigms were chosen in particular, as previous studies have evaluated social discrimination by measuring the relative interaction durations with novel and familiar mice under free-choice conditions (Hitti and Siegelbaum, 2014; Okuyama et al., 2016). The repeated exposures to a particular conspecific result in decreased interaction time. Thus, the interaction time of a novel conspecific compared to the familiar conspecific can be used as an index for social memory. The EPM and the OFT were used as control, since high levels of anxiety, impaired motor function, and hyperactivity have strong comorbidities with ASD and ID (Hayashi et al., 2007; Won et al., 2013). The short and long-term cognitive abilities were measured using the Morris water maze test, to examine spatial learning and memory. Tests for olfaction, including the food burying test and the olfaction habituation/dishabituation test, were conducted to ensure that the social behaviours were not associated with anosmia.

Given that the transgenic mice demonstrated mPAK3-GFP expression in both the EC-DG PP and the BLA, although much weaker, the behavioural experiments primarily targeted PAK expression in the EC, but also the BLA, to rule out its effect on social memory. This was achieved through two approaches. First, DOX was locally infused via cannulas over the EC or the BLA, as control. If the PP is important for the regulation of PAK in social memory, then social recognition memory should be reversibly manipulated by the addition or removal of DOX. Therefore, if the amygdala does not play a role in PAK signaling for the regulation of social memory, then local administration of DOX should not reverse the social recognition memory deficits. Second, the highly-selective group I PAK inhibitor, IPA3, was locally infused in the EC layer II cells or the BLA of WT mice. The infusion of IPA3 in the EC PP should mimic the behavioural deficits observed in the dTg mice. However, if PAK signaling within the BLA does not play a role in social memory processing, then the local infusion of IPA3 into this region, will not result in any behavioural deficits.
In this section, I will discuss the behavioural consequences of the mPAK3-GFP transgene. I expect that the dTg mice (prior to DOX administration) and WT mice infused with IPA3 inhibitors in the EC-DG PP will display social deficits that are not due to deficits in anosmia, motor function, or motivational abilities.

4.2.2 Materials and Methods

4.2.2.1 Behavioural Tests

For behavioural studies, all mice were bred and housed in groups of two to five animals in cages. For transgenic mice, the control animals were always littermates of mutant dTg mice, and both control and mutant groups were sex-balanced. To measure social recognition memory, a three-chamber social interaction test and a five-trial social memory assay were used. To control for novelty recognition, I subjected the mice to a novel object recognition test for two inanimate objects. To examine other behavioural changes, I conducted the EPM and OFT. To test other forms of memory, I used a well-validated Morris water maze paradigm, to measure spatial and contextual memory processes. Furthermore, I evaluated mice in their ability to detect or recognize odours. I used a food burying task, to measure the latency of a fasted subject mouse to uncover a pellet, and an olfaction habituation/dishabituation test, to measure the subject’s ability to detect changes in social and non-social odours. For experiments involving the systemic treatment of DOX to turn off the transgene, subject mice were first tested in the relevant behavioural paradigms. Following this baseline testing, the mice were treated continuously with 0.5 mg/ml DOX in acidified drinking water, replaced every other day for four weeks. After the treatment, the mice were re-tested in the appropriate behavioural paradigms.

4.2.2.2 Stereotaxic Surgery

All stereotaxic surgeries discussed in this section were completed in collaboration with Krutika Joshi from Dr. O.C. Snead laboratory. To limit the effect of DOX locally, bilateral cannulas were implanted above the EC (AP: -3.80mm, ML: ±3.70mm) or the BLA (AP: -1.5mm, ML: ±2.80mm). All animals had a postoperative recovery period of at least 5 days prior to further experimentation. For the local administration of DOX, the drug was dissolved in saline and infused 3.70mm below the cannula for the
EC and 3.45mm below the cannula for the BLA. A volume of 0.1µl at a rate of 0.2µl/minute was infused once per day for a period of 7 days using a Dual Syringe Infusion Pump. For the local administration of the PAK inhibitor, IPA3 (50µM), and the control, PIR3.5 (50µM), the drugs were dissolved in DMSO and infused 3.70mm below the cannula for the EC and 3.45mm below the cannula for the BLA. A volume of 0.1µl at a rate of 0.2µl/minute was infused 30 minutes prior to behavioural testing.

4.2.3 Results

4.2.3.1 dTg CamKIIα-tTA/tetO-mPAK3-GFP mice demonstrate deficits in social recognition memory but normal novelty recognition

WT and dTg CamKIIα-tTA/tetO-mPAK3-GFP mice were evaluated on their performance on a well-validated three-chamber social interaction test (Figure 4.2.1A). During habituation, WT and dTg mice demonstrated a similar preference for the two empty cages indicated by an equal amount of exploration. In the second stage, both WT and dTg mice spent more time interacting with an unfamiliar juvenile mouse that was matched by age and sex over an empty cage (Figure 4.2.1B). Next, in stage 3, I tested the short-term ability of the subject mouse to discriminate and recognize the now-familiar mouse from the second stage with another novel mouse. WT mice displayed preference for the novel mouse, whereas dTg mice showed no significant preference for the novel mouse over the familiar mouse (Figure 4.2.1C and 4.2.1D). To distinguish if the deficits were due to the subject’s inability to recognize the novel mouse or selective to remembering the mouse they already encountered, the novel object recognition test was used (Figure 4.2.2A). The habituation phase showed no differences in the amount of time both WT and dTg mice spent exploring two identical objects (Figure 4.2.2B). In stage 2, similar to WT, dTg mice showed higher levels of exploration of the novel object compared to the familiar one (Figure 4.2.2C). In stage 3, both groups spent more time exploring the second novel object compared to the one they previously encountered (Figure 4.2.2D). To further examine short-term social memory processing, I conducted a five-trial social interaction assay (Figure 4.2.3A). WT mice showed clear habituation to a novel mouse over five 60-second presentations (with 3-minute inter-trial intervals) and demonstrated significant dishabituation (increased interaction time) when a
second novel mouse was presented on the sixth trial (Figure 4.2.3B). However, dTg mice exhibited low levels of interaction with both novel mice across all six trials (Figure 4.2.3C).
Figure 4.2.1 dTg mice exhibit deficits in social recognition memory. (A) Schematic of the social interaction test. (B) The social interaction test showing normal preference for S1 over empty cage during stage 2 in both dTg and WT mice (WT: n = 13, p < 0.0001; dTg: n = 12, p < 0.0001; two-tailed paired t test). (C) The social interaction test showing impaired preference for S2 over S1 during stage 3 in dTg compared to WT mice (WT: p = 0.0026; dTg: p = 0.9117; two-tailed paired t test). (D) Discrimination scores of stage 3 showing lack of discrimination in dTg compared to WT mice (p = 0.0213; two-tailed paired t test).
Figure 4.2.2 dTg mice exhibit normal object recognition memory. (A) Schematic of the novel object test. (B) WT and dTg mice showed preference for novel object 2 versus familiar object 1 (WT: n = 13, p = 0.0001; dTg: n = 11, p = 0.0058; two tailed paired t test). (C) WT and dTg mice showed preference for novel object 3 versus 2 (WT: p = 0.0005; dTg: p = 0.0024; two tailed paired t test). (D) Discrimination scores reflecting preferences for object 3 versus 2 (p = 0.1183; two-tailed unpaired t test).
Figure 4.2.3 dTg mice exhibit deficits in social memory retrieval. (A) Schematic of the five-trial social memory assay. (B) WT, but not dTg mice, showed memory acquisition during Trials 1-5 (WT: n = 15, p < 0.001; dTg: n = 15, p = 0.7423; two-way ANOVA; Post-hoc Holm-Sidak’s multiple comparisons for Trial 1 versus Trial 5). In Trial 6, dTg mice showed significantly decreased interaction time compared to WT mice during the presentation of a novel mouse (p = 0.0015; two-tailed unpaired t-test). (C) Normalized interaction time obtained from the repeated trial memory test. WT, but not dTg mice, showed memory acquisition during Trials 1-5 (WT: n = 15, p = 0.0012; dTg: n = 15, p = 0.7147; two-way ANOVA; Post-hoc Holm-Sidak’s multiple comparisons for Trial 1 versus Trial 5).
4.2.3.2  dTg mice display normal anxiety-like behaviour, spatial learning and memory, and olfaction ability

To confirm if the social memory deficits in the dTg mice were accompanied by hyperactivity or anxiety-like behaviour, I employed the EPM and OFT. I found that dTg mice spend the same amount of time in the open and closed arms suggesting normal anxiety-like behaviour (Figure 4.2.4A). In addition, freezing levels, which is an indication of a mouse’s innate response to fear were not different between WT and dTg mice (Figure 4.2.4B). Hyperactivity in dTg mice were also not different in their total time mobile (Figure 4.2.4C). To investigate whether dTg mice were also impaired in other ASD/ID-associated symptoms, I evaluated short and long-term cognitive memory performances by conducting a Morris water maze test to examine hippocampal-dependent spatial learning and memory. As a control, I conducted a visible test and found no differences in motor function suggesting normal locomotion, motivation, and vision in dTg mice (Figure 4.2.5A). In the hidden platform training, the distance travelled to reach the platform was quantified over three days of training. Similar to WT mice, dTg mice were able to learn by the last day of training (Figure 4.2.5B). The short-term probe test at 2 hours revealed that dTg mice displayed comparable retention of spatial memory similar to WT mice. dTg mice spent a similar amount of time in the target quadrant as WT animals, compared to other zones (Figure 4.2.5C). Furthermore, the long-term probe test (24 hours) also demonstrated that they have normal spatial learning and memory capabilities (Figure 4.2.5D). Since olfaction is essential for social recognition and interaction, I ensured that the deficits in social memory were not due to anosmia. Therefore, I assessed the mice on two behavioural tasks designed to measure their ability to detect and differentiate non-social and social odours. In the buried food test, I measured how quickly a fasted animal could uncover a food pellet that is hidden under a layer of bedding. dTg mice showed normal ability to detect the presence of food buried under a layer of bedding which suggested normal ability to use odour cues to locate the food (Figure 4.2.6A). In the olfactory habituation/dishabituation test, I sequentially presented for three trials (for 90 seconds each trial and inter-trial interval for 30 seconds) vanilla, mixed (vanilla and herbs), and a social cue consisting of bedding from another cage. I measured the amount of time the mouse spent investigating the applicator. The dTg mice also showed normal olfaction habituation to simple and complex non-social odours (Figures 4.2.6B-4.2.6D). Importantly, the dTg mice were significantly impaired in olfaction habituation to social odour presentations, despite the fact that their initial investigation time was similar to WT controls (Figure 4.2.6D).
Figure 4.2.4 dTg mice exhibit normal anxiety-like behaviour. (A) dTg and WT mice displayed a similar amount of time in the closed arms, open arms, and center in an EPM test (WT: n = 13; dTg: n = 12; Closed $p = 0.4078$; Open $p = 0.3876$; Center $p = 0.1951$; two-tailed unpaired t test). (B) dTg and WT mice showed a similar amount of freezing in an OFT (WT: n = 13; dTg: n = 12; $p = 0.1560$; two-tailed Mann Whitney test). (C) dTg and WT mice showed a similar amount of time mobile in an OFT (WT: n = 13; dTg: n = 12; $p = 0.2664$; two-tailed Mann Whitney test).
Figure 4.2.5 dTg mice exhibit normal spatial learning and memory. (A) dTg and WT mice showed similar distance traveled to the visible platform (WT: n = 13; dTg: n = 11; Genotype $F_{(1, 60)} = 0.7074 \; p = 0.4036$; two-way RM ANOVA). (B) dTg and WT mice showed similar learning acquisition in the hidden training (Genotype $F_{(1, 84)} = 1.418 \; p = 0.2371$; two-way RM ANOVA). (C) Probe tests (2 hours) showed that dTg and WT mice spent more time in the target quadrant (Target $p = 0.9837$, Other $p = 0.5883$; two-tailed unpaired Mann Whitney test). (D) Probe tests (24 hours) showed that both dTg and WT mice spent more time in the target quadrant (Target $p = 0.9699$, Other $p = 0.6239$; two-tailed unpaired t test).
Figure 4.2.6 dTg mice exhibit normal social and non-social odour detection. (A) dTg and WT mice displayed similar latency to uncovering buried pellets (WT: n = 10; dTg: n = 9; p = 0.3450; two-tailed Mann Whitney test). (B) dTg and WT mice showed normal habituation to the vanilla cue (WT: n = 9; dTg: n = 10; Genotype $F_{(1, 17)} = 2.744$, $p = 0.1159$; Trial $F_{(2, 34)} = 57.51$, $p < 0.0001$; 2-way RM ANOVA). (C) dTg and WT mice showed normal habituation to the complex non-social cue (WT: n = 9; dTg: n = 10; Genotype $F_{(1, 17)} = 0.2967$, $p = 0.5930$; Trial $F_{(2, 24)} = 110.6$, $p < 0.0001$; 2-way RM ANOVA). (D) dTg mice showed significantly impaired habituation to the social cue compared to WT mice (WT: n = 14; dTg: n = 13; Genotype $F_{(1, 25)} = 4.121$, $p = 0.0531$; Trial $F_{(2, 50)} = 36.26$, $p < 0.0001$; 2-way RM ANOVA; Post-hoc Holm-Sidak’s multiple comparisons for Trial 3 $p = 0.0136$).
4.2.3.3 Deficits in social memory in dTg mice can be restored with systemic DOX administration

To test whether the social memory deficit is reversible, I applied DOX (0.5mg/ml) in the drinking water of dTg mice (and WT mice, as control) for a minimum of four weeks to systemically turn off the expression of the mPAK3-GFP transgene in the entire brain (Figure 4.2.7A). When presented to the modified three-chamber apparatus during habituation, WT and dTg mice with and without DOX showed equal preference for either empty cage. In the second stage, all four groups showed preference for an unfamiliar mouse matched by age and sex over an empty cage compared to dTg mice without DOX (Figure 4.2.7B). In the last stage, WT mice without DOX and WT and dTg mice treated with DOX all displayed preference for the novel mouse over the familiar mouse (Figures 4.2.7C and 4.2.7D).

