PAKs 1 & 3 control postnatal brain development and cognitive behaviour through regulation of axonal and dendritic arborizations

By Wayne Huang

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

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ABSTRACT

The mechanisms underlying the postnatal developments of brain volume and
intellectual function remain an enigma. In this study, I provide evidence that axonal and
dendritic arborizations are key determinants of postnatal brain enlargement, with
functional consequences at the synaptic and behavioral levels. P-21 activated kinases
(PAKs) are key kinase effectors of small family Rho GTPases, the so-called master
regulators of neuronal cytoskeleton. Studies in vitro and in non-mammalian systems
implicated PAKs to play a variety of neurophysiological roles, from neurogenesis to
neuronal morphogenesis. However, in vivo functions of PAK in the development of
mammalian nervous system remain uncovered owing to the presence of multiple
functionally redundant isoforms. I took a genetic approach to address this problem by
generating a double knockout (DK) mice which lack expression of the brain-dominant
Group I PAK isoforms, PAK1 and PAK3. These mice were born healthy with normal
brain size but displayed impaired postnatal brain growth, resulting in a drastic reduction
of brain volume at maturity (by 36% compare to wild type littermates). Stereology
revealed that the secondary microcephaly in the DK mice is accompanied by little change
in total cell count due to a proportional increase in neuronal and glial density. Instead, mature PAK1&3 DK neurons displayed greatly reduced dendritic and axonal arborization, which was shown to underlie the brain volume reduction. Moreover, I provided evidence that actin disorganization through ADF/cofilin downstream of PAK may be responsible for this process. A direct consequence of reduced dendritic complexity in the DK mice was a 50% reduction in total synapse number. This reduction in number was accompanied by abnormal spine morphology, enlargement of individual synapses and altered synaptic architecture. These synaptic morphological deficits translated directly into functional deficits, as the mutant mice displayed enhanced synaptic transmission and impaired bidirectional synaptic plasticity. Of great clinical importance, these mice exhibited phenotypes with striking resemblance to patients with PAK3-associated X-linked mental retardation. These phenotypes include: microcephaly, anxiety, hyperactivity and severe cognitive impairments. Together, this study demonstrates that PAK1&3 signaling mediates dendritic and axonal branching likely through actin, and contributes to the proper development of brain size, synaptic network and cognition. The study further suggests that molecules in PAK signaling may serve as important therapeutic targets for human patients with secondary microcephaly.
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CHAPTER 1
INTRODUCTION

1.1. Learning and memory

A “higher function” of the nervous system of animals and particularly of humans is their ability to alter their neural circuitry and behaviors in response to experience. Learning is the acquisition of information that makes this possible, and memory is the retention and storage of that information. The two are obviously related and are often considered together.

1.1.1. Types of memories

From a physiological perspective, memory can be categorized as either explicit or implicit. Explicit memory, also called declarative or recognition memory, is acquired consciously (or at least with awareness) and is dependent on the hippocampus and other parts of the medial temporal lobe for its retention. Explicit memory includes memories for events (i.e. episodic memory) and memories for words, rules and languages (i.e. semantic memory). On the other hand, implicit memory does not involve awareness. It is a nondeclarative, reflexive type of memory. Retention of implicit memory does not require hippocampus. This type of memory includes skills, habits and conditioned reflexes. Often times, explicit memories initially required for activities like bicycle riding can become implicit once the task is thoroughly learned. (Ganong, 2005) This study investigates the acquisition and retention of explicit memory.
Explicit memory (and also many forms of implicit memory) consists of short-term and long-term memory. Short-term memory lasts seconds to hours, during which processing in the hippocampus and elsewhere induces long-term changes in synaptic strength. Long-term memory can be stored for years and sometimes for life. (Cowan, 2008)

1.1.2. Encoding explicit memory

Encoding explicit memories involves working memory in the frontal lobes and unique processing in the hippocampus. Working memory is a form of short-term memory that keeps information available, usually for a short period of time, while the individual plans actions based on it. Working memory areas are connected to the hippocampus and the adjacent parahippocampal portions of the medial temporal lobe (Ganong, 2005). In humans and other animals, bilateral destruction of ventral hippocampus leading to loss of CA1 neurons causes striking defects in short-term memory (Isaacson, 1972). Interestingly, in these subjects their implicit memory processes are completely intact. The subjects with hippocampal lesion perform adequately in terms of conscious memory as long as they concentrate on what they are doing. However, any distraction for even the shortest period causes loss of all the memories of what they are doing or propose to do (Scoville and Milner, 2000). They are thus capable of new learning and retaining old pre-lesion memories, but fail to form new long-term memories. Also, brain imaging studies using techniques such as magnetic resonance imaging and positron emission tomography characterized blood flow and oxygen use in the hippocampus, and identified fluctuations
in those parameters during learning tasks (Squire, 1992; Squire et al., 1990). In order words, hippocampus is absolutely essential for memory consolidation.

Hippocampus is closely associated with surrounding areas which have been shown to be also important for memory. One such area is the parahippocampal cortex in the medial frontal lobe. fMRI and evoked potential studies in humans showed that when subjects recall explicit memories, brain activity is increased in their parahippocampal area and the contralateral frontal lobe (Rugg, 1998). Another area is the amygdala, which is involved in the coding and retention of emotionally charged memories. During retrieval of fearful memories, for instance, the theta rhythms of the amygdala and the hippocampus become synchronized (Seidenbecher et al., 2003). Consistently, lesions in amygdala have been shown to result in deficits in fear conditioning (Cousens and Otto, 1998; Goosens and Maren, 2001). In humans, events that evoke strong emotions are remembered better than events that are emotionally neutral. However, such distinction is lost in patients with bilateral amygdala lesion (Ganong, 2005).

1.1.3. Long-term memory storage

While hippocampus plays a key role in the consolidation of short-term memory, long-term memories are stored in various parts of the neocortex. Evidence indicates that various parts of the memories – visual, olfactory, auditory, etc. – are located in the cortical regions concerned with those functions (Winters et al., 2008). These various components of the whole memory are tied together by long-term changes in the strength of transmission at relevant synapses. In this way, long-term memories once established can be recalled by a large number of different sensory associations (Ganong, 2005). For
instance, vivid visual memories can be evoked not only by a similar scene but also by sounds, smells, or words associated with the scene.

In summary, to encode explicit memory, sensory informations are temporally stored in various parts of the prefrontal cortex as working memory. It is then relayed to medial temporal lobe, especially the parahippocampal cortex. From there, it enters the hippocampus and is processed and consolidated in ways that are still unclear. During this period, the memory is vulnerable and subject to distraction (McGaugh, 2000). Output from hippocampus via the subiculum and entorhinal cortex alters the strength of synaptic circuits in various parts of the neocortex, leading to the formation of remote long-term memories.