4.2.3.4 Deficits in social memory in dTg mice can be restored with local infusions of DOX in the EC but not BLA

To determine whether the expression of mPAK3-GFP within the EC-DG circuit is responsible for the social memory deficit in the dTg mice, bilateral cannulas were implanted over layer II of the EC for local pharmacological infusion of DOX (0.5mg/ml; 0.1µl bilaterally for 7 days) to turn off the transgene expression specifically in this area alone. In the DOX infused dTg mice, the expression of mPAK3-GFP was largely reduced in the EC and PP pathway to the DG (Figure 4.2.8A). The three-chamber test showed that social memory in the dTg mice was also completely restored to WT levels with no effect on their habituation and sociability (Figures 4.2.8B-4.2.8D). Since the transgene was also mildly expressed in the BLA, I also performed local infusion of DOX in the BLA. Although, it completely switched off the transgene expression in this region, it did not reverse the social memory deficits in the dTg mice (Figure 4.2.8A-4.2.8D).
Figure 4.2.7 Deficits in social memory in dTg mice can be restored with systemic DOX administration. (A) Coronal sections of WT and dTg mice with DOX systemic administration (0.5 mg/ml). Scale bars: 1000 µm. (B) dTg mice with DOX systemic administration showed normal preference for S1 similar to WT mice (WT with DOX: n = 14, p < 0.0001; WT without DOX: n = 8, p < 0.0001; dTg with DOX: n = 14, p < 0.0001; dTg without DOX: n = 7, p < 0.0001; two-tailed paired t test). (C) dTg mice with DOX systemic administration showed normal preference for S2 similar to WT mice (WT with DOX: p = 0.0001; WT without DOX: p < 0.0001; dTg with DOX: p < 0.0001; dTg without DOX: p = 0.0725; two-tailed paired t test). (D) dTg mice with DOX systemic administration showed a significantly increased discrimination score, similar to WT mice with or without DOX, (F(3, 39) = 5.192, p = 0.0041; one-way ANOVA; Post-hoc Holm-Sidak’s multiple comparisons, dTg with DOX vs dTg without DOX p = 0.0068).
**Figure 4.2.8** Deficits in social memory in dTg mice can be restored with local DOX administration to the EC. (A) Horizontal and coronal sections of WT and dTg mice with local DOX infusion into the EC (0.5mg/ml; 0.1µl bilaterally for 5 days) or BLA (0.5mg/ml; 0.1µl bilaterally for 5 days) respectively, showing mPAK3-GFP expression turned-off in dTg. Scale bars: 1000 µm. (B) dTg mice with local DOX infusion into the EC or BLA showed normal preference for S1 similar to WT control mice (WT with DOX in EC: n = 9, p = 0.0039; WT with DOX in BLA: n = 6, p = 0.0004; dTg with DOX in EC: n = 7, p = 0.0156; dTg with DOX in BLA: n = 7, p = 0.0005; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (C) dTg mice with local DOX infusion into the EC, but not in to the BLA showed improved preference for S2 (WT with DOX in EC: p = 0.0117; WT with DOX in BLA: p = 0.0004; dTg with DOX in EC: p = 0.0313; dTg with DOX in BLA: p = 0.8702; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (D) dTg mice with DOX infusion in the EC, but not in the BLA, showed an increased discrimination score (WT vs dTg for EC infusion, p = 0.7446; WT versus dTg for BLA infusion, p = 0.0485; two-tailed Mann Whitney test and two-tailed paired t test).
4.2.3.5 Pharmacological inhibition of PAKs in layer II cells of the EC of WT mice reveals social memory deficits similar to dTg mice

To further confirm that PAK signaling in the PP is indispensable for social memory, bilateral cannulas were implanted over layer II cells of the EC of WT mice for local pharmacological infusion of IPA3 or the control peptide PIR3.5 (5nM, 1µl/side over 2 minutes with an additional 2 minutes for diffusion) (Tada and Sheng, 2006; Wang et al., 2013a) (Figure 4.2.9A). WT mice were treated with IPA3, 30 minutes prior to social memory testing, and demonstrated social memory impairments that mimic dTg mice with IPA3 in the three-chamber social interaction test (Figure 4.2.9B-4.2.9D). However, WT mice with IPA3 infused in the BLA (Figure 4.2.10A) showed no social memory impairments similar to control WT mice with the PIR3.5 control (Figure 4.2.10B-4.2.10D). I chose to target the LEC II cells for two main regions. Firstly, from a technical standpoint, it is easier to accurately target an infusion cannula in this region of the entorhinal cortex as opposed to the MEC. Secondly, previous studies have demonstrated the importance of the LEC in non-spatial “what” processing, whereas the MEC is involved in spatial “where” processing (Canto et al., 2008).
Figure 4.2.9 Acute infusion of the PAK inhibitor within the EC in WT mice impairs social memory. (A) Experimental procedure for infusion of the PAK inhibitor, IPA3, or control, PIR3.5 (5 nM, 1 µl/side over 2 minutes) followed by the social interaction test. (B) WT and dTg mice with IPA3 or PIR3.5 infusion in the EC showed normal preference for S1 (WT PIR3.5: n = 11, *p* = 0.0010; WT IPA3: n = 10, *p* < 0.0001; dTg IPA3: n = 11, *p* = 0.0010; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (C) WT and dTg mice with IPA3 infusion in the EC showed no preference for S2 over S1 compared to WT mice with PIR3.5 infusion (WT PIR3.5: *p* = 0.0029; WT IPA3: *p* = 0.8240; dTg IPA3: *p* = 0.8764; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (D) Discrimination scores of stage 3 showing that WT and dTg mice with IPA3 infusion in the EC were impaired in discrimination compared to WT with PIR3.5 infusion (*F*(2, 29) = 8.583, *p* = 0.0012, one-way ANOVA; Post-hoc Holm-Sidak’s multiple comparisons, *p* = 0.0032).
Deficits in social memory in dTg mice has no effect on local DOX infusion to the BLA. (A) Experimental procedure for drug infusion in the BLA followed by the social interaction test. (B) WT and dTg mice with IPA3 or PIR3.5 infusion in the BLA showed normal preference for S1 (WT PIR3.5: n = 11, p < 0.0001; WT IPA3: n = 6, p < 0.0001; dTg PIR3.5: n = 12, p = 0.0020; dTg IPA3: n = 7, p < 0.0001; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (C) WT mice with IPA3 or PIR3.5 infusion in the BLA showed normal preference for S2 compared to dTg mice with IPA3 or PIR3.5 infusion in the BLA that showed no preference for S2 over S1 (WT PIR3.5: p = 0.0137; WT IPA3: p < 0.0001; dTg PIR3.5: p = 0.5859; dTg IPA3: p = 0.6356; two-tailed Wilcoxon matched-pairs signed rank test and two-tailed paired t test). (D) Discrimination scores of stage 3 showing that WT mice with IPA3 or PIR3.5 infusion in the BLA exhibited normal discrimination compared to dTg mice with IPA3 or PIR3.5 infusion ($F_{(3, 30)} = 3.074; p = 0.0426$; one-way ANOVA).
4.2.4 Discussion

In this section, I demonstrated that CaMKIIα-tTA/tetO-mPAK3-GFP dTg mice have specific deficits in social recognition memory. In addition, the systemic or local EC administration of DOX restored social memory in the dTg mice. Lastly, the acute inhibition of PAK activity in the EC of WT mice through IPA3 impaired social recognition memory. Together, these findings suggest that PAK plays an important role in the EC-DG PP in the regulation of social recognition memory.

From the social interaction tests, the dTg mice demonstrated distinct impairments in discrimination but normal sociability that were not attributed to deficits in novelty recognition. Interestingly, I also observed normal locomotor activity, anxiety-like behaviour, hippocampal-dependent learning and memory, and olfaction which suggests that PAK signaling plays a very specific role in social recognition memory. These social deficits are consistent with behavioural phenotypes observed from PAK KO studies (Huang et al., 2011). However, the KO mice also demonstrated a multitude of other impairments, including hyperactivity, heightened anxiety-like behaviour, and deficits in associative and spatial learning and memory (Asrar et al., 2009; Huang et al., 2011; Meng et al., 2005). The reason that dTg mice displayed selective deficits in social recognition but not in other forms of memory, could be due to the restricted expression of mPAK3-GFP in the PP alone. Compared to the KO models that completely ablate the expression of the gene in the entire brain, the transgenic model is a more precise approach to specifically modify certain populations of neurons in particular brain regions. Therefore, deficits in spatial or associative forms of memory may be associated with the deletion of PAKs in the hippocampus, while social recognition memory may be particularly liable to alterations in PAK signaling within the EC-DG PP circuitry.

In the olfaction habituation/dishabituation test, the dTg mice were presented with an applicator containing a social cue, which was swabbed from an unfamiliar cage. The mice were able to form a memory of the social cue, but their habituation towards the subsequent presentations of the cue was impaired. This was confirmed to be not due to their inability to smell or the complexity of the stimulus. Thus, the social cue presented through an applicator was capable of eliciting the formation of a social
engram, similar to the presence of an actual mouse. This reveals novel insights into the mechanistic features of social memory, in that a social cue, regardless if it is the actual mouse or a swab of an unfamiliar cage, involves the same memory recognition processes through PAKs in the EC-DG PP circuitry.

The fact that PAK is indispensable for social recognition memory in the PP is further corroborated by the restoration of social recognition memory through the systemic administration or the local infusion of DOX into the EC. Previous studies have found that social cognition is a complex process that involves the ability to rapidly identify social cues, the integration of memory through face and name processing, and the anticipation of behaviour in a reciprocal and competitive setting (Adolphs, 2009); and that each aspect may be regulated by a different neural network. For example, in humans and rodents, the BLA has been found to be critical for sociability through interpreting emotions from faces (Adolphs, 2010). However, my findings suggested that PAKs within the BLA do not play a critical role in this process, which was tested in stage 2 of the three-chamber social interaction test. Furthermore, PAK signaling in the BLA also did not affect social recognition memory, since DOX administration to the BLA alone had no effect on dTg mice. Since the EC-DG circuit is the main interface between the hippocampus, amygdala, and various associative cortical regions (Canto et al., 2008), these results suggest that the EC-DG circuit may serve as a specific hub for social recognition memory processing.

It is important to note that some studies have shown that the Tet-off system is leaky, where the background expression of the transgene may not be fully repressed in the “off” state following continued DOX administration (Delerue et al., 2014). Therefore, to ensure that the deficits in social recognition memory were directly attributed to the effects of PAK signaling in the EC, I chose to use an acute pharmacological approach to inhibit PAK activity in WT mice. IPA3 is an isoform-selective inhibitor of the Cdc42-dependent activation of group I PAKs (Deacon et al., 2008). The infusion of IPA3 (just 30 minutes prior to testing) was capable of eliciting a similar behavioural deficit in social recognition memory as the dTg mice. Together, this suggests that the acute inhibition of PAK mimics the effect of the mPAK3-GFP transgene in the EC and is capable of impairing social recognition memory.
4.3 The Synaptic Mechanisms Underlying the Inducible Disruption of PAK Signaling

4.3.1 Introduction and Rationale

The hippocampus is one of the most well-characterized forebrain structures, known to regulate synaptic plasticity and long-term memory processes. The axonal projections from layer II of the lateral and medial EC make up the MPP and LPP. These axon fibers constitute the direct primary projection system relaying sensory information from the neocortex to the hippocampal formation. Although the PP is the origin of input to the hippocampus, the functional and behavioural role of the corticohippocampal circuitry on learning and memory remains unclear. To date, most studies that explore the cellular and molecular mechanisms involved in learning and memory have focused on the examination of the hippocampus, specifically the CA3-CA1 Schaffer collateral pathway (Takahashi and Magee, 2009). The correlation between LTP and memory formation is corroborated by evidence that both processes share similar induction and maintenance mechanisms (as discussed in Section 1.1.5.2). However, the role PAKs in synaptic plasticity and social memory within the EC-DG PP remains unknown.

As discussed earlier, PAKs play an important role in the hippocampus in the regulation of synaptic transmission, plasticity, and learning and memory (Asrar et al., 2009; Meng et al., 2005). Studies have shown that double PAK1 and PAK3 KOs have altered basal synaptic responses due to an enhanced individual synaptic potency which impaired bidirectional synaptic plasticity within the Schaffer collateral pathway (Huang et al., 2011). Changes in presynaptic function in the double KO mice were also observed through decreased paired pulse facilitation (PPF). Given that the PAK1 and PAK3 double KO mice display deficits in neurotransmitter release that are accompanied by impairments in various modes of memory, it is reasonable to investigate whether social memory may be associated with neurotransmitter release and synaptic plasticity. Specifically, this aim will examine whether the social recognition memory impairments in dTg mice can be explained by altered synaptic transmission and plasticity and/or morphological abnormalities in the structural properties of the EC-DG synapse.
PPF experiments are representative of a short-term form of homosynaptic facilitation that lasts from hundreds of milliseconds to several minutes. It can result in bidirectional changes in synaptic strength. For example, a second larger postsynaptic response is attributed to the residual calcium in the presynaptic terminal following the first action potential. However, the response to a second stimulus pulse can also be smaller compared to the first. This short-term synaptic depression can be due to vesicle depletion, inactivation of release sites, and decreased presynaptic calcium influx (Fioravante and Regehr, 2011). The EC-DG PP is comprised of the MPP and LPP, which can be differentiated pharmacologically and electrophysiologically. Specifically, the EC-DG MPP synapse typifies paired-pulse depression (PPD), while the LPP synapse results in PPF. Also, the MPP synapse has been referred to as the “where pathway” and shown to be important for conveying spatial information whereas, the LPP or “what pathway” is responsible for novel object information (Witter, 2007). Despite that the transgene was expressed in both the MPP and LPP (Figure 4.1.4) in the dTg mice, I focused on the MPP pathway for the electrophysiological recordings as it can be easily distinguishable through its characteristic PPD.

Based on the biochemical characterization of the dTg mice (Results section 4.1), the expression pattern of the mPAK3-GFP transgene in the EC-DG PP was used as a reference to examine the functional consequences of this specific pathway. Given that the mPAK3-GFP transgene was not expressed in other areas of the hippocampus, extracellular recordings along the Schaffer collaterals was used as a control to compare basal synaptic transmission and short and long-term plasticity in these pathways. To further study the intrinsic biophysical effects of the transgene expression, miniature excitatory postsynaptic activity was conducted in the DG, BLA, and CA1 regions using whole-cell recordings. Given that numerous neuropsychiatric diseases are characterized by morphological defects in dendritic spines (Chapleau et al., 2009b), electron microscopy techniques were also used to examine the structural properties of the PP in dTg mice. Given that PAK KO mice demonstrated synaptic and morphological deficits (Huang et al., 2011), I hypothesized that the dTg mice would similarly display alterations in synaptic plasticity and dendritic spine morphology.
4.3.2 Materials and Methods

4.3.2.1 Extracellular Field Recording

Electrophysiological field potentials (fEPSPs) were measured through the synaptic activation of the EC-DG and CA3-CA1 pathways. Within the hippocampus, the apical dendrites of the PP and Schaffer collateral pathways are all arranged in a parallel direction which allows them to be activated synchronously. fEPSPs represent local current sinks or sources due to the collective activity of many cells. The current sink is due to the relative flow of positive charges (mostly sodium) entering the cell (away from the electrode). The current loop is completed by current flowing through the cell, out across the membrane, and back to the site of the current sink. The ionic flow across the membrane results in current flow from source (adjacent regions with lower membrane resistance) to sink. Therefore, the extracellular field recordings in the PP and Schaffer collaterals of dTg mice will examine the synaptic activity in these circuits through measuring the amplitude, frequency, and polarity of the field responses.

Hippocampal slices from the WT and dTg mouse brains were prepared as described in the Experimental Procedures chapter. A stimulating electrode was positioned in the hippocampus at the apex of the DG blade or the CA3 Schaffer collateral pathway. A recording glass electrode (3-4MΩ) was placed in the MPP or the CA1 region of the hippocampus. The slices were stimulated at a voltage that evoked 60% of the maximum response. The response for field EPSPs were calculated from measuring the slope between 5% and 60% of the peak response, which was normalized to the mean of the baseline response. For input/output field recordings, the stimulus intensities were measured from 2, 3, 5, 7, 10, 20, 40, 60, 80, 100, 120, and 140mV. For the paired-pulse field recordings, the inter-stimulus intervals were measured at 25, 50, 100, 200, 300, 400, 500, and 1000ms. LTP at the CA1 synapse was induced by four trains of 100 Hz lasting 1 second each, with a 20-second intertrain interval. The baseline period was recorded for 30 minutes prior to the induction of LTP, followed by a post-induction phase of 50 minutes.
4.3.2.2 Intracellular Whole-cell Recording

All intracellular recordings were done in collaboration with Feng Cao from our laboratory. In comparison to extracellular recording, intracellular whole-cell techniques measure slower graded voltages, such as receptor or synaptic potentials. Intracellular patch clamp recordings insert glass microelectrodes into the cell membrane with minimal damage. Cells within the DG, BLA, and CA1 of WT and dTg mice were clamped at -65mV throughout the experiment. For EPSC\textsubscript{AMPAR}/EPSC\textsubscript{NMDAR} ratio experiments, EPSC\textsubscript{AMPAR} was the peak value obtained at -70mV, whereas EPSC\textsubscript{NMDAR} was the response amplitude at +40mV taken 40ms after the onset of the response. For current/voltage relationship, EPSC\textsubscript{AMPAR} (6 responses averaged) were recorded at various holding potentials including -80, -60, -40, -20, 0, +20, +40, and +60 mV) with 100µM D-APV. The EPSC amplitudes were normalized to those recorded at -60mV. For mEPSCs, the responses were recorded at -70mV with 100µM picrotoxin and 1µM TTX.

4.3.2.3 Electron Microscopy

Electron microscopy was used to examine the EC-DG synapse at an ultrastructure precise resolution of 50pm and a magnification of up to 10,000,000x. This technique uses a finely focused beam of accelerated electrons to bombard and illuminate the specimen. The transcardially perfused brains from WT and dTg mice were cut into 1x1mm blocks covering the molecular layer area of the DG. For each block, 1µm thick sections were cut and stained with 1% toluidine blue to guide further trimming to isolate equivalent DG molecular layer regions. From the chosen blocks, thin sections (60nm) were cut and stained with uranyl acetate and Reynolds lead citrate and subsequently mounted on hydrophilic carbon coated grids. Electron micrographs were taken at a final magnification of 25,000x.

4.3.3 Results

4.3.3.1 Impaired presynaptic function at the EC-DG synapse of CamKII\textsubscript{α}-tTA/tetO-mPak3-GFP mice

To elucidate the synaptic basis underlying the social memory deficit in the dTg mice, I conducted extracellular electrophysiological field recordings along the medial PP across a wide range of stimulus
intensities (Figure 4.3.1A). I found that the input-output curve of fEPSPs evoked by given stimulus intensities, showed significantly reduced basal synaptic responses in dTg slices (Figure 4.3.1B). Accordingly, I also compared the size of the presynaptic fiber volley which was proportional to the number of presynaptic neurons recruited by the stimulus. Similarly, I observed a significantly decreased basal synaptic transmission in dTg mice despite the same number of presynaptic axon fibers being activated by the stimulation (Figures 4.3.1C and 4.3.1D). This could be attributed to deficits in the response of the postsynaptic neuron or diminished presynaptic release probability. Therefore, to tease apart these possibilities, I examined PPF, which is a presynaptic form of short-term plasticity. In WT mice, the medial PP typifies PPD, where the synaptic response of a second closely spaced stimuli is decreased compared to the first, which may be due to the fact that the PP has a higher probability of release and/or a larger readily releasable vesicle pool. However, dTg mice displayed an increased PPF as opposed to PPD (Figure 4.3.1E). To corroborate this, the miniature excitatory postsynaptic activity in the DG granule cells was recorded using TTX which blocks action potential formation and propagation (Figure 4.3.2A). mEPSC amplitude was unchanged suggesting no differences in the postsynaptic response (Figure 4.3.2B). On the other hand, mEPSC frequency was reduced (Figure 4.3.2C). Our results also revealed no changes in the ratios of AMPA/NMDA types of glutamate receptors which contribute to the evoked excitatory responses in the DG compared to WT mice (Figure 4.3.3A and 4.3.3B). Furthermore, the current/voltage relation of EPSC\textsubscript{AMPA} was not altered (Figure 4.3.3C).
Figure 4.3.1 Impaired presynaptic function at the EC-DG synapse in dTg mice using extracellular field recording. (A) Schematic of stimulation and recording electrode placement in the hippocampal slice. (B) Input/output curves of fEPSP vs stimulation intensity (WT: n = 10; dTg: n = 8; Group $F_{(1, 208)} = 49.95, p < 0.0001$; Intensity $F_{(12, 208)} = 34.73, p < 0.0001$, two-way RM ANOVA). Scale bar: 0.35 mV/4 ms. (C) Input/output curves of fEPSP vs prefiber volley (WT: n = 10; dTg: n = 8; Group $F_{(1, 208)} = 49.95, p < 0.0001$, two-way RM ANOVA). (D) Prefiber volley vs stimulation intensity. (WT: n = 10; dTg: n = 8; Group $F_{(1, 208)} = 0.3841, p = 0.5361$, two-way RM ANOVA). (E) Altered paired-pulse ratio in dTg mice (WT: n = 6; dTg: n = 8; Group $F_{(1, 108)} = 39.06, p < 0.0001$; ISI $F_{(8, 108)} = 2.340, p = 0.0233$; 2-way RM ANOVA; Post-hoc Holm-Sidak’s multiple comparisons: $p = 0.0439$ for ISI 25, $p = 0.0065$ for ISI 40, $p = 0.0372$ for ISI 100). Scale bar: 0.2 mV/5ms.
Figure 4.3.2 Impaired presynaptic function at the EC-DG synapse in dTg mice using intracellular whole-cell recording. (A) Schematic of recording electrode placement in the DG granule cell layer in a hippocampal slice. (B) mEPSC amplitude is unchanged in dTg mice (WT: n = 11; dTg: n = 12; Amplitude $p = 0.7737$, two-tailed unpaired t test). Scale bar: 10 pA/1s. (C) mEPSC frequency is reduced in dTg mice (WT: n = 11; dTg: n = 12; Frequency $p = 0.0002$, two-tailed unpaired t test). Scale bar: 10 pA/1s.
Figure 4.3.3 Normal AMPA and NMDA receptor contributions to the EC-DG synapse in dTg mice using intracellular whole-cell recording. (A) Schematic of recording electrode placement in the DG granule cell layer in a hippocampal slice. (B) EPSC\textsubscript{AMPA}/EPSC\textsubscript{NMDAR} ratios in dTg mice (WT: n = 9; dTg: n = 8; \( p = 0.6076 \); two-tailed unpaired t test). Scale bar: 100 pA/50 ms. (C) Current/voltage relationship of AMPAR-mediated synaptic currents in dTg mice. (WT: n = 9; dTg: n = 8; Group \( F_{(1, 120)} = 0.1107, p = 0.7399 \); Potential \( F_{(7, 120)} = 625.0, \ p < 0.0001 \); two-way RM ANOVA). Scale bar: 100 pA/50 ms.
4.3.3.2 Increased number of synaptic vesicles closer to the axon terminal at the PP in dTg mice via EM

To further examine the structure of individual synapses in the PP, I obtained and analyzed EM thin sections of the PP synapse at the granule cell layer of the DG of the hippocampus. The density of asymmetric synapses, identified by the presence of the postsynaptic density (PSD) apposed to the presynaptic vesicles, was unchanged in the dTg sections compared to the WT (Figure 4.3.4A). Although the number of synapses as well as the length of the PSD were not altered, a greater number of synaptic vesicles were found closer to the axon terminal in dTg mice compared to WT mice (Figures 4.3.4B-4.3.4D), consistent with a deficit in presynaptic neurotransmitter release.

4.3.3.3 Normal synaptic function at the CA1, EC, and BLA synapse in dTg mice

Schaffer collaterals have been previously shown to play an important role in the regulation of synaptic transmission, plasticity, and learning and memory (Asrar et al., 2009; Huang et al., 2011; Meng et al., 2005). However, in contrast to the EC-DG synapse, synaptic transmission at the Shaffer collateral CA3-CA1 synapses was not altered in the dTg mice (Figure 4.3.5A), as evidenced by normal input/output curves of fEPSPs (Figure 4.3.5B), paired-pulse facilitation (Figure 4.3.5C), frequency or amplitude of mEPSCs (Figure 4.3.6A and 4.3.6B), AMPA/NMDA ratio (Figure 4.3.6C), current/voltage relationship of AMPA currents (Figure 4.3.6D), and LTP induced by HFS (4x100Hz) (Figure 4.3.5D). These results were consistent with the lack of transgene expression in the CA1 and CA3 regions of the hippocampus (Figure 4.1.4). Although I focused my analysis on the cellular mechanisms governing the function of the EC, no differences were found in the frequency or amplitude of mEPSCs in the EC layer II neurons (Figure 4.3.7A). It is possible that projections other than those of the PP may contribute to the behavioural and synaptic deficits observed. Since, the EC layer V cells form connections with the BLA (Canto et al., 2008) and that a low level of mPAK3-GFP transgene expression was observed in the BLA, I also examined the spontaneous activity in this region. However, the frequency and amplitude of mEPSCs in the BLA neurons from dTg slices were comparable to the WT (Figure 4.3.7B).
Figure 4.3.4 Impaired distribution of synaptic vesicles at the EC-DG terminals of dTg mice. (A) Representative electron micrographs of the DG molecular layer. Scale bars: 500 µm (15000x), 250 µm (30000x). (B) Synapse density in dTg mice (WT: n = 25; dTg: n = 23; $p = 0.5035$; two-tailed unpaired t test). (C) PSD length distribution in dTg mice (WT: n = 25; dTg: n = 24; Group $F_{(1, 237)} = 0.3064$, $p = 0.5804$; Length $F_{(2, 237)} = 130.3$, $p < 0.0001$; two-way ANOVA). (D) Number of synaptic vesicles in dTg mice (WT: n = 99; dTg: n = 94; $p = 0.0756$; two-tailed unpaired t test). (E) Altered distribution of synaptic vesicles in dTg mice (WT: n = 98; dTg: n = 84; Group $F_{(1, 1920)} = 3.651$, $p = 0.0562$; Distance from terminal $F_{(9, 1920)} = 140.0$, $p < 0.0001$; two-way ANOVA, Post-hoc Holm-Sidak’s multiple comparisons between genotypes, Distance 0-49 nm: $p = 0.0005$; 50-99nm: $p = 0.0012$).
Figure 4.3.5 Normal basal synaptic transmission and plasticity in the Schaffer collateral synapse in dTg mice. (A) Schematic of stimulation and recording electrode placement in the hippocampal slice. (B) Similar input/output curves of fEPSP vs stimulation intensity at CA1 synapse in dTg and WT mice (WT: n = 6; dTg: n = 12; Group $F_{(1, 192)} = 2.795, p = 0.0962$; Intensity $F_{(11, 192)} = 23.90, p < 0.0001$; two-way RM ANOVA). Scale bar: 0.25 mV/3.5 ms. (C) Normal PPF at CA1 synapse in dTg and WT mice (WT: n = 11; dTg: n = 6; Group $F_{(11, 120)} = 1.160, p = 0.2836$; ISI $F_{(7, 120)} = 8.730, p < 0.0001$; two-way RM ANOVA). Scale bar: 0.15 mV/16 ms. (D) Intact LTP induced by high frequency stimulation (4 trains of 100 Hz, 1s each with a 20 s inter-train interval) in dTg mice. (WT: n = 4; dTg: n = 7; $p = 0.1404$; two-tailed unpaired t test). Scale bar: 0.1 mV/5 ms.
Figure 4.3.6 Normal AMPA and NMDA receptor contributions in the Schaffer collateral synapse in dTg mice. (A) Normal mEPSC amplitude recorded from CA1 pyramidal neurons (WT: n = 10; dTg: n = 10; Amplitude $p = 0.6852$; two-tailed unpaired t test). Scale bar: 10 pA/1 s. (B) Normal mEPSC frequency recorded from CA1 pyramidal neurons (WT: n = 10; dTg: n = 10; Frequency $p = 0.6689$; two-tailed unpaired t test). Scale bar: 10 pA/1 s. (C) EPSC\textsubscript{AMPAR}/EPSC\textsubscript{NMDAR} ratios recorded from CA1 pyramidal neurons (WT: n = 7; dTg: n = 5; $p = 0.8165$; two-tailed unpaired t test). Scale bar: 100 pA/50 ms. (D) Current/voltage relationship of AMPAR-mediated synaptic currents recorded from CA1 neurons. (WT: n = 13; dTg: n = 9; Group $F_{(1, 160)} = 0.8189, p = 0.3669$; Potential $F_{(7, 160)} = 111.7, p < 0.0001$; two-way ANOVA). Scale bar: 100 pA/50 ms.
Figure 4.3.7 Normal mEPSC amplitude and frequency in the EC layer II and the BLA of dTg mice. (A) mEPSC amplitude and frequency recorded from the EC layer II neurons in dTg and WT mice (WT: n = 14; dTg: n = 10; Amplitude $p = 0.2031$, Frequency $p = 0.8209$; two-tailed unpaired t test). Scale bar: 10 pA/1 s. (B) mEPSC amplitude and frequency recorded from the BLA neurons in dTg and WT mice (WT: n = 19; dTg: n = 20; Amplitude $p = 0.7402$; Frequency $p = 0.1976$; two-tailed unpaired t test and two-tailed Mann Whitney test). Scale bar: 10 pA/1 s.
4.3.4 Discussion

In this section, I demonstrated that synaptic transmission, specifically presynaptic neurotransmitter release, was impaired at the EC-DG synapse of dTg mice. Interestingly, the distribution of synaptic vesicles was closer to the presynaptic membrane at the PP terminals in dTg mice compared to WT mice. Ultimately, these findings provide novel insight into the cellular mechanisms by which PAK signaling regulates presynaptic neurotransmitter release and basal synaptic response in the EC-DG PP.