1.2. Synapses

Impulses, or action potentials (APs), are transmitted from one cell to another through synapses. In the central nervous system, synapses are most commonly found where axon of one neuron terminates on the dendrite of another (can also terminate on soma or axon, however). Synaptic transmission is different from simply jumping action potential from one neuron to another. It is a complex process that enables grading and adjustment of neural activity in response to external signals. The effect of discharge at the presynaptic neuron can be either excitatory or inhibitory on the postsynaptic neuron, and the summation of effects of all the inputs determines whether and how rapidly action potential will be fired in the postsynaptic neuron.
1.2.1. Chemical synapses

Transmission at most synapses is chemical; the AP in the presynaptic axon causes secretion of neurotransmitters from the axonal terminal. These neurotransmitters diffuse across the synaptic cleft (20-40nm wide) and binds to receptors on the surface of the postsynaptic cell. Receptor activation triggers events that lead to opening or closing of membrane channels, and generation or inhibition of AP. It can also lead to signaling cascades that alter gene expression and synaptic properties. Rarely in selected population of neurons, synapses can also be electrical. In this case, AP reaching the presynaptic terminal spreads to the postsynaptic cell through low-resistance gap junctions, directly triggering an excitatory postsynaptic potential (EPSP) with much shorter latency than chemical synapse. In this study, the discussion in focused solely on the chemical synapses.

1.2.2. Synaptic architecture

The anatomical structure of the synapses varies according to the region of the mammalian nervous system. The ends of the presynaptic fibers are enlarged to form terminal boutons, or synaptic knobs. In the cortex and hippocampus, it has been calculated that 98% of the excitatory synapses are on dendrites and 2% on cell bodies (Kandel, 2000). Axon terminals commonly form synapses on dendritic spines, small knob-like projections from dendrites. On average, each primary axon of a neuron divides to form over 2000 synaptic endings, and it is estimated that there are about $2 \times 10^{14}$ synapses in the human CNS (Blinkov, 1968). This alone makes communications between
neurons, or neuronal networks, extremely complex. In addition, synapses are dynamic structures and are able to change their properties and number with use and experience.

At the active zone of the presynaptic terminal are many membrane-enclosed vesicles which contain neurotransmitters. The vesicles and neurotransmitters are synthesized in the neuronal cell body and transported along the axon to the endings (Ganong, 2005). AP reaching the axonal terminal activates voltage-gated $\text{Ca}^{2+}$ channels, and the subsequent influx of $\text{Ca}^{2+}$ triggers a cascade of signaling that leads to vesicle fusion with the presynaptic membrane and neurotransmitter release. Vesicles can either fuse completely or partially (i.e. “kiss and run”) to discharge their content (Kandel, 2000). The details of the process by which the synaptic vesicles fuse is not entirely known, but involves Soluble NSF Attachment Protein Receptors (SNAREs). The SNARE proteins can be divided into two categories: those on vesicles or v-SNAREs, such as synaptobrevin; and those on the target membrane or t-SNAREs, such as syntaxin found on the presynaptic cell membrane (Ganong, 2005). Active zone also contain several proteins that interact with v- and t-snares; the function of these proteins remain unknown. One such protein is synaptophysin, a synaptic vesicle glycoprotein that is present in virtually all synapses and interacts with synaptobrevin. Its ubiquity and specificity at the synapses has led to its use in immunostaining for quantification of synapses (Calhoun et al., 1996).

Across the synaptic cleft is the postsynaptic membrane, and usually a postsynaptic thickening called the postsynaptic density (PSD). Originally identified as an electron-dense region at the postsynaptic membrane, PSD contains many proteins involved in the regulation of synaptic function. These include: postsynaptic density-95 (PSD95),
neuroligin (a cellular adhesion molecule), neurotransmitter receptors, calcium/calmodulin-dependent protein kinase II, and actin (Ziff, 1997). Binding of neurotransmitter activates the receptor, leading to opening of ion channels or activation of a secondary messenger system, which leads to changes in the post-synaptic potential. The same neurotransmitter can have different and even opposite effects on the postsynaptic cell, depending on the receptor subtype (Ganong, 2005). The resulting postsynaptic potential can be either depolarizing and excitatory (called excitatory PSP or EPSP), or hyperpolarization and inhibitory (called inhibitory PSP, or IPSP). PSPs from summates both spatially and temporally, and their net effect at the axonal hillock of the postsynaptic neuron will determine whether AP is fired (and at what frequency) or inhibited.

The fusion of synaptic vesicles is a probabilistic event. Stimulation of the presynaptic neuron (physiologically or through electrode) can increase the probability of release. However, even without stimulation, single vesicles will occasionally fuse and release their content, generating miniature EPSPs (mEPSPs). Bernard Katz pioneered the study of these mEPSPs at the neuromuscular junction in 1951, revealing the quantal nature of synaptic transmission (FATT and KATZ, 1952). Quantal size is defined as the synaptic response to the release of neurotransmitter from a single vesicle, and each vesicle in the same synapse always contains the same amount of neurotransmitters.

1.2.3. Glutaminergic synapse

The amino acid glutamate is the main excitatory neurotransmitter in the brain and spinal cord, and it has been estimated that glutaminergic synapses are responsible for
75% of excitatory transmissions in the brain (Ganong, 2005). Glutamate receptors are of two types: metabotropic receptors and ionotropic receptors.

The metabotropic glutamate receptors (mGluRs) are serpentine G protein-coupled receptors that increase intracellular IP$_3$ and DAG levels or decrease intracellular cAMP levels (Dhami and Ferguson, 2006). mGluRs are found both presynaptically and postsynaptically (Shigemoto et al., 1997). They appear to be involved in the production of synaptic plasticity, particularly in the hippocampus and the cerebellum. mGluR1 knockout in mice, for example, causes deficits in spatial learning and severe motor incoordination (Conquet et al., 1994).

The ionotropic receptors are ligand-gated ion channels. There are three types, each named based on a potent agonist of the receptor. The ionotropic glutamate receptors consist of: the kainate receptors, the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptors, and the NMDA (N-methyl-D-aspartate) receptors. Each glutamate receptor is composed of multiple subunits; kainate has five, AMPA has four, and NMDA has six identified to date (Ganong, 2005). The glutamate receptors are pentamers of these subunits. The kainate receptors are simple ion channels that permit Na$^+$ influx and K$^+$ influx when open. Majority of AMPA receptors contain GluR2 subunit, and are only permeable to Na$^+$. A subpopulation of AMPA receptors lacking GluR2 subunit allows both Na$^+$ and Ca$^{2+}$ influx. The balance between the two at a given synapse can change in response to activity (Liu and Cull-Candy, 2002).

The NMDA receptors are cation channels permeable to both Na$^+$ and Ca$^{2+}$, and they are unique in several ways (Paoletti and Neyton, 2007). First, glycine is required as a co-agonist to glutamate to enable gating. Second, despite been gated open these channels
are blocked at resting membrane potential by extracellular Mg\textsuperscript{2+} ions. This blockage is removed when the postsynaptic membrane is partially (and sufficiently) depolarized by the activation of AMPA receptors. Finally, NMDA receptors activation allows large amounts of Ca\textsuperscript{2+}, a potent intracellular signaling molecule, to enter the cell. Ca\textsuperscript{2+} influx through NMDA receptor has been established to play a key role in inducing synaptic plasticity. In this way, these receptors play critical roles in molecular mechanism underlying learn and memory.