Specifically, the extracellular recording techniques showed that PPF experiments, a presynaptic form of short-term plasticity, was impaired in the PP of dTg mice, which may be due to reductions in neurotransmitter release. The reductions in paired pulse experiments are consistent with that of the PAK1 and PAK3 double KO mice (Huang et al., 2011). The intracellular recordings in the dTg mice demonstrated that mEPSC frequency was reduced, which further confirmed that presynaptic neurotransmitter release was impaired with no changes in synapse number. These results also indicated that the neurotransmitter content per quanta and the number or type of postsynaptic receptors at the terminals in the dTg mice were unchanged. Ultimately, the decreased mEPSC frequency may be due to a reduced rate of spontaneous vesicle fusion and release, which resulted in an increased number of synaptic vesicles at the axon terminals. Neurotransmitter release may be due to changes in presynaptic axon terminal morphology involved in the fusion and release of synaptic vesicles. This finding is inconsistent with the double KO mice, as the frequency and amplitude of mEPSCs were significantly elevated, suggesting that the PAK1 and PAK3 have profound effects on the potency of synapse function, through regulating neurotransmitter release and postsynaptic responses (Huang et al., 2011). The disparity between these results may be explained by the differences in genetic targeting. Compared to the KO mice which had significantly impaired postnatal brain development and neuronal complexity, the transgenic mice had a highly specific expression of the mPAK3-GFP transgene in the EC-DG terminals of mature mice (beginning at 3 weeks). Since PAKs are downstream targets of several key signaling pathways at the synapse that regulate basal synaptic transmission, including Rho GTPases, cell-adhesion molecules, and calcium channels (Daniels and Bokoch, 1999; Humeau et al., 2009; Manser et al., 1994), these results support the fact that PAKs play a critical role in regulating basal synaptic transmission in both developing and mature synapses.
The EM findings further indicated that presynaptic neurotransmitter release was impaired in the dTg mice. As mentioned, studies have shown that Rho GTPases are well-known organizers of the actin cytoskeleton through actin severing proteins that mediate vesicle trafficking, docking, and priming (Doussau et al., 2000). During prolonged synaptic activity, the readily releasable pool of synaptic vesicles must be replenished to maintain the high levels of neurotransmitter exocytosis (Bajjalieh, 1999; Betz, 1970; Schweizer et al., 1995). The actin cytoskeleton provides the main structural support within the presynaptic terminal and may serve as a track for the translocation of synaptic vesicles from the reserve pool to replenish the readily releasable pool at the active zone (Doussau and Augustine, 2000). Specifically, it is the balance between the assembly and disassembly of the actin filaments that may control the mobilization of the synaptic vesicles between the reserve and readily releasable pools. Furthermore, actin also plays a highly coordinated role in the fusion of synaptic vesicles to the plasma membrane and in the release of their content into the extracellular space. This is supported by studies that demonstrate that F-actin directly interacts with SNAREs (Porat-Shliom et al., 2013). Furthermore, Cdc42-dependent polymerization of actin promotes the docking of secretory vesicles in a process that is dependent on intersectin-1L, an actin binding protein that binds directly to SNAREs (Wen et al., 2011). Since PAKs are essential for proper cofilin-mediated actin distribution in neuronal processes, it is possible that the perturbations in PAK signaling may affect downstream actin-binding proteins such as LIMK, which may lead to the collapse of the actin cytoskeleton network within the presynaptic terminal. This can result in impaired trafficking, docking, and priming and hence, the accumulation of synaptic vesicles close to the presynaptic membrane of the EC axon terminal. Specifically, enhanced levels of cofilin-induced actin depolymerization (to be discussed in detail in section 4.4) may affect the structural dynamics of the active zone and subsequent fusion and release of the synaptic vesicles from the readily releasable pool, which could result in the observed reductions in neurotransmitter release and basal synaptic transmission. This is supported by evidence that Rac1, which is present in synaptic vesicles, activates LIMK and inhibits cofilin-induced actin depolymerization (Arber et al., 1998). Thus, the reorganization of the actin filaments in the presynaptic terminal through the PAK-LIMK-cofilin signaling pathway may be an important mechanism in the regulation of neurotransmitter release.
4.4 The Molecular Mechanisms Underlying the Inducible Disruption of PAK Signaling

4.4.1 Introduction and Rationale

Early studies that have examined the molecular mechanisms underlying the role of PAKs have employed cultured cell lines. These in vitro studies have shown that PAKs are critical for regulating actin and microtubule networks downstream of Rac1 and Cdc42. Specifically, the inactivation of upstream Rac1 led to dendritic spine anomalies and alterations in synaptic function in developing networks using hippocampal slice culture (Boda et al., 2004; Tashiro and Yuste, 2004). Furthermore, the pharmacological or genetic inhibition of PAK1 in cultured cortical and hippocampal neurons impaired synaptic transmission, LTP, and altered dendritic arborization and spine formation (Hayashi et al., 2004; Hayashi et al., 2007). With respect to PAK3, its suppression through the transfection of a small interfering RNA or a kinase-dead mutant in hippocampal slice cultures, also resulted in the formation of abnormally elongated dendritic spines, filopodia-like protrusions, and a decrease in mature synapses that failed to express postsynaptic densities connected to presynaptic terminals (Boda et al., 2004). In particular, the R67C mutation in the regulatory domain of PAK3, reduced spine density in hippocampal organotypic slice cultures (Kreis et al., 2007). More recently, the long and immature dendritic spines that were associated with a loss of function of PAK3, were rescued with the expression of constitutively active PAK1, suggesting the compensatory roles of PAKs in spine morphogenesis (Boda et al., 2008). Together, these results stress the critical role of PAKs in dendritic spine morphogenesis and synaptic plasticity, yet the molecular mechanisms through which PAKs regulate the structural and synaptic processes in vivo remains unclear.

PAK signaling through its interaction with the active form of Rac and Cdc42 have been shown to be important in the regulation of cofilin through the Rho signaling pathway (Asrar et al., 2009; Huang et al., 2011; Manser et al., 1994). Previous studies in our lab demonstrated that PAK1 and PAK3 double KO mice have reduced phosphorylated cofilin levels and that blocking cofilin activity rescued spine deficits, suggesting PAK plays an important role in cofilin regulation (Huang et al., 2011). Other studies that have focused on the downstream effectors of PAK, including LIMK1 KO and LIMK1/2 double KO mice, which were found to be severely affected in cofilin phosphorylation and exhibited
significant abnormalities in spine morphology and LTP (Meng et al., 2002). However, these deficits were observed through the widespread deletion of PAKs in the KO models. Thus, whether the mPAK3-GFP transgene in the dTg mice impairs cofilin regulation in dendritic spine formation and synaptic plasticity is unknown.

As mentioned previously, actin goes through continuous treadmilling between filamentous and globular actin (Patterson and Yasuda, 2011). A shift in the equilibrium contributes to basal spine motility and activity dependent structural plasticity. Actin affects presynaptic neurotransmitter release, postsynaptic receptor properties, and synaptic plasticity (Krupp et al., 1999; Meng et al., 2004). Based on the findings from our lab, PAKs are critical for synaptic plasticity and learning and memory, via regulating cofilin activity through the reorganization of the actin cytoskeleton (Huang et al., 2011). Therefore, I hypothesize that the impairments in synaptic transmission, short-term plasticity, and social memory in the dTg mice, may be due to altered cofilin phosphorylation through an actin-dependent manner in the EC-DG terminals. In this section, I will discuss the changes observed in cofilin and the actin cytoskeleton in the EC-DG PP between WT and dTg mice.

### 4.4.2 Materials and Methods

#### 4.4.2.1 Molecular and Biochemical Analysis

To identify if targets in PAK signaling are changed in dTg mice compared to WT mice, antibodies specific to cofilin and p-cofilin were used in Western blot analysis and immunohistochemistry. Protein lysates were microdissected from the hippocampus and cerebellum and used to compare the total levels of cofilin and p-cofilin in WT and dTg mice. In addition, brain sections from WT and dTg mice were stained with appropriate primary antibodies including anti-GFP and phalloidin to visualize the regional expression of actin in the EC-DG and the CA3 as control. Both Western blot and immunostaining analyses for each genotype were quantified using ImageJ software and are described in the Experimental Procedures chapter.
4.4.3 Results

4.4.3.1 Enhanced total cofilin activity in dTg mice

Through Western blot analysis of hippocampal or cerebellar lysate, I compared the activity of cofilin in WT and dTg mice. The basal level of the inactive phosphorylated cofilin (pCofilin) was unchanged, however, the cofilin activity was enhanced in the dTg compared to the WT hippocampal fraction (Figures 4.4.1A-4.4.1C). Overall, the dTg mice were reduced in the relative ratio of pCofilin compared to total cofilin levels (Figure 4.4.1D). The basal level of pCofilin and cofilin were unchanged in the cerebellum of dTg mice (Figure 4.4.1A).

4.4.3.2 Perturbed F-actin network in dTg mice as visualized by phalloidin

Given the elevated amount of basal cofilin in dTg mice, I further investigated the effects on actin assembly. I observed a marked increase in phalloidin intensity along the axons projecting to the DG which is representative of greater filamentous actin (F-actin) content in the PP compared to the CA3 region of the hippocampus in dTg mice (Figures 4.4.2A and 4.4.2B).

4.4.4 Discussion

In this section, I demonstrated that mPAK3-GFP transgene expression enhanced the activity of cofilin (active), without altering the level of pCofilin (inactive) in hippocampal lysates of dTg mice. Furthermore, the amount of F-actin in the EC-DG PP of dTg mice was increased compared to WT mice. Thus, this suggests that perturbations in PAK signaling enhances cofilin activity which severs existing actin filaments, resulting in the increase in overall F-actin levels.

The Western blot results demonstrated the increased activation of cofilin within the hippocampus. Although, pCofilin levels were unchanged, the overall pCofilin to cofilin ratio was reduced in this region. This is consistent with PAK1 and PAK3 double KO mice that similarly showed an increase in cofilin activity in the hippocampus (Huang et al., 2011). However, in the double KO mice, the
enhanced cofilin activity was associated with a reduction in phosphorylated but not total cofilin. Since the R67C transgene decreases both Cdc42 binding and induced PAK3 activity, it is expected that the activities of the downstream signaling molecules would also be altered. The effect on the total level of cofilin may be explained by the differences in transcription, translation, or post-translational modifications between the acute expression of the mPAK3-GFP transgene versus the chronic KO approach. Furthermore, there were fewer developmental compensations in the dTg mice compared to the global double KO mice. Notably, the single KO of PAK1 or PAK3 revealed no changes in the basal level of pCofilin or total cofilin, which further suggests that PAKs serve a redundant role in the regulation of cofilin phosphorylation. Thus, the localized disruption of PAK in the PP expression was a less disruptive approach to investigate the molecular mechanisms governing PAK function in this circuit.

To specifically examine the EC-DG PP, I used immunohistochemistry to examine the actin properties with the F-actin dye, phalloidin. I showed that the PP was highly enriched in F-actin in the PP of dTg mice compared to WT mice. This is consistent with the interpretation that enhanced cofilin activity severs existing actin filaments resulting in the generation of free barbed ends that polymerizes actin and ultimately, increases F-actin content (Ghosh et al., 2004; Shekhar and Carlier, 2017). It is important to note that other studies have shown that increased cofilin activity is also associated with decreased F-actin under some conditions (Bamburg, 1999). For example, previous single PAK1 KO and PAK1 and PAK3 double KO studies have revealed reductions in F-actin, through phalloidin, in the CA1 region of the hippocampus (Asrar et al., 2009; Huang et al., 2011). Compared to these KO studies, the transgenic mice may show conformational changes in actin dynamics through differences in the PAK signal transduction pathways. Specifically, the expression of the mPAK3-GFP transgene can lead to the severing and uncapping of preexisting actin filaments and the generation of free barbed ends, which are the site for actin filament assembly. Therefore, this suggests that PAKs regulate cofilin activity and thereby F-actin dynamics in the EC-DG PP. However, given the role of PAK on other effectors, the secondary effects that this may have on cofilin activity cannot be discounted. Hence, future studies will aim to elucidate the exact signaling mechanism through which PAKs affect the actin cytoskeleton.
Figure 4.4.1 Enhanced cofilin activity in dTg mice. (A) Western blots of cerebellar (CR) and hippocampal (HP) protein lysates showing changes in pCofilin/cofilin in the hippocampus, but not in cerebellum in dTg mice (mutant band probed with anti-PAK3). (B) Summary graph showing significantly increased cofilin in the HP, but not in the CR (CR WT: n = 9; dTg: n = 9; p = 0.9765; two-tailed unpaired t test; HP WT: n = 10; dTg: n = 12; p = 0.0057; two-tailed unpaired t test). (C) Summary graph showing total pCofilin in the CR and HP (CR WT: n = 13; dTg: n = 13; p = 0.3026; two-tailed Mann Whitney test; HP WT: n = 13; dTg: n = 13; p = 0.7442; two-tailed Mann Whitney test). (D) Summary graph showing a significantly decreased pCofilin/cofilin ratio in the dTg HP (WT: n = 12; dTg: n = 12; p = 0.0121; two-tailed unpaired t test).
Figure 4.4.2 Altered actin assembly in dTg mice. (A) Confocal images of transverse sections from WT and dTg mice stained with DAPI, anti-GFP and phalloidin. Region of interest (ROI) is marked on the PP with a white box. Scale bars: 100 µm. (B) Summary of corrected total fluorescence of the PP ROI (WT: n = 10; dTg: n = 15; p = 0.003; two-tailed unpaired t test).
4.5 The Role of the EC-DG Circuit in Social Memory

4.5.1 Introduction and Rationale

Sensory information from the different modalities is first processed in distinct cortical association areas and then relayed through the MPP and LPP to the DG of the hippocampus (Eichenbaum and Lipton, 2008). The EC is critical for declarative and associative memories and forms subcortical connections to the basal forebrain, claustrum, amygdala, basal ganglia, thalamus, hypothalamus, and brainstem (As discussed in the section 1.2.2; Figure 1.2) (Canto et al., 2008). In rodents, the EC forms a neural map consisting of grid cells that define the spatial environment (O'Keefe and Burgess, 2005). Studies have shown that neurotoxic lesions to the EC in mice impaired contextual fear learning and visual recognition memory but not spatial learning (Burwell et al., 2004; Gaffan and Murray, 1992; Moss et al., 1981). Therefore, as the major input and output structure of converging sensory modalities, the EC may be the central signaling hub responsible for relaying memory engrams to downstream subcortical targets for further processing. Despite the extensive studies documenting the critical involvement of the entorhinal cortical-hippocampal circuit in memory processing and storage, particularly for spatial and contextual memories, surprisingly very few studies exist to specifically elucidate the role of this circuit in social behavior.

As discussed earlier, recent studies employing acute optogenetic approaches have implicated various brain regions and circuits, including the amygdala, hippocampus, and ventral tegmental area to play a critical role in the different facets of social behaviour, including social interactions, approach, and discrimination memory (Felix-Ortiz and Tye, 2014; Gunaydin et al., 2014; Hitti and Siegelbaum, 2014; Okuyama et al., 2016). However, there is little overlap between the fundamental mechanisms and circuitry underlying social memory proposed in these studies. Furthermore, the precise cell types and molecular substrates involved remain elusive. Thus, I used the CaMKIIα-tTA/tetO-mPAK3-GFP transgenic mice to study the role of PAK in excitatory reelin-positive EC cells that project to the DG in social memory.
Since the results from section 4.3 revealed that dTg mice had impairments in basal synaptic transmission, I wanted to investigate whether the social memory deficits were due to reduced neurotransmitter release. Thus, to address whether PAK activity in the PP was indispensable and/or inducing to regulate social memory, I used an acute optogenetic approach. I expressed either ChR2 or ArchT in the EC layer II cells, to activate or silence the PP projections to the DG. This targeted approach allowed for improved spatial, temporal, and neurochemical precision in the bidirectional control of the EC-DG axon terminals. More importantly, compared to the acute pharmacological approaches discussed in section 4.2, optogenetic targeting allowed for the selective manipulation of the EC-DG circuit during each stage of the social memory test, including memory formation and retrieval.

In this section, I will discuss the specific experiments used to investigate the necessity and sufficiency of the EC-DG pathway in the different stages of social memory. First, to distinguish if the EC was indispensable for social memory formation or retrieval, the PP was artificially inhibited in the WT mice during each stage of the test respectively. Next, to determine if PAK activity in the EC was sufficient for inducing social memory, the PP was activated within the WT mice. Lastly, to investigate if the social deficits in dTg mice were due to reduced neurotransmitter release in the PP, the EC-DG terminals were transfected with ChR2 and acutely activated. If social recognition memory is dependent on proper neurotransmitter release, then I expect that the acute inhibition of the PP in WT mice will reveal social memory deficits that are similar to dTg mice. Furthermore, I hypothesize that activation of the PP will restore social memory in the dTg mice. Together, these findings will elucidate the necessity and sufficiency of the EC-DG in social memory.

4.5.2 Materials and Methods

4.5.2.1 Stereotaxic Surgery

All stereotaxic surgeries were completed in collaboration with Robin Nguyen and Afif Aqrabawi from Dr. Jun Chul Kim’s laboratory. Anesthetized mice were virally injected with rAAV5-CaMKIIα: eArchT3.0-EYFP (with a titer of 3.3*10^{12}), rAAV5-CaMKIIα-EYFP (with a titer of 3.3*10^{12}), or rAAV5-CaMKIIa-hChR2 (H134R)-mCherry-WFRE-PA (with a titer of 4.5*10^{12}). The virus was targeted into the EC layer II cells bilaterally and optic fibers were subsequently implanted above the
dorsal DG or the ventral CA1. The surgically operated mice were recovered for 4 and 6 weeks to allow for sufficient ChR2 and ArchT viral transduction, respectively, before behaviour tests were performed.

4.5.2.2 Immunohistochemistry

The expression pattern of ChR2-mCherry and ArchT-eYFP, and the placement of the optic fibers were confirmed via histological staining of fixed coronal brain sections following all behaviour tests. For dTg mice, the colocalization of the mPAK3-GFP transgene and the ChR2-mCherry was confirmed with anti-GFP antibodies. Subject mice with the optical fibers misplaced or viral mistargeted were excluded from the analysis.

4.5.2.3 Behavioural Tests

4.5.2.3.1 Three Chamber Social Interaction Test

For ArchT activation, ArchT or control EYFP mice were assessed with or without continuous bilateral 532nm green-light, counterbalanced over two days of testing. The green-light was presented during either the 5-minute sociability stage (stage 2) or the 5-minute discrimination/memory stage (stage 3). To test the effect of ChR2 activation on social discrimination/memory recognition, ChR2 expressing WT or dTg mice were given bilateral 473nm blue-light pulses in a 30 second light-on followed by 30 second light-off pattern presented during the 5-minute discrimination/memory stage. To further test the effect of ChR2 in facilitating sociability/memory, the three-chamber testing protocol was slightly modified in which the sociability period was reduced to 2 minutes, which was followed by a 5-minute social memory or discrimination stage. In this paradigm, WT or dTg mice were given bilateral 473nm blue-light pulses delivered during stage 2 or 3 in a 30 second on/off pattern only when the test mice were interacting with the previously encountered stranger. For ChR2 experiments, 589nm yellow-light pulses with a 30 second light-on followed by 30 second light-off pattern was used as control.
4.5.2.3.2 Five-Trial Social Memory Assay

In the modified five-trial social memory assay, social memory/habituation for a stranger mouse was measured across six consecutive trials prior to the presentation of a novel mouse during dishabituation in the seventh trial. Half of the ArchT or EYFP expressing mice were assessed under 532nm green-light off conditions while the other half, under green-light on conditions in the fifth trial (familiar mouse) and seventh trial (novel mouse).

4.5.2.3.3 Novel Object Test

ArchT or control EYFP mice were subjected to three stages where a novel object was presented. Continuous 532nm green-light was delivered bilaterally for 5 minutes during the presentation of the new object. Mice were tested with and without light in a counter-balanced manner.

4.5.2.3.4 Elevated Plus Maze Test

ArchT and control EYFP expressing mice with the optical fibres were allowed to explore the apparatus without light for 2 minutes. Continuous 532 nm green-light was delivered bilaterally for 2 minutes and the procedure was repeated with light off for 2 additional minutes.

4.5.2.3.5 Fear Conditioning

4.5.2.3.5.1 Contextual Fear Conditioning

ArchT or control EYFP expressing mice with optical fibers attached received a 3-minute home cage habituation prior to the test. The fear conditioning training allowed individual subjects to explore the chamber for 3 minutes before the onset a continuous 300Hz tone lasting 30 seconds. During the last 2 seconds, the subject was exposed to a continuous foot shock. Three of such training sessions were delivered in succession, with 30 second intertrain intervals. 2 and 24 hours after the training, contextual memory (4 minutes in the training chamber) was tested with continuous 532nm green-light delivered bilaterally.
4.5.2.3.5.2 Trace Fear Conditioning

EC-CA1 ArchT and ChR2 mice with optical fibers were placed in the conditioning chamber denoted as “Context A” for 3 minutes, before the onset of a discrete 2000Hz tone lasting 20 seconds. This was followed by a 20-second trace period and a 2-second foot shock. Two more of such training sessions were delivered in succession, with a 2-minute intertrain interval. Mice received continuous green-light during the 3 training periods. 24 hours after the training sessions, subjects were placed in a modified conditioning chamber denoted as “Context B” for 3 minutes, followed by the same tone as presented during training for 60 seconds and a post-tone period of 3 minutes. This was repeated two additional times.

4.5.2.4 Intracellular Whole-cell Recording

To confirm the effect of ArchT expression at the EC-DG projection, EPSCs evoked by stimulating the MPP were recorded for 5 minutes; before the green-light was applied, during the green-light illumination and after the green-light was turned off. To confirm ChR2 expression, whole-cell recordings were obtained from the EC layer II or adjacent somatosensory cortical neurons and action potentials were triggered by blue-light pulses (5 ms, 473 nm, 1-4 mW) using a Dual OptoLED delivered through a 40x objective lens. To confirm the effect of ChR2 activation on synaptic transmission at the EC-DG synapses, 1ms pulses of blue-light (473nm, 1-4mW) were delivered to the DG molecular layer area and the light-evoked EPSPs or EPSCs were recorded from the DG granule cells. To confirm that DG granule cells did not express ChR2, NBQX (10µM) and picrotoxin (100µM) were added to ACSF to block EPSP, action potentials or EPSC recorded from the DG granule cells.
4.5.3 Results

4.5.3.1 Inhibition of the EC-DG circuit impairs social recognition memory retrieval

To determine whether the neuronal activity of the EC-DG circuit is required for social recognition memory, I used an optogenetic approach to inhibit the EC-DG. The superficial layer II cells of the ventral EC of WT mice were injected with adeno-associated viruses containing ArchT fused with EYFP or EYFP alone under the control of the CaMKIIα promoter to restrict their expression to excitatory neurons (Figure 4.5.1A and 4.5.1B). In the hippocampus, ArchT was seen in the molecular layer, but not in the granule cells of the DG (Figure 4.5.1C). Optic fibers were implanted bilaterally above the dorsal DG to allow for the optical inhibition of the EC-DG terminals via the continuous delivery of 532nm green-light. I first conducted a three-chamber social interaction test (Figure 4.5.2A) to assess sociability (stage 2) and social recognition memory (stage 3). In the EYFP control group, when constant illumination with 532nm green-light was presented either during stage 2 or 3, the mice showed a similar amount of interaction or preference during memory encoding and retrieval compared to those without light (Figure 4.5.2B-4.5.2D).

However, in ArchT-EYFP mice, when the green-light was delivered either during stage 2 or 3, social recognition memory, but not sociability was significantly impaired (Figure 4.5.2E-4.5.2H). The effect of optical inhibition during discrimination in stage 3 was significantly greater than during sociability (Figure 4.5.2H), suggesting that the memory retrieval process was particularly liable to perturbations in the EC-DG circuit. To test this further, I performed a modified version of the five-trial social memory assay to evaluate social habituation and dishabituation (Figure 4.5.2I-4.5.2K). Without optical inhibition, both ArchT-EYFP and EYFP expressing mice showed a gradual reduction in interaction time across four successive presentations to the same stranger mouse (Figure 4.5.2J). This indicated that both groups were able to form the memory of the stranger. However, in the fifth trial with the same mouse but now with constant green-light being presented to inactivate the EC-DG terminals, there was a marked increase in the exploration time in the ArchT-EYFP group compared to the EYFP control group. The ArchT-EYFP group behaved as if a novel stranger mouse was presented, suggesting impairments in social memory retrieval (Figure 4.5.2K). The light-induced increase in exploration
time was reversed when the green-light was turned off in trial 6. Specifically, the interaction time was decreased to the same level as in trial 4. In the following trial (trial 7) when a novel stranger was introduced, both ArchT-EYFP and EYFP groups showed a similar increase in exploration time. The EC-DG terminal inhibition had no effect on anxiety-like behaviour (Figure 4.5.3A-4.5.3C), novel object recognition (Figure 4.5.4A-4.5.4C), or contextual fear learning and memory (Figure 4.5.5A and 4.5.5B).

4.5.3.2 Activation of the EC-DG circuit facilitates social recognition memory retrieval

To examine if the activation of the EC-DG circuit is sufficient to facilitate social recognition memory, I expressed ChR2 in the terminals of EC-DG excitatory neurons (similar to the technique as described for ArchT in Figure 4.5.1A and 4.5.1B). As discussed in the previous results section, the effect on memory retrieval was more pronounced when the EC-DG circuit was inhibited during discrimination (Figure 4.5.2H). Thus, the effect of illumination epochs of 473nm blue-light pulses during memory retrieval of the social interaction test was investigated. However, social recognition memory was similar between the blue-light and 589nm yellow-light control pulses (Figure 4.5.6A-4.5.6D).

However, it was possible that the five-minute sociability period in the three-chamber social interaction test I used, may have saturated the memory encoding/discrimination system, so that the effect of further activating the circuit was occluded. Therefore, the duration of sociability was reduced from five to two minutes. The mice were no longer able to discriminate between the novel and familiar mouse (Figure 4.5.6E-4.5.6H), which suggested that the two-minute interaction was insufficient for memory encoding and/or retrieval. When 473nm blue-light pulses were applied to activate the EC-DG terminals during the two-minute interaction period, ChR2 expressing mice showed no preference for either mouse (Figure 4.5.6E-4.5.6H). In contrast, when the blue-light pulses were applied during the discrimination stage, the ChR2 mice showed a significant preference for the novel S2 mouse (Figure 4.5.6I-4.5.6L).
Figure 4.5.1 Expression of ArchT and ChR2 in the EC-DG circuit. (A) The placement of the optical fiber in the dorsal DG and site of viral injections in the ventral EC. (B) Inhibition and activation of neurotransmitter release at the EC-DG synapse. (C) Immunostaining images of dorsal and ventral brain sections showing the expression of ArchT-EYFP in the EC and the PP pathway in the DG. Scale bars: 1000 µm (Coronal); 500 µm (DG); 100 µm (EC).
Figure 4.5.2 Inhibition of the EC-DG circuit blocks social recognition memory retrieval. (A) The three-chamber social interaction test with continuous 532 nm green-light presented during sociability or discrimination. (B) Inhibition during stage 2 or 3 had no effect on preference for stranger 1 (S1) over empty cage (E) in EYFP mice (EYFP: n = 8, p < 0.0001; EYFP-Stage 2: n = 8, p < 0.0001; EYFP-Stage 3: n = 9, p = 0.0039; two-tailed paired t test and two-tailed Wilcoxon matched-pairs signed rank test). (C) Inhibition during stage 2 or 3 had no effect on normal preference for stranger 2 (S2) over S1 in EYFP mice (EYFP: p = 0.0156; EYFP-Stage 2: p = 0.0002; EYFP-Stage 3: p = 0.0113; two-tailed paired t test and two-tailed Wilcoxon matched-pairs signed rank test). (D) Inhibition during stage 2 or 3 had no effect on discrimination scores of stage 3 preferences in EYFP mice \(F(2, 22) = 0.7585, p = 0.4802\); one-way ANOVA). (E) Representative track plots and heat maps showing the effect of ArchT
on social discrimination. (F) Inhibition of the EC-DG circuit by ArchT during stage 2 or 3 had no effect on sociability compared to control ArchT mice (ArchT: n = 11, p < 0.0001; ArchT-Stage 2: n = 12, p < 0.0001; ArchT-Stage 3: n = 11, p < 0.0001; two-tailed paired t test). (G) Inhibition of the EC-DG circuit by ArchT during stage 2 or 3 abolished preference for S2 over S1 (ArchT: p = 0.0001; ArchT-Stage 2: p = 0.1790; ArchT-Stage 3: p = 0.6455; two-tailed paired t test). (H) Decreased discrimination scores for stage 3 in ArchT-Stage 3 compared to ArchT-Stage 2 or ArchT control mice (F(2, 31) = 4.235, p = 0.0237, one-way ANOVA; Post-hoc Holm-Sidak’s multiple comparisons with ArchT, ArchT-Stage 2 p = 0.2538, ArchT-Stage 3 p = 0.0198). (I) A modified, repeated multiple-trial social memory assay with continuous 532nm light presented during Trial 5 and 7. (J) ArchT and EYFP mice showed memory acquisition during Trials 1-4. ArchT mice with inhibition on Trial 5 showed an increased interaction time compared to EYFP mice (EYFP: n = 8, ArchT: n = 10; p = 0.0226; two-tailed unpaired t test). In Trial 6 (light off), both ArchT and EYFP mice showed memory for the first stranger mouse. ArchT and EYFP mice with light showed similar preference for a novel mouse on Trial 7. (K) Normalized interaction time obtained from the repeated trial memory test.
Figure 4.5.3 Inhibition of the EC-DG Circuit by ArchT had no effect on anxiety-like behaviour. 

(A) Distance travelled between ArchT and EYFP control mice with (On) and without (Off) 532nm green-light (EYFP: n = 8; ArchT: n = 11; Off1 p = 0.1351; On p = 0.4472; Off 2 p = 0.8949; two-tailed unpaired t test). (B) Number of entries into open arms between ArchT and EYFP control mice with or without light (Off1 p = 0.2219; On p = 0.2397; Off2 p = 0.6465; two-tailed unpaired t test). (C) Number of entries into closed arms between ArchT and EYFP control mice with or without light (Off1 p = 0.5289; On p = 0.1315; Off 2 p = 0.9542; two-tailed unpaired t test).
Figure 4.5.4 Inhibition of the EC-DG Circuit by ArchT had no effect on object recognition memory. (A) The habituation phase of the novel object recognition test showing no differences in the preference for object 2 versus object 1 in ArchT and EYFP mice with and without light (EYFP Off: n = 8, p = 0.2430; EYFP On: n = 7, p = 0.0652; ArchT Off: n = 11, p = 0.5201; ArchT On: n = 11, p = 0.7759; two-tailed unpaired t test). (B) Preference for novel object 3 versus familiar object 2 for ArchT and EYFP mice with and without 532 nm green-light during discrimination stage (EYFP Off: p = 0.0273; EYFP On: p = 0.0003; ArchT Off: p = 0.0062; ArchT On: p < 0.0001; two-tailed unpaired t test). (C) Discrimination scores of preferences for object 3 versus 2 \((F_{(3, 33)} = 1.084, p = 0.3694, \text{ one-way ANOVA})\).
Figure 4.5.5 Inhibition of the EC-DG Circuit by ArchT had no effect on contextual fear memory.