1.2.4. Synaptic plasticity

Short-term and long-term changes in synaptic function and strength can occur in response to certain stimulation pattern, reflective of learning and experience. These changes, collectively termed synaptic plasticity, are of great interest because they represent the molecular and cellular basis of learning and memory. Synaptic plasticity can occur both pre- and post-synaptically. (Ganong, 2005)

Posttetanic potentiation (PTP), habituation, and sensitization are different types of synaptic plasticity. In PTP, synaptic response is enhanced for a short period (up to 60 seconds) following a brief tetanizing train of stimuli in the presynaptic neuron (Bao et al., 1997). Such stimuli causes Ca\textsuperscript{2+} to accumulate in the presynaptic terminal such that intracellular binding proteins that keep cytoplasmic Ca\textsuperscript{2+} low are temporary overwhelmed (Ganong, 2005). Habituation occurs when a benign stimulus is repeated over and over, causing the evoked response to gradually weaken and disappear. During habituation, the presynaptic Ca\textsuperscript{2+} channels are gradually inactivated upon repeated stimulation, leading to decreased intracellular Ca\textsuperscript{2+} and transmitter release (Bear, 2001). Sensitization is the
prolonged occurrence of augmented postsynaptic responses after a habituated stimulus is paired with a noxious stimulus. Shown in *Aplysia*, this type of plasticity is due to presynaptic facilitation involving presynaptic serotonergic neurons (Brunelli et al., 1976).

Two types of synaptic plasticity critical to hippocampal memory consolidation are long-term potentiation (LTP) and long-term depression (LTD). In LTP, a persistent increase in EPSP is achieved after a brief period of rapidly repeated stimulation of the presynaptic neuron. Unlike posttetanic potentiation, this form of plasticity is initiated by increase in intracellular Ca\(^{2+}\) in the postsynaptic neuron, instead of presynaptic neuron. LTP occurs in many parts of the nervous system, but it is best studied in the hippocampus. There are actually two forms of LTP found in hippocampus: mossy fiber LTP, which is presynaptic and NMDA receptor-independent, and Schaffer collateral LTP, which is postsynaptic and NMDA receptor-dependent (MacDonald et al., 2006). The established basis of Schaffer collateral LTP is summarized in Fig 1 (Ganong, 2005). The basis of mossy fiber LTP is still unsettled, but evidence suggests it involves cAMP and I\(_h\), a hyperpolarization-activated cation channel (Huang et al., 1994).

A great amount of evidence indicates that NMDA receptor activation, critical to LTP induction, plays an essential role in the acquisition of spatial memory. First evidence to support this hypothesis was provided by Morris and collaborators in 1986. The group found that blocking NMDA receptor with AP5 inhibited both spatial learning and LTP, thereby suggesting an overlap in the mechanism by which LTP was sustained and spatial learning was consolidated (Morris et al., 1986). Subsequently, same conclusions were derived using genetically manipulated mice. Both NR2A subunit knockout mice and CA1-specific NMDA receptor knockout mice exhibited impaired spatial learning coupled
with a deficit in LTP (Tsien et al., 1996; Sakimura et al., 1995). Conversely, overexpression of NR2B subunit in mice enhanced both LTP and learning and memory (Tang et al., 1999).
Fig 1. Established mechanism of Schaffer Collateral LTP. (1) Glutamate is released from the presynaptic neuron and binds to postsynaptic AMPA and NMDA receptors. (2) AMPA receptor activation causes $\text{Na}^+$ influx, causing depolarization. (3) Partial depolarization removes $\text{Mg}^{2+}$ block on NMDA receptors. (4) $\text{Ca}^{2+}$ enters the neurons along with $\text{Na}^{2+}$ through unblocked activated NDMA receptor, leading to a rise in intracellular $\text{Ca}^{2+}$. (5) $\text{Ca}^{2+}$ activates calmodulin (CaM), which in turn activates $\text{Ca}^{2+}$/calmodulin kinase ii (CaMKII). Activated CaMKII can phosphorylate AMPA receptors (6a) to increase their conductance, and recruit AMPA receptors into the synaptic membrane from cytoplasmic storage sites (6b). In addition, retrograde signal may pass to presynaptic neurons (6c), producing long-term increase in quantal release of glutamate. (Ganong, 2005)
LTD was first discovered in hippocampus but was subsequently shown to be present throughout the brain in the same fibers as LTP (Ganong, 2005). LTD is the opposite of LTD, characterized by a decrease in EPSP amplitude in response to low frequency stimulation. Such stimulation also triggers Ca^{2+} influx, but in a smaller quantity compare to LTP, resulting in the activation of different pathways (Bear, 2003). In the cerebellum, LTP induction appears to require the phosphorylation of the GluR2 subunit of the AMPA receptor (Chung et al., 2003).

1.3. Development of the nervous system

Normal development of nervous system is essential for proper brain function. However, the process of development can be disrupted by both genetic and environmental factors, leading to the possibility of severe mental disorders. Even after birth, the first few years of an infant’s life are very important for the proper development of the nervous system. During this period, the brain is still susceptible to mental and neurological disorders affecting development.

The nervous system is one of the first structures to appear in the human embryo. By third week of development, ecotodermal plate thickens to form the neural plate, whose lateral edges elevate to form neural folds. The notochord induces neural folds to fuse into neural tube, which becomes separated from the rest of ecotoderm. During the fusion of neural plate, a subgroup of neural plate cells escape into the space between the tube and ectoderm. These neural crest cells give rise to neurons and glia of the peripheral nervous system. The cranial end of the neural tube expands to form the three main divisions of the developing brain separated by two constrictions. These brain vesicles are:
prosencephalon, which becomes forebrain; mesencephalon, which becomes midbrain; and rhombencephalon which becomes hindbrain. (Bear, 2005)

The walls of newly enclosed neural tube consist of neuroepithelial cells – a group of multipotent progenitor cells. They form a thick layer over the entire wall of the neural tube and divide rapidly, resulting in the neuroepithelial layer. The cells in this layer give rise to primitive nerve cells, or neurblasts. Neuroblasts migrate away from the lumen and settle into other portions of the neural tube. The neurons from the developing cerebral and cerebellar cortices migrate from the mantle zone through the marginal zone and form a layered sheet of gray matter. In the cerebral cortex, two populations of neurons are generated from distinct proliferative zones. The excitatory pyramidal neurons are derived from neuroblasts in the cortical ventricular zone and use radial glial fibers to migrate into the cortex. In the cortical plate, they accumulate in an inside-out sequence to form six layers. In contrast, inhibitory nonpyramidal neurons are mainly derived from the ganglionic eminence of the ventral telecephalon. These cells use tangential migratory pathways along the axon bundles of the corticofugal fibers to reach the cortex (Nadarajah et al., 2003). Once the migration is completed, neurons sprout dendrites and axons that must be guided towards the correct target through a number of guidance cues. This process called neuronal morphogenesis begins prenatally and continues after birth. (Conn, 2008) See section 1.3.1. for the different stages of neuronal morphogenesis.

The growth and survival of neurons are determined by the presence of a family of intrinsic factors (Bennet et al., 2002). These include, but are not limited to: nerve-growth factors (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins (NT-3, NT-4/5, and NT-6). These factors mediate their actions on neurons by binding to cell-surface
receptors. The effects of these factors are not restricted to specific neuron populations, and each neuron is influenced by several growth factors. A neuron’s requirement for tropic factors may change during the course of development (Conn, 2008).

During embryonic neurogenesis, approximately 50% more neurons are formed than present in the mature nervous system. Thus, neurogenesis is accompanied by the elimination of a large number of neurons through a genetically program cell death, or apoptosis. This form of neuronal loss is proposed to serve serveral key functions. First, it eliminates neurons that have made inappropriate synaptic connections. Second, it removes neurons that serve transient developmental functions, such as radial glia. Finally, it represents an innate control mechanism to retain only the healthiest neurons that make the most connections and, thus, receives the most trophic factors. All these ensure that the nervous system tends towards an optimal configuration. (Conn, 2008)

The brain does not stop developing after birth. In fact, the brain quadruples in size following birth. This is due to dendritic growth, axon myelination and formation of new synapses. These changes are most carefully documented in the maturation of the frontal and temporal cortex, amygdala and hippocampus. They are accompanied by a decrease in grey matter in the cortex, and an increase in the density of white matter as the infant matures. Formation of synapses (synaptogenesis) continues postnatally and well into adulthood. Synapses are constantly added, eliminated and refined throughout an individual’s life. (Ganong, 2005)
1.3.1. Neuronal morphogenesis in primary culture

Neurons possess a highly polarized structure that allows integration and one-way flow of information. A neuron typically has many dendrites that branch extensively, receiving multiple inputs from presynaptic neurons. In addition, it possesses a single axon that forms multiple collaterals to replay information to postsynaptic neurons. The morphogenesis of neurons has been studied extensively in primary hippocampal neuron cultures and is shown to consist of 5 principle stages (Dotti et al., 1988; Higgins et al., 1997) (Fig 2). Upon plating, round neuron attaches to the substratum and forms lamellipodia (stage 1). Within 12 hours, typically neurites begin to sprout (stage 2). Neurites are cylindrical processes with growth cones at the distal end. They continuously extend and retract, but there is no net elongation of neurites during this period. Within 24 hour, one of these neurites will elongate rapidly without retraction and develop into an axon (stage 3). The remaining neurites continue to grow and branch as well but at a slower rate to form dendrites (stage 4). Axons develop further and form presynaptic terminals, from which neurotransmitters are released. Dendrites form dendritic spines, small compartmentalized postsynaptic protrusion where most of the excitatory synaptic connections are formed (stage 5). At this point, synaptic contacts are made and spontaneous electrical activity arises in the neuronal network.
Fig 2. Five stages of neuronal morphogenesis in primary hippocampal neuron culture. Pictures modified from Nikolic, 2008.
1.4. P-21 activated kinases

Rho family GTPases are molecular switches that cycle between inactive GDP-bound state and active GTP-bound state in response to a variety of extracellular signals including neuronal activity, neurotrophic factors and contact with extracellular matrix. In turn, they regulate a wide range of downstream effectors and create a complex signaling network that influences cytoskeletal dynamics and gene expressions (Fig 3). The best-studied Rho GTPases are Rho A (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP, thereby switching Rho GTPases on. In contrast, GTPase activating proteins (GAPs) increase endogenous GTPase activity of Rho GTPases, thereby promoting the off state. (Luo, 2000)

The p-21 activated kinases, or PAKs, are a family of key effectors of Rac1 and Cdc42, shown to regulate many aspects of cellular function, such as: cytoskeletal dynamics, cell motility, migration, proliferation, differentiation, and gene expression. PAK1, PAK2 and PAK3 constitute the group I PAKs based on their high amino-acid sequence identity. Over the last few years, growing evidence indicate PAK of group I to play unique roles in brain ontogenesis, neuronal differentiation and synaptic plasticity (Kreis et al., 2007). Moreover, defects of group I PAK signaling have been implicated in neurodegenerative diseases, with PAK3 being specifically implicated in mental retardation.
Fig 3. Rho GTPase activation and its substrates. Rho GTPases respond to extracellular signals such as neural activity and neurotrophins. They cycle between active GTP-bound state and inactive GDP bound state, catalyzed by Guanine Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs) respectively. The three Rho GTPases – Rac, Cdc42 and Rho – activate an array of downstream substrates, through which they regulate cytoskeletal dynamics and gene expressions. Some immediate downstream substrates of Rho GTPases include: PAK, Wiskott-Aldrich syndrome protein (WASP), WASP-family verproli-homologous protein (WAVE), cyclin-dependent kinase 5 (CDK5), insulin receptor tyrosine kinase substrate p53 (IRSp53), and Rho Kinase. Modified from Luo, 2002 and Gevek, 2009.
1.4.1. PAK structure and expression

PAKs are characterized by the presence of a conserved carboxy-terminal kinase domain and a non-catalytic N-terminal region which contains a p21-binding domain (PBD) where Rac1 and Cdc42 GTPases can bind. PAKs of higher eukaryotic organisms are classified into two groups, based on sequence homology, structure and biochemical properties. Group 1 PAKs consists of PAKs 1, 2 and 3; their regulatory domain contains an auto-inhibitory domain (AID) that overlaps partially with PBD. GTPase can only bind to PBD when AID is released. In addition, Group I PAKs contain highly conserved proline-rich domains responsible for interaction with adaptors Nck and Grb2 and the guanine nucleotide exchange factor PIX/Cool (Bagrodia et al., 1995; Manser et al., 1998). The regions between these proline rich domains are where PAKs 1, 2 and 3 differs, and may allow for isoform-specific signaling (Rashid et al., 2001; Walter et al., 1998). For instance, PAK1 contains a unique T212 site and PAK2 contains a D212 site sensitive to caspase cleavage. A schematic illustration of PAK1 structure is shown in Fig 4.

Northern blot and RT-PCR analysis have shown that group I PAKs have different tissue expression patterns: PAK1 is highly expressed in the brain and spleen, PAK2 is ubiquitously expressed, and PAK3 is mainly expressed in the brain (Kreis and Barnier, 2009). In the brain, PAK1 is highly expressed in the hippocampus and in the cortex, especially in cell layers IV and V (Manser et al., 1995). PAK3 is expressed in the hippocampus and cortex, and the highest expression is found in the post-mitotic neurons of dentate gyrus and cortical layers II/III and V. Furthermore, this protein is also expressed in the amygdala and olfactory bulb (Allen et al., 1998; Manser et al., 1995;
McPhie et al., 2003). However, no PAK3 immunoreactivity was detected in the proliferating neurons in the ventribular and subventribular zone.

Fig 4. Schematic diagram of PAK1 structure. The PAK1 backbone is shown in orange and the catalytic domain (aa 255-259) in bright blue. The p21 (Rac/Cdc42)-binding domain or PBD is shown in purple, overlapping with the pale green autoinhibitory domain (AID). Yellow boxes are the five PXXP putative SH3-binding motifs, the green box is the noncanonical prorich Pix/Cool SH-3 binding motif, and the red box is the ED-rich region of unknown significance. The white cycles represent identified sites of phosphorylation by Akt (Ser21), Cdc/Cdk5 (Thr212) and PDK (Thr423). From review by Bokoch, 2003.
Since PAK2 is ubiquitously expressed, its role in brain has not been studied significantly. A recent study comparing expressions of PAKs in various cell types in the brain revealed that PAK1 is enriched in neurons but also oligodendrocytes, whereas PAK3 is mostly found in neurons (Cahoy et al., 2008).

During neuronal development, expressions of PAKs 1 and 3 are highly regulated. PAK1 expression increases at E14-16 and is maintained throughout adulthood (Causeret et al., 2009a). As for PAK3, studies have demonstrated a correlation between its upregulation and neuronal differentiation in two different animal models, in *Xenopus laevis* neurons and in mouse cortical GABAergic interneurons (Cobos et al., 2007; Souopgui et al., 2002). Together, evidence suggests that both PAKs may play important roles in the development of nervous system and of neurons.