(A) ArchT mice showed no difference in freezing before and after shock training compared to EYFP mice (EYFP: n = 8; ArchT: n = 10; Before $p = 0.7255$; After $p = 0.8101$; two-tailed Mann Whitney test and two-tailed unpaired t test). (B) ArchT and EYFP mice with 532nm green-light during contextual test showed similar short and long-term contextual fear memory (2 hours $p = 0.4638$; 24 hours $p = 0.8945$; two-tailed unpaired t test).
Figure 4.5.6 Activation of the EC-DG circuit facilitates social recognition memory retrieval. (A) The social interaction test with 473 nm blue-light during stage 3. (B) Activation of the EC-DG circuit by ChR2 during stage 3 had no effect on preference for S1 over E (ChR2: n = 8, \( p < 0.0001 \); ChR2-Stage 3: n = 10, \( p < 0.0001 \); two-tailed paired t test). (C) Activation of the EC-DG circuit by ChR2 during stage 3 had no effect on preference for S2 over S1 (ChR2: \( p = 0.0015 \); ChR2-Stage 3: \( p = 0.0065 \); two-tailed paired t test). (D) No differences in discrimination scores between ChR2-Stage 3 and control mice (\( p = 0.6410 \); two-tailed paired t test). (E) A modified SI test with stage 2 being reduced to 2 minutes. The 473 nm light was presented during stage 2 only when the test mouse was interacting with S1. (F) Activation of the EC-DG circuit by ChR2 during stage 2 had no effect on preference for S1 (ChR2: n = 7, \( p < 0.0001 \); ChR2-Stage 2: n = 7, \( p = 0.0156 \); two-tailed paired t test and Wilcoxon matched-pairs signed rank test). (G) Activation of the EC-DG circuit by ChR2 during
stage 2 had no effect on the lack of preference for S2 over S1 (ChR2: $p = 0.4790$; ChR2-Stage 2: $p > 0.9999$; two-tailed paired t test). (H) No differences in discrimination scores between ChR2-Stage 2 and control mice ($p = 0.9414$; two-tailed paired t test). (I) A modified SI test with 473nm light being presented during stage 3. (J) Activation of the EC-DG circuit by ChR2 during stage 3 had no effect on preference for S1 over empty cage (ChR2: $n = 7$, $p < 0.0001$; ChR2-Stage 3: $n = 8$, $p = 0.0078$; two-tailed paired t test and Wilcoxon matched-pairs signed rank test). (K) Activation of the EC-DG circuit by ChR2 during stage 3 facilitated preference for S2 over S1 (ChR2: $p = 0.2202$; ChR2-Stage 3: $p = 0.0059$; two-tailed paired t test). (L) Discrimination scores showing significantly increased discrimination in ChR2-Stage 3 compared to control group ($p = 0.0034$; two-tailed paired t test).
4.5.3.3 Activation of the EC-DG circuit rescues social recognition memory in dTg mice

To directly test whether the social memory deficit in mPAK3-GFP transgenic mice was due to impaired presynaptic neurotransmitter release at the EC-DG synapse, rescue experiments were performed in dTg mice by acutely activating the PP terminals. ChR2 was expressed in ventral EC II neurons in the dTg mice (Figure 4.5.7A) and further confirmed using staining techniques for the expression pattern of ChR2. The expression of ChR2-mCherry colocalized with mPAK3-GFP transgene expression in the dTg mice (Figure 4.5.7B and 4.5.7C). In the dTg mice, 589nm yellow-light control pulses did not rescue the deficit in social recognition memory. However, when 473nm blue-light pulses were used during stage 3, the dTg mice showed a strong preference for the novel S2 stranger which was comparable to WT mice (Figure 4.5.7D-4.5.7G). To further dissect the mechanisms underlying this behavioural reversal, the effect of optical activation in the modified three-chamber social interaction test was tested, where the duration of sociability was reduced to 2 minutes. When the blue-light pulses were presented during memory encoding (stage 2), the memory deficit in the dTg mice was not rescued (Figure 4.5.8A-4.5.8D). However, when the blue-light pulses were presented in during memory retrieval (stage 3), the dTg mice showed a significant preference for the novel S2 stranger (Figure 4.5.8E-4.5.8H).

4.5.3.4 Inhibition or activation of the EC-vCA1 circuit has no effect on social recognition memory

EC layer II/III axons that mainly project to the outer two-thirds of the molecular layer of the DG, also form the origin of the excitatory projections that directly innervate the stratum lacunosum-moleculare of CA1 neurons (Witter, 2007). A recent study demonstrated that the CA1 region of the hippocampus is required for social memory; specifically, the dorsal CA2 - ventral CA1 - nucleus accumbens shell circuit may act to process, encode, and store salient social information (Hitti and Siegelbaum, 2014; Okuyama et al., 2016). To test whether the social memory deficits observed in WT mice are caused by perturbations in the ECIII-ventral CA1 pathway, ArchT-EYFP was injected under the control of the CaMKIIα promoter into ventral ECIII cells. Optic fibers were subsequently implanted bilaterally above the ventral CA1 to allow for the inhibition of the ECIII-vCA1 terminals via the continuous delivery 532nm green-light (Figure 4.5.9A-4.5.9C). In the three-chamber social interaction test (Figure
ArchT mice with continuous 532nm green-light displayed normal sociability and social discrimination similar to the controls (Figure 4.5.9E-4.5.9G). Next, ECIII-CA1 projections were activated via the viral delivery of ChR2 in to these projection fibers (Figure 4.5.10A-4.5.10C). In the modified three-chamber test (where the sociability stage was reduced to 2 minutes), the ChR2 mice (with 473nm blue-light pulses) displayed impaired memory retrieval, despite normal sociability similar to the controls (Figure 4.5.10D-4.5.10G).
Figure 4.5.7 Optical activation of the EC-DG terminals reverses social memory deficits in dTg mice. (A) The placement of the optical fiber in the dorsal DG and site of injections of ChR2 viruses in the ventral EC layer II in the dTg mice. (B) Schematic of the EC-DG synapse by optical stimulation of ChR2 in dTg mice. (C) Immunostaining images showing restricted expression of ChR2 in the EC and the PP pathway in the DG that colocalizes with mPAK3-GFP in dTg mice. Scale bar: 1000 µm (coronal), 500 µm (DG). (D) The social interaction test with 473nm blue-light during stage 3. (E) Activation of the EC-DG circuit by ChR2 during stage 3 had no effect on normal sociability in dTg mice (ChR2: n = 13, p = 0.0002; ChR2-Stage 3: n = 16, p < 0.0001; two-tailed Wilcoxon matched-pairs signed rank test). (F) Activation of the EC-DG circuit by ChR2 during stage 3 was sufficient to rescue the impaired preference for S2 in dTg (ChR2: p = 0.8316; ChR2-Stage 3: p < 0.0001; two-tailed paired t test). (G) Discrimination scores of stage 3 showing that ChR2 activation during stage 3 improved discrimination in dTg mice (p = 0.0114; two-tailed paired t test).
Figure 4.5.8 Optical activation of the EC-DG terminals is sufficient for social memory retrieval.

(A) The modified SI test with the duration of stage 2 being reduced to 2 minutes and 473 nm light being presented during stage 2 only when interacting with S1. (B) Activation of the EC-DG circuit by ChR2 during stage 2 had no effect on normal sociability in dTg mice (ChR2: n = 13, p < 0.0001; ChR2-Stage 2: n = 13, p = 0.0002; two-tailed paired t test and two-tailed Wilcoxon matched-pairs signed rank test). (C) Activation of the EC-DG circuit by ChR2 during stage 2 had no effect on the impaired preference for S2 over S1 in dTg mice (ChR2: p = 0.4712; ChR2-Stage 2: p = 0.2943; two-tailed paired t test). (D) Activation of the EC-DG circuit by ChR2 during stage 2 had no effect on the discrimination scores of stage 3 in dTg mice (p = 0.9734; two-tailed paired t test). (E) The modified social interaction test with 473 nm blue-light presented during stage 3. (F) Activation of the EC-DG circuit by ChR2 during stage 3 had no effect on normal sociability in dTg mice (ChR2: n = 12, p = 0.0005; ChR2-Stage 3: n = 12, p = 0.0005; two-tailed Wilcoxon matched-pairs signed rank test). (G) Activation of the EC-DG by ChR2 during stage 3 rescued the impaired preference for S2 over S1 in dTg mice (ChR2: p = 0.7281; ChR2-Stage 3: p = 0.0019; two-tailed paired t test). (H) Activation of the EC-DG circuit by ChR2 during stage 3 significantly increased discrimination scores of stage 3 in dTg mice (p = 0.0338; two-tailed paired t test).
Figure 4.5.9 Inhibition of the EC-CA1 circuit has no effect on social recognition memory retrieval. (A) The placement of the optical fiber in the ventral CA1 and site of viral injections in the ventral EC. (B) Inhibition of neurotransmitter release at the EC-CA1 synapse. (C) Immunostaining image of a ventral brain section showing the expression of ArchT-EYFP in the CA1. Scale bar: 1000 µm. (D) The three-chamber social interaction test with continuous 532 nm green-light presented during social discrimination. (E) Inhibition of the EC-CA1 circuit by ArchT during stage 3 had no effect on sociability similar to control ArchT mice (ArchT: n = 12, p < 0.0001; ArchT-Stage 3: n = 11, p < 0.0001; two-tailed paired t test). (F) Inhibition of the EC-CA1 circuit by ArchT during stage 3 had no effect on discrimination preference for S2 over S1 (ArchT: p < 0.0001; ArchT-Stage 3: p < 0.0001; two-tailed paired t test). (G) Normal discrimination scores for stage 3 in ArchT-Stage 3 similar to ArchT control mice (p = 0.2870; two-tailed paired t test).
Figure 4.5.10 Activation of the EC-CA1 circuit does not facilitate social recognition memory retrieval. (A) The placement of the optical fiber in the ventral CA1 and site of viral injections in the ventral EC. (B) Activation of neurotransmitter release at the EC-CA1 synapse. (C) Immunostaining image of a ventral brain section showing the expression of ChR2-mCherry in the CA1. Scale bar: 1000 µm. (D) A modified social interaction test with stage 2 being reduced to 2 minutes. The 473 nm light was presented during stage 3 only when the test mouse was interacting with S1. (E) Activation of the EC-CA1 circuit by ChR2 during stage 3 had no effect on preference for S1 over the empty cage (ChR2: n = 11, p = 0.0010; ChR2-Stage 3: n = 11, p = 0.0010; two-tailed Wilcoxon matched-pairs signed rank test). (F) Activation of the EC-CA1 circuit by ChR2 during stage 3 had no effect on preference for S2 over S1 (ChR2: p = 0.8836; ChR2-Stage 3: p = 0.2732; two-tailed paired t test). (G) Discrimination scores showing no discrimination in ChR2-Stage 3 similar to the control group (p = 0.3886; two-tailed paired t test).
4.5.3.5 Inhibition of the EC-vCA1 circuit enhances trace fear memory

Previous studies have shown that excitatory neurons in the EC layer III activate interneurons in the distal dendrites in the CA1, which suppresses the EC input in trace fear memory (Kitamura et al., 2014). Similarly, the WT mice injected with ArchT and ChR2 were subjected to a trace fear conditioning paradigm while inactivating the ECIII-vCA1 projections during training with continuous 532nm green-light (Figure 4.5.11A-4.5.11C). Though no differences were observed in freezing during training, the test revealed that the ArchT group displayed enhanced freezing during the post-tone periods compared to the controls. This is consistent with the observation that the ECIII-vCA1 is a feed-forward inhibitory circuit involved primarily in temporal association memory (Figure 4.5.11C).

4.5.3.6 Electrophysiological confirmation of the effect of ArchT and ChR2 in EC layer II neurons

To confirm the efficacy of ArchT inhibition in the EC-DG circuit, action potentials were recorded from layer II EC neurons expressing ArchT-EYFP in ventral brain slices taken from mice following behavioural testing. The firing of these neurons was completely blocked by the application of 532nm green-light (Figure 4.5.12A). In contrast, the firing properties of adjacent primary somatosensory cortical neurons that did not express ArchT were not affected by the green-light illumination (Figure 4.5.12B). To further verify that synaptic transmission at the EC-DG terminals was inhibited, EPSCs were recorded from DG granule cells evoked by the electrical stimulation of the MPP and showed that the size of EPSCs was reversibly inhibited by the green-light illumination (Figure 4.5.12C). To examine the efficacy of ChR2 activation, whole-cell recordings in brain slices were prepared from the WT mice following the behaviour tests. 5ms pulses of 473nm blue-light reliably triggered action potentials in the EC layer II neurons expressing ChR2, but not in the adjacent primary somatosensory cells that did not express ChR2 (Figure 4.5.13A and 4.5.13B). To verify that blue-light stimulation induced synaptic release at the EC-DG terminals, EPSCs were evoked by the blue-light pulses from the DG granule cells and the responses were completely blocked by NBQX (10µM) and picrotoxin (100µM) (Figure 4.5.13C), which further confirmed that ChR2 was not expressed in the DG granule cells.
Figure 4.5.11 Inhibition of the EC-CA1 Circuit by ArchT enhances trace fear memory. (A) ArchT and control mice with 532nm green-light during training showed no differences in freezing before, during, and after shock training compared to control mice (Control: n = 8; ArchT: n = 8; p = 0.7335; two-way RM ANOVA). (B) ArchT and control mice showed similar fear memory at 24 hours post-training (p = 0.3441; two-way RM ANOVA). (C) ArchT and control mice showed similar freezing during the baseline and tone presentation but enhanced fear memory post-tone (Baseline p = 0.1879; Tone p = 0.9377; Mann Whitney test; Post-tone p = 0.0442; two-tailed unpaired t test).
Figure 4.5.12 Electrophysiological verification of optogenetic inactivation of the EC-DG circuit by ArchT. (A, B) Whole-cell current clamp recordings of action potentials from the EC layer II neurons expressing ArchT (A) and adjacent S1 somatosensory cortical control neurons without ArchT expression (B) in acute brain slices, showing that 532nm green-light illumination blocked action potentials in the EC neurons, but not in the control somatosensory cortical neurons. 7 neurons were recorded for each of the two brain regions with similar results. Scale bars: 50mV/5s. (C) Whole-cell voltage clamp recordings of EPSCs from the DG granule neurons in acute hippocampal slices showing that 532nm green-light illumination diminished synaptic responses evoked by stimulating the MPP pathway. 8 neurons were recorded with similar results. Scale bar: 50pA/20ms.
Figure 4.5.13 Electrophysiological verification of optogenetic activation of the EC-DG circuit by ChR2. (A, B) Whole-cell current clamp recordings of action potentials from the EC layer II neurons expressing ChR2 (A) and adjacent S1 somatosensory cortical control neurons without ChR2 expression (B) in acute brain slices, showing that 473nm blue-light illumination (5ms pulse) triggered an action potential in the EC, but not S1 neurons. 7 and 4 neurons were recorded for the EC and S1, respectively, with similar results. Scale bars: 40mV/50ms. (C) Whole-cell current (top two traces) and voltage (bottom two traces) clamp recordings from the DG granule cells in acute hippocampal slices, showing that 473nm blue-light illumination (5ms pulse for current clamp, 1ms pulse for voltage clamp) evoked EPSPs and EPSCs that were completely blocked by addition of NBQX (10 µM) and picrotoxin (100µM). 7 neurons were recorded with similar results. Scale bars: 40mV/50ms (top), 100pA/20ms (bottom).
4.5.4 Discussion

In this section, I demonstrated that the EC-DG PP was indispensable and inducing for social memory retrieval. In addition, PAKs within the EC-DG circuit, specifically at the presynaptic terminals from EC layer II, is an essential molecular substrate in neurotransmitter release which subserves this social function. Since PAKs are common targets of several neurodevelopmental and neuropsychiatric disorders, these results provide a potential circuit and synaptic mechanism underlying social memory impairments associated with these disorders.

The inhibition of the EC-DG terminals with ArchT prevented social discrimination, but not sociability, in the three-chamber social interaction test. Since the inhibitory effect during discrimination was more potent compared to that during sociability ($p = 0.0198$), it is the retrieval rather than the encoding of the social memory that is particularly tuned to the manipulations of the EC-DG circuit. This possibility was further supported by the five-trial habituation test where I showed that the retrieval of the learned habituation is impaired by the acute inhibition of the EC-DG terminals, while the formation of memory was unaffected. Therefore, the process of memory formation is less susceptible to EC-DG perturbations compared to memory recall. It is important to note that these experiments examined the short-term retrieval of social discrimination memory which has previously been found to persist from 30 minutes to 24 hours following the familiarization stage (Okuyama et al., 2016). In these other studies, long-term social memory requires CREB-mediated transcription and protein synthesis to occur within 1-2 hours following the initial interaction (Kogan et al., 1997; Kogan et al., 2000; Richter et al., 2005). Given that my experiments only tested social recognition ability within 1-2 minutes following pairing, the observed deficits in retrieval are associated with short-term social memory that do not require de novo protein synthesis. Thus, it suggests that the EC-DG PP plays a critical role in the processes that regulate the immediate retrieval of short-term social memory traces.