Group 1 PAKs have been demonstrated to have different subcellular localization in neuronal cells. In neuroblastoma N1E-115 cells, PAK1 was found predominantly within the cell body and along dendritic shafts, PAK2 was found in areas with filopodia, and PAK3 was found in areas with lamellipodia and membrane ruffling (Marler et al., 2005). In hippocampal and cortical neurons however, PAK1 is expressed in axons and dendrites, while PAK3 is concentrated in cell bodies and large diameter axons (Ong et al., 2002; Hayashi et al., 2002). PAK is also found in dendritic spines where its activated form (marked by phospho-threonine 421) is highly enriched in the post-synaptic density. Distinct temporal expression and spatial localization of different PAK members suggest they may be involved in different neuronal functions.
1.4.2. Roles of PAKs in neurophysiology

Various studies from mostly in vitro and non-mammalian systems have implicated PAKs to play distinct roles in various aspects of neurophysiology. Broadly, these roles can be categorized as: 1) cell fate regulation (i.e. division, apoptosis, differentiation), 2) neuronal migration, 3) neuronal morphogensis (i.e. polarity, neurite outgrowth, axon guidance) and 4) synaptic plasticity and spine morphology, which are associated with cognitive functions (Kreis et al., 2009). Table 2 summarizes implicated neurophysiological roles of PAK homologs in various invertebrate models.

Existence of three different group I PAK in mammals, with two being predominantly expressed in the brain, suggests they may have specific individual functions in neurophysiology. However, specific functions of each PAK isoform in the mammalian system remain unclear despite numerous studies. It has been a major challenge to develop methods that specifically manipulate one isoform without affecting the others. Given that there’s a high degree of structural homology between the group I PAKs, most techniques used in in vitro studies such as small interfering RNA (siRNA) and dominant negative protein expression may not be specific for any one isoform. Moreover, because group 1 PAKs are often coexpressed in the brain during development, they may share redundant functions that may be preserved (and thus masked) in PAK single knockout mice. In these mice, the loss of one PAK may be compensated functionally by presence of other isoforms, thereby posing significant challenge to uncovering the roles of group 1 PAKs in vivo. Indeed, there appears to be a huge gap between in vivo and in vitro studies regarding the roles of PAK in neurophysiology. While a multitude of roles are uncovered in in vitro studies that acutely disrupt PAK
function (discussed below), phenotypes of PAK1 or PAK3 single knockout mice are very mild; these mice are present with deficit in late-phase LTP, but are otherwise normal in brain and neuronal structure as well as in cognitive behavior (Asrar et al., 2009; Meng et al., 2005).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>General role</th>
<th>Specific finding / functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Pak1</td>
<td>Xenopus</td>
<td>CNS development</td>
<td>Control cellular distribution of Tumorhead protein involved in neural tube morphogenesis during CNS development</td>
<td>Delsert. 2007 Dev. Biol.</td>
</tr>
<tr>
<td>Pak3</td>
<td>Xenopus</td>
<td>Neurogenesis</td>
<td>During embryogenesis Pak3 expression activated by proneural regualtors X-Ngnr-1 and X-NeuroD, and inhibited by Notch. Loss of X-Pak3 function in embryo inhibited neuronal differentiation. Constitutive expression of membrane targeted Pak3 led to cell cycle arrest and premature neuronal differentiation.</td>
<td>Souopgui. 2002 EMBO J.</td>
</tr>
<tr>
<td>Ste20</td>
<td>Yeast</td>
<td>Cell polarity</td>
<td>Cell polarization during yeast budding</td>
<td>Otilie. 1995 EMBo J.</td>
</tr>
<tr>
<td>D-Pak</td>
<td>Drosophila</td>
<td>Axon guidance</td>
<td>PhotoR cell projection requires PAK binding to Rac/Cdc42 GTPases.</td>
<td>Hing. 1999 Cell</td>
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</table>
Table 2. Roles of PAKs in invertebrate neurophysiology. Summary based on review by Kreis et al., 2009.

<table>
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<th></th>
<th>Species</th>
<th>Function</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>D-Pak</td>
<td>Drosophila</td>
<td>Axon guidance</td>
<td>D-Pak KO lethal, but heterozygous larvae show anomalies in photoR cell axon projection: extend normally, but fail to find target.</td>
<td>Ang. 2003</td>
</tr>
<tr>
<td>Ce-Pak1</td>
<td>C. elegans</td>
<td>Axon guidance</td>
<td>Targeting of olfactory axons in brain</td>
<td>Lucanic. 2006</td>
</tr>
<tr>
<td>D-Pak</td>
<td>Drosophila</td>
<td>Axon guidance</td>
<td>Targeting of ventral cord commisural motorneurons</td>
<td>Fan. 2003</td>
</tr>
<tr>
<td>Pak</td>
<td>Drosophila</td>
<td>Axon guidance</td>
<td>During repulsion of commissural axons from the midline of fly embryo, Slit stimulation of Robo receptor led to recruitment of DOCK/Nck and D-Pak to Robo inducing Rac1 activation.</td>
<td>Hu. 2001</td>
</tr>
<tr>
<td>Pak1</td>
<td>Aplysia sensory neuron</td>
<td>Plasticity</td>
<td>Pak can antagonise repulsive guidance signaling of Plexin-B1 in motor axon pathways by competing for Rac binding. Reciprocally, Plexin-B1 can inhibit Rac-induced Pak activation by recruiting active Rac, resulting in Pak inactivation and growth cone collapse/turning.</td>
<td>Vikis. 2002</td>
</tr>
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</table>

Genes Dev.
1.4.2.1. Neuronal cell fate

Cell division requires coordinated actin and microtubule dynamics. Evidence indicates that PAK, especially PAK1, may play an important role in cell division cycle. In mouse fibroblast, expression of AID of PAK1 induced cell cycle arrest and led to inhibition of cell cycle progression regulators, cyclins D1 and D2 (Thullberg et al., 2007). PAK1’s role in nuclear processes have been shown to be mediated through CtBP (C-terminal binding protein) and Aurora A, a centrosome kinase. PAK1 directly phosphorylates CtBP which results in its nuclear to cytosol translocation, and in doing so blocks transcription repressor activity of CtBP (Barnes et al., 2003). During mitosis, PAK1 phosphorylates Aurora A on Thr288 and Ser342, leading to its activation (Zhao et al., 2005).

Interestingly, PAKs have been studied extensively in tumorogenesis in the central nervous system. For example, PAKs have been shown to be involved in neurofibromatosis, a genetic disorder that causes formation of benign tumors in the central and peripheral nervous system. Merlin / Schwannomin, the product of neurofibromatosis 2 tumor suppressor gene, can bind directly to PBD of PAK1 to suppress its interaction with Rho GTPase and thus its activation (Kissil et al., 2003). PAK1 can, in turn, phosphorylate Merlin on S518 to cause its inactivation. In glioblastoma, increased prevalence of activated phosphorylated forms of PAK1 in the cell cytoplasm has been associated with shorter survival time of the patients, possibly owing to the role of PAK1 in the invasiveness of the tumor (Aoki et al., 2007).

PAKs have been shown to be involved in apoptotic pathways, both promoting cell death and cell survival. PAK2 is well-studied for its proteolytic cleavage by casases
during end stages of apoptosis (Rudel and Bokoch, 1997). Cells lines that express dominant negative PAK mutant were resistent to Fas-induced formation of apoptotic bodies. On the other hands, PAK1 promotes cell survival by phosphorylating BAD (Bcl-2 antagonist of cell death). However, to little data concerning the apoptotic role of PAK in neuronal physiology has been reported.

Several studies have indicated PAK plays a role in neuronal differentiation. The Notch signaling pathway is important for determining a variety of cell fates. Sharp, a corepressor of Notch target genes, was identified as PAK-interacting protein that is phosphorylated by PAK1. This phosphorylation strengthens the transcriptional repression of Notch target genes (Vadlamudi et al., 2005). In *Xenopus*, X-PAK1 controls for cellular distribution of Tumorhead protein involved in neural tube morphogenesis, and it is proposed that X-PAK1 can regulate neural plate cell proliferation through Tumorhead activation (Wu et al., 2007). A separate line of study showed that PAK3 may play a special role in neuronal differentiation. PAK3 expression, but not PAK1 or 2 expressions, is upregulated by proneural regulators X-Ngnr-1 and X-NeuroD and inhibited by Notch signaling implicated for glial cell differentiation. Furthermore, loss of X-PAK3 in *Xenopus* embryo inhibited neuronal differentiation, whereas its over-expression led to premature differentiation (Souopgui et al., 2002).

1.4.2.2 Neuronal migration

A few studies have described a role of PAK in regulating neuronal migration. Cobo and collaborators used DLx knockout mice to provide indirect evidence that repression of PAK3 promotes tangential migration of immature interneurons generated in...
the medial ganglionic eminence of the basal telecepha lone into the cortex (Cobos et al., 2007). This group demonstrated that Dlx homeobox transcription factors are essential for the migration of interneurons, through their suppression of neurite outgrowth, until they have reached the cortex. Once interneurons have migrated, PAK3 is up-regulated to allow differentiation. In a more recent study, PAK was shown to be required for neuronal migration in vivo in mouse embryo cortices (Causeret et al., 2009a), utilizing in utero electroporation to deliver various mutated PAK constructs and small hairpin RNA (shRNA). Expression of hyperactivated PAK1 caused neurons to arrest in the intermediate zone with misoriented and disorganized leading projections. Suppressing PAK1 protein expression using shRNA disrupted the morphology of migrating neurons. Interestingly, a significant number of neurons with reduced PAK1 expression aberrantly entered into the normally cell-sparse marginal zone, suggesting a loss of their ability to cease migrating or to dissociate from radial glia.

1.4.2.3. Neuronal morphogenesis

Neuronal polarity

In neurons, cell polarization is especially important because it is required for proper migration and specification of axons and dendrites. During early development, neurons polarize to form many dendrites and a single axon. In order to establish this polarity, the centrosome and Golgi must be recruited to the base of the leading process of the migrating neuron. Many studies have implicated PAK to play an important role in neuronal polarity. Initial study that showed PAK was important in cell polarity is done in Saccharomyces cerevisiae. The founding member of PAK, Ste20, was implicated in the
correct cell polarization during yeast budding (Ottilie et al., 1995). A large group of conserved proteins called PAR proteins have been found to regulate cell polarization in many different contexts in diverse animals, and they have been proposed to form part of the fundamental mechanism for cell polarization (Goldstein and Macara, 2007). In mammals, indirect evidence places PAK upstream of PAR proteins through PAK substrate LIM domain kinase (LIMK). In primary hippocampal neuron culture, overexpression of LIMK1 accelerates axons formation and enhances the accumulation of PAR3/PAR6 at growth cones (Rosso et al., 2004). In another study, localized activation of PAK has been shown to be essential for axon specification in vitro (Jacobs et al., 2007). In hippocampal neurons, activation of PAK1 was spatially restricted to the immature axon despite its uniform presence in all neurites. Hyperactivation of PAK1 at the membrane of all neurites of loss of PAK1 disrupted axon-dendrite distinction, and led to formation of multiple axons.

**Neurite extention**

Both Rac1 and Cdc42, direct upstream regulators of PAK, have been shown to play important roles in neurite initiation and outgrowth (Govek et al., 2005). In PC12 cells, membrane targeting of PAK1 induced neurite outgrowth (Daniels et al., 1998). While cytoplasmic PAK1 constructs did not cause efficient neurite outgrowth from PC12 cells, targeting of these constructs to plasma membrane via a C-terminal isoprenylation sequence induced PC12 cells to extend neurites. This effect was shown to be independent of PAK1 ser/thr kinase, but dependent on structural domains within both N- and C-terminus. Application of N- and C-terminal regions of PAK1 as dominant negative
inhibited normal neurite outgrowth stimulated by NGF. The role of PAK in neurite outgrowth was also studied in N1E-115 neuroblastoma cells, in a model based on neurite outgrowth on laminin in the presence of repulsive molecules (Marler et al., 2005). In these cells, outgrowth of neurite avoided crossing repulsive substrates, but the avoidance response was partially overcome by overexpression of membrane-targeted and kinase-inactive form of PAK. Also, when N-terminal PAK sequence was introduced to interfere with PAK function, significantly more neurites crossed into the repulsive barrier.

In addition to the localization of PAK, the regulation of its kinase activity plays an important role in neurite outgrowth. Expression of active PAK1 induced an increase in the number of dendrites, whereas expression of dominant negative PAK1 caused a reduction of dendrites (Hayashi et al., 2002). Downstream of PAK, several studies reported LIMK as important players in neurite extension, through cofilin phosphorylation (Endo et al., 2007). Knockdown of cofilin/ADF by siRNA almost completely inhibited NGF-induced neurite extension in PC12 cells. One study suggests PAK3 may play an indirect role in interneuron neurite outgrowth (Cobos et al., 2007). In the mouse telecephalon, Dlx homeobox transcription factors are essential for tangential migration of GABAergic interneurons to neocortex. It is shown that in immature interneurons, Dlx1/2 repression of PAK3 is critical in restraining neurite growth and promoting tangential migration.

**Axon guidance**

Several studies have implicated PAK in axonal guidance. The first of such studies was done in photoreceptor cells of drosophila omatides. Hing and collaborators showed
that PAK functions downstream of SH2/SH3 adaptor protein Dock to regulator photoreceptor axon guidance (Hing et al., 1999a). Normal pattern of R cell connectivity required PAK’s kinase activity and Dock plus Cdc42/Rac binding in drosophila. Although D-PAK loss of function is lethal, transheterozygous larvae show severe anomalies in axon projection of R cells. Their axons extend normally into the brain but fail to acquire target. Other data reported an important role of D-PAK and Dock in regulating olfactory axon pathfinding in *drosophila* (Ang et al., 2003). In *C. elegans*, two PAK homologs, max-2 and Ce-PAK1, are required for netrin-mediated commissural motor axon guidance (Lucanic et al., 2006).