The requirement for the EC-DG circuit in social memory retrieval is also supported by the ChR2 experiments, which showed that optical activation of the EC-DG terminals during social discrimination, but not during sociability, facilitated memory recognition. Although I demonstrated that
in WT mice, a 2-minute pairing was insufficient for the retrieval of short-term social recognition memory, another study has demonstrated that a 2-minute interaction period was sufficient to elicit long-term social memory retrieval for up to 7 days; given that the animals were group-housed (Kogan et al., 2000). In this same study, socially isolated mice (chronic isolation for 3 weeks) demonstrated normal social discrimination at 30 minutes but a complete lack of social recognition at 24 hours. The inconsistency with my findings may be explained by differences in the protocols for housing and the social recognition memory paradigms (home cage interaction vs the three-chamber test). Nevertheless, my results provide novel insight into the mechanistic features of short-term social memory recall. Specifically, working memory in the context of social recognition memory can be rapidly encoded and consolidated in less than 2 minutes.

Finally, I showed that acute ChR2 activation along the PP restored social memory in dTg mice, which suggests that PAK signaling at the EC-DG terminals is involved in the retrieval, but not encoding, of social recognition memory. It is important to note that previous studies have identified the role of the ventral CA1 neurons and their nucleus accumbens shell projections to be important for social memory (Okuyama et al., 2016). However, my results indicated that the inhibition of the ECII-CA1 circuit had no effect on social memory retrieval and furthermore, the activation of the circuit was insufficient to facilitate memory retrieval. Thus, the ECII-CA1 circuitry may be an important storage site of the memory trace. Given that the LEC and MEC layer II neurons send (via the DG) and receive reciprocal inputs from distal and proximal CA1 through the subiculum and ECV (Canto et al., 2008; Witter, 2007), it is possible that the ECII neurons aid in the retrieval of the social memory held within the ventral CA1. During retrieval, the activation of the EC-DG circuit may re-access the social memory stored in the CA1, which projects to the subiculum and then ECV and/or ECII. This is consistent with previous studies showing that the CA1-Subiculum-EC5 circuitry is required for the retrieval of episodic fear memory (Roy et al., 2017). During the replay of the social encounter, a pattern of neural activity associated with the memory is relayed via the PP back into the hippocampus for reconsolidation. Therefore, the EC reelin-positive pyramidal neurons projecting to the molecular layer of the DG may be a novel cortical connection that completes the reciprocal hippocampal and entorhinal circuitry which is essential for the encoding, storage and retrieval of social memories. Together, these results demonstrated that either acute optogenetic or molecular manipulations of the EC-DG terminals was
sufficient to rescue the social memory deficits in the PAK transgenic mouse model, providing a new therapeutic strategy and potential target to treat social disorders.
5 Overall Discussion

Data Attribution- Figure 5.1 and ideas discussed in this chapter were adapted from the following original publication:


In this thesis project, I first characterize a novel transgenic mouse model and show that the mPAK3-GFP transgene is predominantly expressed in the reelin-positive neurons within the EC and their projections to the DG. Second, I perform various behavioral tests on these transgenic mice and show that they are impaired in social recognition memory, but normal in spatial, fear, and object recognition memory. I further demonstrate that this deficit in social memory in the transgenic mice can be restored by selectively turning off the transgene expression in the EC layer II neurons. Third, we perform electrophysiological recordings in the EC, DG, and BLA and show that presynaptic neurotransmitter release is impaired at the EC-DG terminals. Fourth, I conduct analyses of downstream effectors of PAKs and show that cofilin and F-actin are significantly altered in the EC-DG circuit. Finally, I employ an optogenetic approach and show that inhibition of the EC-DG terminals blocks social memory retrieval, but the activation of the terminals facilitates social memory in WT mice and rescues social memory deficit in the mPAK3-GFP transgenic mice. Taken together, these results suggest that the activation of the EC-DG circuit is indispensable and inducing for social memory retrieval and PAK signaling may play a critical role in the circuit activation in these processes. These results are significant for our understanding of social memory in both health and disease.

Imaging studies in social cognition implicate the temporal lobe as part of the “social brain” responsible for processing higher level social behaviours (Olson et al., 2013). Specifically, damage to the anterior temporal lobe in humans, which encompasses the EC, affects affiliative group behaviours (Frith and Frith, 2010; Olson et al., 2013; Olson et al., 2007). Thus, it is hypothesized that social memory processing occurs via a selective circuit within the medial temporal lobe specialized for processing
social information and independent from general cognition. Although previous studies in humans (Adolphs, 2009; Frith and Frith, 2010; Olson et al., 2013; Olson et al., 2007) and animal models (Bannerman et al., 2001; Bannerman et al., 2002; Petrulis et al., 2005; Petrulis and Eichenbaum, 2003) have implicated the EC in social behaviour, definitive assessment of the circuitry has not been possible due to the limited control of precise brain regions and the corresponding cellular populations involved in these studies. Recent rodent studies have shown that both the CA1 and CA2 regions within the hippocampal circuit are involved in social memory (Hitti and Siegelbaum, 2014; Okuyama et al., 2016). Specifically, these authors proposed a circuit of how social information is processed and stored; which starts in the olfactory epithelium and vomeronasal organ, to the dorsal CA2, then to the ventral CA1, and the nucleus accumbens shell circuit (Hitti and Siegelbaum, 2014; Okuyama et al., 2016; Spehr et al., 2006). However, how social information is retrieved from these storage sites is unknown. Through the recent advances in methods that allow for the combined use of optogenetics and restricted transgene expression, I selectively target the EC-DG circuit more definitively to understand its role in the various stages in social memory. I show that the inhibition of the EC-DG terminals prevents social discrimination, but not sociability, in the three-chamber social interaction test (Figure 4.5.2). Specifically, the inhibition of the EC-DG PP during social discrimination but not encoding impair recognition memory, which suggests that memory retrieval is particularly liable to perturbations in this circuit. Furthermore, the activation of the EC-DG PP facilitates social memory discrimination (Figure 4.5.4). Together, these findings highlight the importance of the EC-DG circuit in social memory retrieval. These optogenetic results are consistent with those obtained from the inducible transgenic mice showing that the reversible disruption of PAK signaling specifically targeting the EC-DG circuit impairs social recognition memory, while sociability or other forms of memory remain intact (Figure 4.2.1-4.2.5). Importantly, acute optogenetic activation of the EC-DG terminals during discrimination, but not during sociability, promotes social memory retrieval in the transgenic mouse model (Figure 4.5.7). Alternatively, the observed social memory deficits may be attributed to differences in the complexity of the three-chamber social tests as compared to the simplicity of novel object recognition task, which was used as control. Since social memory is a complex task that involves both novelty and spatial recognition, further tests should be conducted to examine both contextual and associative aspects simultaneously. These findings would dramatically strengthen the conclusion of the role of PAK in the EC-DG circuit in only social recognition memory.
Although our molecular genetic and optogenetic targeting of the EC-DG is fairly specific, it is possible that the social memory deficits observed in mPAK3-GFP transgenic mice are caused by perturbations in the ECIII-ventral CA1 or ECII-dorsal CA2 pathways. However, this is unlikely because I observe no transgene expression in either the dorsal and ventral CA1 or CA2 areas in the dTg mice. In addition, the optogenetics experiments use an acute approach that enable the precise cell-specific targeting of PP terminals only in the dorsal DG (light reaching an area of \(\sim1\text{mm}^2\)), excluding the delivery of light to the dorsal CA2 and ventral CA1. To further rule out the possibility of alternate EC projections to the CA1 or the BLA, approaches including extracellular field and intracellular whole-cell recordings, structural analyses using EM, and local DOX infusion experiments demonstrate that there are no changes in synaptic transmission and plasticity, structural properties, and behavioural tests of social discrimination. Interestingly, given that the PP is the primary input to the hippocampus, the deficits observed in basal synaptic response at this synapse may affect the signaling in latter mossy fiber and Schaffer collateral connections to the CA3 and CA1 pathway. However, my findings reveal no synaptic, morphological, or behavioural impairments associated with these regions. Therefore, the mPAK3-GFP transgene specifically perturbed the EC-DG synapse alone.

The present study provides novel insight into the cellular and subcellular mechanisms by which PAK signaling affects synaptic function required for social memory retrieval. Although previous studies have shown that PAKs regulate dendritic spine morphology, basal synaptic strength, and synaptic plasticity through a cofilin-dependent actin mechanism (Allen et al., 1998; Asrar et al., 2009; Huang et al., 2011), the precise site of their action is unknown due to a lack of cell type- or compartment-specific approaches. In addition, the relative contribution of the synaptic effects of PAKs to behaviour is unknown. The development of mPAK3-GFP transgenic mice with precise perturbations of PAK signaling in the presynaptic terminals of EC-DG synapses allows the investigation of the specific role of PAKs in presynaptic function (Figure 4.3.1 and 4.3.2) and its contribution to social memory recall without the confounding effects of postsynaptic dendritic spines. The social recognition memory impairments in the transgenic mice, which are tested in the order of a few minutes, are consistent with the synaptic alterations in short-term plasticity that last for milliseconds. However, the precise
physiological mechanisms that underlie short-term plasticity, including PPD, in learning and memory are still unclear. Potentially, the EC-DG PP plays a role in the rapid information processing of social cues during the initial encounter with the stranger mouse. The alterations in PAK signaling may impair the normal integration of the presynaptic activity for social information processing, which ultimately results in the memory deficits. Thus, the findings that the dTg mice are severely impaired in synaptic strength due to a reduced frequency of neurotransmitter release and an abnormal accumulation of synaptic vesicles at the EC-DG terminals reveal a definitive contribution of PAKs to the presynaptic regulation. Furthermore, this suggests the role of short-term plasticity in social memory.

The results demonstrate that mPAK3-GFP transgene disrupts coflin-mediated actin reorganization within the EC-DG terminals, which may lead to impairments in the replenishment and/or release of synaptic vesicles. Other studies have demonstrated that the activation of synaptic receptors during fear and drug-related learning have led to alterations in actin cytoskeleton dynamics and neuronal structure within the amygdala and ultimately, are also essential for memory formation (Fischer et al., 2000). In these earlier studies, the changes in actin polymerization were important for glutamate receptor trafficking and the reorganization of neuronal morphology. The actin cytoskeleton has also been shown to play a critical role in the dynamic translocation of synaptic vesicles from the reserve pool to the readily releasable pool as well as vesicular fusion at the active zone (Cingolani and Goda, 2008). Since PAK perturbations affect LIMK and coflin-mediated actin reorganization (Zhao and Manser, 2012), disruption of PAK signaling may lead to an altered actin network within the presynaptic terminal, resulting in impaired replenishment and/or release of synaptic vesicles. The fact that acute optogenetic activation of the EC-DG terminals alone is sufficient to facilitate social memory recall in WT mice (Figure 4.5.6) and to reverse the social memory deficit in the transgenic mice (Figure 4.5.7), strongly suggests that PAK-mediated presynaptic release at the EC-DG synapse is the synaptic basis that underlies social recognition memory recall. Thus, the results are the first to suggest that perturbations in PAK signaling and the actin cytoskeleton affect basal synaptic transmission and short-term plasticity in the EC-DG terminals, during social memory retrieval.
This study also represents a major milestone towards understanding the role of disease-linked genes, such as PAK, in social cognition. Since PAKs are central regulators of the cytoskeletal structure via coflin-dependent actin regulation, perturbations in the Rho signaling pathway may collapse actin dynamics at the axon terminals resulting in decreased probability of neurotransmitter release and a subsequent accumulation of synaptic vesicles at the active zone, which result in reductions in basal synaptic transmission and plasticity. Hence, normal synaptic function and axonal structure of PP projections to the DG may be particularly sensitive to the retrieval of social memories. The study provides insight into the understanding and treatment of social impairments associated with brain diseases. It is known that mutations in the PAK genes are directly linked to various neurodevelopmental and neuropsychiatric disorders, including ID, ASD, and schizophrenia (Allen et al., 1998; Bokoch, 2003; Gilman et al., 2011; Kim et al., 2017; Zhao and Manser, 2012). Similar genetic alterations are also found in several proteins that interact with PAKs, including LIMK1 and Rho family regulators (Meng et al., 2002; Todorovski et al., 2015; Zhang et al., 2005). Moreover, recent studies have shown that manipulations of PAK proteins can ameliorate the cognitive and social deficits in several animal models, including fragile X syndrome, neurofibromatosis, and schizophrenia (Chen et al., 2011; Dolan et al., 2013; Hayashi et al., 2007; Molosh et al., 2014). Although these studies have established PAKs as a major risk factor for these brain disorders, the pathogenic processes underlying the PAK effects is unknown. The present study, by revealing the precise neural circuit and synaptic mechanisms through which PAKs affect social memory retrieval, has important clinical implications. Based on our results, it is reasonable to postulate that selective activation of the EC-DG circuit either optically or pharmacologically through targeting PAK signaling may be beneficial to improve social memory in patients with brain disorders (Figure 5.1). Thus, the EC-DG circuit may provide a new target for novel acute and pharmacological therapeutic approaches to the treatment of social disorders.
Figure 5.1 Restoration of social recognition memory in dTg mice via pharmacological or acute manipulations. This project has identified the role of the EC-DG circuit in the regulation of social memory. Specifically, the inducible disruption of PAK signaling in the EC-DG circuit leads to impairments in social recognition memory. Furthermore, synaptic function is altered through reduced neurotransmitter release due to perturbed cofilin-dependent actin reorganization. However, the social memory in dTg mice can be restored via a pharmacological or acute manipulation. First, the administration of DOX can turn off the expression of the transgene and restore the actin cytoskeleton. Second, the optical activation of the EC-DG terminals enhances neurotransmitter release. These results provide compelling evidence that activation of the EC-DG pathway underlies social recognition memory and that PAK signaling may play a critical role in modulating this process.
6 Future Directions

Social cognition is an extraordinarily complex memory process that shapes the formation and maintenance of social bonds essential for our lives. An increasing number of patients with ASD, ID, and other neuropsychiatric disorders share underlying impairments in social cognition. Currently there is no cure or any effective treatments due to a lack of understanding of the fundamental mechanisms underlying social memory and how it is affected in brain disorders. This thesis project is the first to establish a novel molecular pathway and cellular mechanisms through which PAK regulates social memory with a specific focus on the structural and functional properties of synapses. Furthermore, this work identifies new therapeutic approaches through the pharmacological or acute manipulation of the EC-DG PP neural circuitry in treating disorders associated with social dysfunction. Thus, the manipulation of PAKs within the EC-DG circuit may provide a new strategy to improve social cognition in general. The following sections discuss improvements and alternatives to existing experiments, as well as potential applications of the project.