In the developing nervous system, axons are guided towards their appropriate targets by a series of attractive and repulsive guidance cues. These cues consist of four main types of ligands and their corresponding receptors: Slit/Robo, Sema/Plexin, Netrin/DCC/UNC, and Ephrine/Eph. All these receptors’ signaling converges on Rho GTPases to alter cytoskeletal regulation. Fan and collaborators reported that PAK, Dock, and Rac1/2 function together during Robo repulsion (Fan et al., 2003). During the repulsion of commissural axons from the midline of the fly embryo, activation of Robo receptor by Slit caused recruitment of Dock and D-PAK to the receptor to induce Rac1 activation. In *drosophila*, Plexin B mediates axon guidance by simultaneously inhibiting Rac activation of PAK and enhancing RhoA signaling (Hu et al., 2001). Together, these results demonstrate an essential role of PAK in the balance between repulsive and attractive axon guidance. However, no data regarding the role of PAK in mammalian axon guidance exist, and no isoform specificity has been identified in this process.
1.4.2.4. Synaptic plasticity and spine morphology

Several studies have indicated that PAKs play important roles in synaptic plasticity and dendritic spine morphology. The first of such studies reported that forebrain-specific dominant negative PAK transgenic mice showed altered cortical synaptic morphology and impaired memory consolidation (Hayashi et al., 2004). Cortical neurons in these mice displayed fewer dendritic spines, shorter average spine length, and an increased proportion of larger synapses compared to wild-type controls. These alterations in synaptic morphology correlated with enhanced mean synaptic strength (increased AMPA and NMDA receptor mediated synaptic transmission) and impaired bidirectional synaptic modifiability (enhanced LTP and reduced LTD) in the cortex. In addition, presynaptic structure of the mutant mice contained increased number of docked vesicles. Later, the same group showed that expression of this dominant negative PAK transgene in Fmr1 (fragile X mental retardation 1) KO mice partially rescued the abnormalities of dendritic spines (increased spine density and spine length), and fully rescued the deficits in cortical long-term potentiation (Hayashi et al., 2007). The inhibitory PAK transgene expression also ameliorated the behavioral abnormalities (locomotor activity, anxiety, trace fear conditioning) in FMR1 KO mice. In addition, Hayashi and collaborators showed that PAK interacts with FMRP. These results suggest that PAK and FMRP could complement each other to properly regulate synaptic functions. It is proposed that FMRP and PAK could inhibit each other to form an inactive complex. PAK activation by Rho GTPases could dissociate the complex, and free FMRP to regulate translation. Based on this hypothesis, the AID transgene might have inhibited association between PAK and FMRP, enabling FMRP to inhibit mRNA translation.
Consistent with model, FMR1 KO mice exhibit decreased LTP and increased LTD – the phenotypes opposite to those of dominant negative PAK expressing mice (Bear et al., 2004; Li et al., 2002).

Several other genetic approaches supported the role of PAKs in synaptic plasticity and spine morphogenesis. Studies of mice lacking PAK1 or PAK3 revealed that these two PAKs are especially critical for hippocampal synaptic plasticity (Asrar et al., 2009; Meng et al., 2005). Both PAK1 KO and PAK3 KO mice exhibited normal basal and presynaptic function, but were profoundly impaired in long-term potentiation at CA3-CA1 synapse. NMDA-induced change in cofilin activity was abolished in PAK1 KO hippocampal slices while the total cofilin level was unaffected. Consistent with disrupted cofilin activation, the distribution of filamentous actin (F-actin) in the dendrites and spines was subsequently shown to be abnormal (Asrar et al., 2009). Interestingly, a drastic reduction in the active form of transcription factor cAMP-responsive element-binding protein (CREB) was found in the PAK3 KO mice, implicating a potential novel signaling mechanism by which Rho-PAK3 signaling can regulate cognition (Meng et al., 2005). No learning deficits were reported in PAK1 KO mice. However, PAK3 KO mice were presented with mild deficits in conditioned taste aversion test, although they showed normal spatial learning in Morris water maze. Neither KO mice showed deficits in dendritic spines. Nonetheless, acute in vitro expression of PAK1 and PAK3 mutants proteins (devoid of kinas activity, constitutively active, or containing mental retardation mutations) in hippocampal neuron or slice culture led to abnormalities in spineogenesis (Boda et al., 2004; Zhang et al., 2005). The morphological discrepancies between in vivo studies in KO mice and in vitro studies are likely to be accounted by 1) developmental
compensation in vivo by the other PAK isoforms, and 2) the non-specific experimental approaches used in in vitro studies (i.e. shRNA and dominant negative expression has the potential to influence multiple PAK isoforms and related proteins). Downstream of PAK, actin cytoskeleton regulation by LIMK-1 is fundamental for spinogenesis. LIMK-1 KO mice are present with dendritic spine abnormalities characterized by small spine heads and thin necks, and are associated with enhanced LTP and deficits in fear and spatial learning (Meng et al., 2002).

Evidence for strong role of PAK in spinogenesis and synaptic plasticity has led to investigation of PAK localization and activity in dendritic spines. Penze and collaborators showed that treatment of hippocampal neurons with clustered ephrinB1 induces dramatic increase in phosphorylated PAK in spines (Penzes et al., 2003). They proposed that activation of EphB receptor induces recruitment of Rho-GEF kalirin to spines to activate Rac1 and PAK. One study demonstrated that induction of LTP by theta-burst stimulation drastically increases the number of spines containing phosphorylated PAK and its downstream target cofilin (Chen et al., 2007). Double immunostaining for post-synaptic density protein PSD95 revealed that spines with high pPAK and pCofilin levels had larger synapses. BDNF infusion of hippocampal slices caused rapid phosphorylation of both PAK and cofilin in the spines (Rex et al., 2007). This is interesting because brain-derived neurotropic factor (BDNF) has been shown to be a potent, positive modulator of theta burst induced LTP in adult hippocampus. These studies suggest that PAK-cofilin mediated actin regulation in the dendritic spines plays an important role in regulating LTP.
1.4.3. Downstream of PAK signaling

There are a number of PAK substrates identified that can account for the effect of PAK to modulate cytoskeletal dynamics and gene expression in neurophysiology. These substrates include, but are not limited to: LIM Kinases, regulatory myosin light chain (R-MLC) and myosin light chain kinases (MLCK), stathmin/Op18, filamin A, and MAP kinase. Their pathways are briefly summarized in Fig 6.

**Fig 6. PAK substrates implicated in cytoskeletal and gene regulation.** PAKs can influence transcription through MAPK activation. They can influence actin dynamics through LIMK, MLCK and Filamin A. Lastly, they can influence microtubule dynamics through Stathmin/Op18.
1.4.3.1. LIM Kinases

LIM Kinases (LIMK) -1 and –2 are serine kinases implicated to regulate actin cytoskeletal dynamics through their ability to phosphorylate members of the actin depolymerization factor (ADF) / cofilin. LIMKs are about 70 kDA in size, and have 2 N-terminal LIM domains followed by a PDZ domain and a C-terminal kinase domain. ADF/cofilin, the only known substrate of LIMK, is phosphorylated at Ser3 by LIMK. Phosphorylated cofilin loses its ability to bind effectively to F-actin, and thus its ability to catalyze F-actin depolymerization and severing is inhibited. These processes are essential for proper actin dynamics and a variety of cellular processes requiring rapid actin rearrangement (Bamburg, 1999; Stanyon and Bernard, 1999). LIMK are abundant in neural tissues, and deletion of LIMK gene has been linked to Williams syndrome, a human visuospatial cognitive disorder associated with mild mental retardation.

Edwards and collaborators identified PAK1 as the upstream mediator of LIMK (Edwards et al., 1999). Active PAK1 binds to LIMK-1 and phosphorylates it at Thr508, resulting in ten-fold increase in LIMK-1 activity towards cofilin in vitro. Expression of PAK autoinhibitory domain in vivo blocked LIMK-1’s ability to modulate cofilin activity. Expression of dominant negative LIMK-1 blocked the ability of PAK1 to stimulate dorsal ruffle formation, an actin-mediated process, in BHK cells. These data suggest that LIMK-1 is an important downstream mediator of PAK activity.