6.1 Improvements for DOX Administration in the Tetracycline Inducible System

The creation of a tetracycline transgenic mouse line expressing a dn PAK mutation enabled the precise spatiotemporal genetic targeting of excitatory layer II EC neurons. Specifically, the generation of this novel mouse model allowed for the selective labelling of mature excitatory outputs; at the presynaptic terminals of EC-DG synapses but not in the granule cells of the DG or the pyramidal cells of the CA1. The expression of the transgene was regulated in a DOX-dependent manner, which was capable of completely silencing the mutant-GFP gene expression through the administration of DOX in the drinking water. Despite the advantages of using a well-characterized tet-off system, there are various disadvantages for administering DOX through the drinking water (Redelsperger et al., 2016). Firstly, these inducible systems are often perceived to be leaky, where residual levels of gene expression can be observed even in the presence of DOX. Secondly, studies have reported toxicity of the drug, DOX, following chronic administration. Lastly, the system relies on the transcription and subsequent translation of a target gene, which is therefore relatively slow. Especially, the administration of DOX through the drinking water took approximately 4 weeks to turn off the mPAK3-GFP transgene
completely. Recent studies have shown that this route of administration can result in animal dehydration and weight loss over time (Cawthorne et al., 2007). These authors found that DOX administered through feed (200 mg/kg) avoids dehydration, weight loss, and toxicity, while inducing gene expression in a shorter period of time. Therefore, adapting DOX administration through feed in the CaMKIIα-tTA/tetO-mPAK3-GFP transgenic mouse model can improve the existing tetracycline inducible tet-off system to be more efficient, specific, and safe.

6.2 Investigation of Long-term Social Recognition Memory

Other studies have used behavioural paradigms to explore the long-lasting retrieval of social discrimination memory which have been shown to persist from 30 minutes to 24 hours following the familiarization stage in Crawley’s three chamber social interaction test (Okuyama et al., 2016). In this thesis, social memory was measured using two well-validated social interaction tests, including the three-chamber social interaction test and the five-trial social memory test. Both tests rely on the subject’s free choice and innate social motivations. The tests measure social affiliation and preference for social novelty or social memory. Compared to other behavioural tasks, such as the resident-intruder test (two-trial social memory test) or the tube dominance test, the paradigms prevent aggressive behaviour and eliminate fighting between the subject and the unfamiliar conspecific. In the paradigms conducted in this thesis project, the subject mouse is given the choice to approach and initiate the interaction during the test, which allows for the mouse’s natural inclinations to be quantified in a relatively stress-free manner. The three-chamber apparatus was used for both tests and is larger in size compared to the subject’s home cage. Thus, the subject mouse was able to spontaneously explore and freely engage in the task. Importantly, both social tests that we used followed a strict protocol with a period designed to habituate the subject to the apparatus (24 hours before and 10 minutes prior to the testing). This allowed the mice to familiarize themselves to the novel testing environment. The mice were also handled for a period of a week prior to testing, to minimize the stress-levels that may be associated with the testing. Although, our tests examined short-term working memory, the behavioural paradigms can be easily modified to examine long-term memory retrieval. Therefore, it would be interesting to also examine the effect of PAKs in both short- and long-term social recognition memory. For example, the testing for social recognition memory (stage 3) of the subject mouse can be tested 24
hours following the pairing with the novel mouse (stage 2). Given that the dTg mice demonstrated short-term impairments in social memory, they should also demonstrate long-term social memory impairments. Similarly, using optogenetic techniques, the EC-DG PP can be acutely activated with ChR2 to facilitate the retrieval of social recognition memory. Therefore, the retrieval mechanisms underlying short-term social memory can be compared with those from long-term social memory.

6.3 Manipulation of Other Molecular Targets in PAK Signaling to Rescue the Structural, Synaptic, and Behavioural Deficits

Previous studies have suggested that PAK and Rac1 are bidirectionally regulated (Byrne et al., 2016). Impaired PAK signaling may result in reduced Rac1 activity, which in turn further downregulates PAK and its effectors through feedback inhibition, leading to structural and functional changes at the synapse. From my results, the dTg mice were reduced in the relative ratio of phosphorylated cofilin compared to total cofilin levels, confirming the disruption of the PAK signaling pathway. I further observed a marked increase in phalloidin intensity along the PP which is representative of greater F-actin content at the EC-DG terminals of dTg mice. This is consistent with the interpretation that enhanced cofilin activity severs existing actin filaments resulting in the generation of free barbed ends that polymerizes actin and ultimately, increases F-actin content (Ghosh et al., 2004; Shekhar and Carlier, 2017). Therefore, other downstream molecular targets through which PAK regulates cofilin and actin can be identified and subsequently, pharmacologically manipulated to rescue the behavioural, electrophysiological, and morphological impairments observed in the dTg mice.

For example, previous studies have demonstrated that LIMK KO mice are severely affected in cofilin phosphorylation exhibiting morphological and synaptic abnormalities (Meng et al., 2003b; Todorovski et al., 2015). Thus, to test if LIMK activity is altered in dTg mice, antibodies specific to phosphorylated LIMK and total LIMK can be used in Western blot analysis. In addition, immunohistochemistry and confocal imaging techniques can determine the colocalization of these molecular targets with the PAK transgene in dTg mice. I expect that that the mPAK3-GFP transgene will impair PAK activity, which will reduce the activation of LIMK, and subsequently affect cofilin activity. This will also be
demonstrated through a reduction in pLIMK colocalization with mPAK-GFP cells in the EC layer II and the PP. As control, the CA1 region of dTg mice have no alterations in PAK activity and will reveal that LIMK activity is unchanged.

To determine if LIMK or other targets are functionally downstream effectors of PAK, the behavioural, structural, and functional deficits will be targeted in rescue experiments. Various phosphopeptides or acute inhibitors of the PAK family can block coflin activity or other molecular targets. The short cell-permeable peptide, pS3, blocks coflin phosphatases and LIMKs, and therefore, reduces coflin activity. Similar to our previous studies, the peptide can be tested in vitro in hippocampal dTg slices, to observe their effect through Western blot analysis (Huang et al., 2011). I expect slices treated with pS3 peptide (40ug/ml, 2h) to have increased pLIMK and pCofilin content that is comparable to untreated WT levels. Alternatively, in vivo experiments could involve the stereotaxic implantation of bilateral cannulas into layer II cells of the EC for local pharmacological infusion of fluorescent pS3 (5nM, 1µl/ side over 2 minutes with an additional 2 minutes for diffusion) or control peptides (Tada and Sheng, 2006; Wang et al., 2013). To ensure the effectiveness of the pS3 peptide, brain histology, synaptic plasticity, coflin activation, and spine morphology can be analyzed. If PAK signaling through LIMK and coflin is responsible for regulating social memory; inhibition with pS3 (but not the control peptide) will restore neurotransmitter release, basal synaptic transmission and plasticity, structural abnormalities, and social memory deficits. The experimental protocol and methods are similar to the local infusions of DOX (5µM, 1µl/ side over 2 minutes for 7 days) in the EC that were discussed above. Together these results will dissect the precise role of PAK signaling mediated by LIMK and coflin in social memory via in vivo pharmacological manipulation. An alternative method would be to use short-hairpin RNAs (shRNA) against various molecules in the PAK signaling pathway to reverse abnormalities and identify possible molecular targets to elucidate signaling pathways responsible for structural changes in dendritic spines. By inhibiting Rac signaling or any molecule in the Rho signaling pathway by expressing shRNA against Rac or any molecular target, structural changes can be used as a proxy to observe the effects. This method can target and identify other possible molecules in the Rho signaling pathway or other complementary pathways that may be important for regulating PAK signaling in synaptic and structural plasticity. Similarly, as discussed above, the data focuses on the Rho signaling pathway in social memory. It is possible that other signaling pathways, through Akt, Raf,
or MEK, may be important in the behavioural and synaptic regulation of PAKs. Future experiments can explore if these targets are altered in the dTg mice and whether pharmacological or genetic manipulations are capable of restoring the social memory deficits. Therefore, these alternate mechanisms and corresponding molecular targets may facilitate in the understanding of the precise role of PAK signaling in social memory.

6.4 Alternative Neural Circuits and Signaling Pathways

Although this project focuses on the cellular mechanisms governing the function of the EC, it is possible that secondary projections other than those to the DG may contribute to the behavioural and synaptic deficits observed. For our transgenic model, the genetic targeting strategy is dependent on the CaMKIIα-tTA forebrain promoter which limits the expression of the transgene to excitatory pyramidal neurons in the forebrain. Though the mPAK3-GFP transgene is mostly observed within layer II cells of the EC, sparse labelling in the deeper layers can be observed. The EC is a multimodal limbic association area within the medial temporal lobe that receives numerous projections from cortical association areas and secondary association areas, including the olfactory, visual, and perirhinal cortices. Previous tracing studies have also identified that the deeper layers of the EC have output connections with the BLA, thalamus, postrhinal cortex, retrosplenial cortex, and parietal cortex (Canto et al., 2008). Based on the presence of the transgene expression in the BLA, I conducted control experiments to test the functional and behavioural significance of this area on social memory. However, I was unable to observe any electrophysiological impairments nor restore the social memory deficits when manipulating this area. The other EC downstream regions, including the thalamus and other associative cortices, are unlikely to be involved in the regulation of social memory due to the lack of expression of the mPAK3-GFP transgene seen throughout the brain. However, this does not rule out the possibility that the EC-DG circuit may function in concert with these alternative target/pathways during more complex social memory processing including reconsolidation or extinction. Thus, optogenetics can test the activation or inhibition of specific pathways that may be involved in different facets of social memory. Furthermore, to test the molecular involvement of PAKs in these processes, pharmacological (IPA3 or pS3 etc.) or genetic approaches (through alternate promoters to drive mPAK3-GFP transgene expression in different cell types or regions) can be used.
The PP is composed of the direct and indirect projections from EC layer II and III cells to the DG or the CA1, respectively. Although our optogenetic experiments stereotaxically target the viral opsin into layer II cells, the clear distinction between the two layers is hard to achieve without the use of cell-specific markers. Previous studies have injected retrograde tracers, such as cholera toxin subunit B (CTB), into the DG to label EC layer II cells that are part of this projection. These studies have classified these particular EC layer II Ocean cells that project to the DG to be excitatory and reelin positive (Kitamura et al., 2014). Whereas, EC layer II Island cells that project mainly to the CA1 between the stratum radiatum and stratum lacunosum-moleculare, are pyramidal, CTB-negative, and express Wolfram syndrome 1 (Wfs1) and calbindinD-28K (Kawano et al., 2009). Thus, future studies can use more stringent optogenetic manipulations of Ocean or Island cells on social memory. The specific labelling of the EC layer II DG-projecting cells can be targeted using an AAV that is retrogradely transported from the DG axons back into the somas of Ocean cells. Whereas, ECII Island cells can be targeted using a Wfs1 promoter. The specific Cre-dependent AAV opsin can then be injected into the EC of Wfs1-Cre mice. These specific targeting techniques have been validated by various studies (Kitamura et al., 2014; Kitamura et al., 2015b) and can aid in the distinction between the direct and indirect PP projections. Thus, future studies can focus on improving the viral and transgene targeting techniques to ensure that only specific populations of neurons and their projections are being investigated.

6.5 Applications of EC-DG Manipulations to Other Mouse Models with Social Deficits

Future experiments can also aim to explore whether activating the EC-DG pathway can rescue social deficits in other disease models. To date, numerous studies have explored various mouse models that have identified social impairments. For example, the Fragile X mental retardation gene 1 (FMR1) KO model has been created to model the most commonly identified cause of inherited ID and ASD. The FMR1 protein (an mRNA-binding protein) interacts with many proteins that can potentially regulate the translation of a large number of mRNAs. Interestingly, numerous targets are involved in PAK signaling and actin regulation. The FMR1 KO mice display abnormal hyperactivity and fear response,
which are mitigated by altering the level of PAK1 (Dolan et al., 2013). Additionally, these mice display a host of deficits related to reduced social interactions (Chadman et al., 2008; Ferguson et al., 2000; Jamain et al., 2008; Spencer et al., 2005). These include reduced reciprocal social interactions, low sociability, lack of preference for social novelty, and reduced ultrasonic vocalizations. Therefore, it would be interesting to also examine these phenotypes using a variety of other assays including social preference tests through a Y-maze or partitioned cage. However, with respect to the social domain, the circuits and molecular mechanisms involved in the FMR1 KO mice remain unknown. Other models with social deficits that can be similarly examined include; Nf1, Shank3, neurexins, and neuroligins mouse models. Therefore, possible future experiments will aim to test whether the restoration of PAK signaling (pharmacologically through PAK activators/inhibitors and peptides) or through acute manipulations (optogenetically through ChR2 or ArchT) of the EC-DG can improve social and cognitive performance in these various mouse models. Similar to the approach with the dTg mice, the first step will be to examine if there are any cellular and molecular alterations in PAK signaling in various brain regions. Next, the EC-DG circuit in these mouse models can be functionally analyzed via electrophysiological recordings. If indeed, the EC-DG pathway is altered in these mouse models, then the EC-DG circuit may be optogenetically, pharmacologically, or even genetically targeted in the attempt to restore proper synaptic and behavioural deficits.

6.6 Applications of Engram Labeling Systems

The optogenetic rescue experiments in the dTg mice focused on the activation of all of the EC-DG terminals. In this sense, the retrieval of the social memory was facilitated by the activation of the entire population of neurons, including those cells that do not store the social memory engram. Thus, future experiments can employ new genetic systems that label certain engram cells within the brain. The Cfos-hTA mice (Cfos-tTa/Cfos-shEGFP) have two co-injected transgenes that segregate together, resulting in the expression of the tTA and the two-hour half-life GFP directed to activate the Cfos promoter (From Jackson Laboratory). These mice confer several advantages; including the quantification of Cfos expression via the GFP fluorescence following neuronal activation and the inducible expression of genes of interest based on a Tet-off system. These Cfos/tTa mice can be crossed with mice carrying a gene of interest under the regulatory control of a tetracycline-responsive
promoter element (tetO). The expression of the transgene in the double mutant offspring can be regulated through the administration of the tetracycline analog, DOX in the drinking water.

Future experiments can focus on generating a new transgenic mouse line by crossing the existing tetO mPAK3-GFP transgenic (Tg-1) mice with the new Cfos/tTa mice to generate double mutant offspring (Cfos-tTa/tetO-mPAK3-GFP) mice. These new dTg mice will limit the expression of the mPAK3-GFP transgene in specific social memory engram cells. These new transgenic mice will need to be on DOX since birth to prevent active cells that express Cfos from being labelled. Once off DOX (for a period of 24 hours), the mice can be subjected to a behavioural encounter, such as home-cage interactions with a stranger mouse. The pairing should induce the activity of Cfos-tTa in particular social engram cells within different regions of the brain. Immediately following the home-cage pairing, the transgenic mice will be placed back on DOX to prevent any further labeling. Ultimately, the activity of Cfos-tTa will drive the transcription of the tetO-mPAK3-GFP transgene within the engram cells in 24 hours. To confirm this, brain sections from the transgenic mice can be stained with anti-GFP antibodies to identify engram cells expressing the mPAK3-GFP transgene. To corroborate the findings in this thesis project, the expression of the transgene in the EC II engram cells and their corresponding projections to the DG can be selectively labelled, identified, and tested to see if social recognition memory is impaired. Furthermore, the targeted Cfos-tTa engram cells can be used as a proxy to limit the infection of opsins, including ArchT or ChR2, to inhibit or activate only relevant neuronal ensembles. These future experiments may allow for the detailed manipulation of particular social memory engram cells in various parts of the brain, including regions outside of the EC and hippocampus that may play a role in different aspects of social memory processing.
7 References


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