1.4.3.2. R-MLC and MLCK

Myosins are actin-activated Mg-ATPases that utilize the energy from ATP hydrolysis to power actin displacement along myosin filaments, resulting in either
contraction or tension. Typical myosins that form filaments (i.e. myosin II) consist of 2 heavy chain (MHC) which are actin-activated ATPases, and 2 light chains, one essential and one regulatory (R-MLC). Phosphorylation of R-MLC at Ser-19 by myosin light chain kinase (MLCK) is an important physiological means to modulate myosin contractility. Previous studies have shown that PAK1 and/or PAK2 are able to directly phosphorylate R-MLC at Ser-19, leading to increased contractility (Chew et al., 1998; Ramos et al., 1997; Zeng et al., 2000). PAK1 can also modulate R-MLC function through inhibition of MLCK (Sanders et al., 1999). Phosphorylation of MLCK by PAK1 decreases MLCK activity towards R-MLC by over 50%. Expression of active PAK inhibits R-MLC phosphorylation at Ser-19 and cell spreading. PAK2 is also shown to exhibit similar effect. Constitutively active PAK2 phosphorylates MLCK at Ser439 and Ser991 (Goeckeler et al., 2000). Interestingly, binding of calmodulin to MLCK inhibited phosphorylation by PAK2 at Ser991. Existing data indicates that regulation of myosin by PAK may be complex and involves interplay between PAK and other physiological regulators (e.g. Ca$^{2+}$/calmodulin).

Regulation of multiple myosins is likely an important part of PAK-mediated cytoskeletal signaling. PAK1 phosphorylates the motor domain of the unconventional myosin I (Wu et al., 1996), positively regulating its ability to promote actin assembly via Arp2/3 complex, a seven unit protein that plays a major role in the regulation of actin cytoskeleton. PAK3 phosphorylates myosin VI on Ser406 in vitro to significantly enhance its actin-translocating activity (Yoshimura et al., 2001). Myosin VI is unique from other myosin in that it translates along actin filament towards pointed ends (other
myosin towards barbed ends). It is implicated in hair cell development and movement of stercocilia in the inner ear.

1.4.3.3. Stathmin/Op18

In addition to its regulation of the actin cytoskeleton, more recently several lines of evidence have implicated PAKs as possible regulators of microtubule dynamics. Its role in microtubule regulation is proposed to be important in coordinating events of cell division. For instance, injection of active PAK2 into frog embryo inhibited blastomere division (Neudauer et al., 1998), and introduction of Xenopus PAK into oocytes blocked progesterone-induced maturation (Faure et al., 1997). Shk1, PAK homolog in S. pombe, is necessary for normal interphase and mitotic microtubule organization. Shk localizes to interphase microtubules and mitotic spindles (Qyang et al., 2002). A possible link between PAK and microtubule dynamics is the protein stathmin/Op18. Op18 binds αβ tubulin dimers to inhibit tubulin polymerization and promote microtubule catastrophe. PAK1 phosphorylates Op18 at Ser16 to inhibit microtubule destablizing function of Op18, both in vitro and in vivo in anaphase cells. Microtubule plays a key role in formation and maintenance of the leading edge of the cell. PAK activation promotes stabilization and growth of microtubule into the leading edge.

1.4.3.4. Filamin A

Filamin A is a 280kDa actin-binding protein that induces high-angle cross-linking of actin filaments (Stossel et al., 2001). Cells lacking filamin exhibit reduced cytoplasmic elasticity and unstable membrane. These cells are unable to perform effective
locomotion. It is proposed that filamin A and Arp2/3 complex act together to promote actin structure at the leading edge of cells. Filamin A was identified as PAK1 binding partner in a two-hybrid screen, and their interaction was confirmed in vivo by co-immunoprecipitation (Vadlamudi et al., 2002). Filamin A binds to PAK1 at amino acids 52-132 in such a way that binding relieves autoinhibition and, thus, leads to PAK1 activation. PAK1, in turn, binds to and phosphorylates Ser2152 in filamin A. Evidence indicate that filamin A is required for PAK1-mediated signaling to induce membrane ruffles. Membrane ruffles induced by PAK1-activating stimuli (e.g. heregulin, sphingosine) or by expression of constitutively active PAK was nearly completely blocked in filamin A deficient M2 cell lines, but can be rescued by restoring filamen A expression (Vadlamudi et al., 2002).

1.4.3.5. MAP kinase

PAK’s ability to regulate MAP kinase was first proposed because the yeast PAK homolog, Ste20, was found to act as a MAP kinase kinase kinase kinase (MEKK kinase) (Dan et al., 2001). PAKs 1, 2 and 3 have subsequently been shown to stimulate activation of JNK and p38 MAP kinases, while the dominant negative versions of PAK blocked JNK and p38 MAP kinase activation in response to upstream stimuli (Zhang et al., 1995). However, activation of JNK and p38 MAP kinase only occurs in certain cell types, and the exact mechanism remains unclear. PAK has also been shown to stimulate ERK MAP kinase. An early report showed that PAK1 activation by membrane targeting led to stimulation of p38, JNK and ERK1 (Lu et al., 1997).
1.5. Clinical relevance

Mutations in PAKs have been associated with tumor progression and cognitive and mental disorders. In somatic cell types, PAKs are well-known regulators of cytoskeletal remodelling and cell motility. More recently, they have also been shown to promote tumor formation and cell invasiveness by promoting cell proliferation, regulating apoptosis and accelerating mitotic abnormalities. For instance, increased PAK1 expression has been associated with breast and colorectal carcinomas (Pavey et al., 2006). Pavey and collaborators demonstrated that siRNA-mediated PAK1 knockknown was able to decrease cancer cell invasiveness by five-fold in uveal melanoma. Furthermore, the same group showed that blocking RAS-induced activation of PAK1 almost completely suppressed the growth of sarcoma allografts in mice.

In the nervous system, mutations of PAKs and their downstream substrates have been associated with cognitive diseases (Fig 7).
Fig 7. Hypothesis for PAK signalling in synaptic plasticity and cognitive diseases.

PAK regulates actin cytoskeleton and gene expression through direct and indirect substrates such as LIMK, Raf Kinase or RSK2. These kinases along with PAK itself are specifically implicated in neural pathologies. The disorder associated with mutation of each PAK-related gene is highlighted in red. Figure modified from review by Kreis et al., 2009.
1.5.1. PAK3-associated XLMR

Nonsyndromic X-linked mental retardation (XLMR) syndromes are clinically homogeneous, but genetically heterogeneous disorders. Their genetic bases are largely unknown. Mutation in PAK3 gene has been identified as a causal gene for XLMR. Five mutations have been identified in PAK3 gene affecting different domains of the kinase. The first mutation was reported in 1998. It is a point mutation at R419X in the catalytic region, which led to a truncated protein with disrupted kinase function (Allen et al., 1998). Since then, four other mutations have been identified in mental retardation patients: R67C mutation in the p21 binding domain, A365E mutations located in the kinase domain, W446S mutation in the catalytic domain, and a splice mutation at 5’ end of the intron 6 (Bienvenu et al., 2000; Gedeon et al., 2003; Peippo et al., 2007; Rejeb et al., 2008). The latter results in a disruption of reading frame with a premature stop codon at position 128.

Biochemical analysis showed different mutations had different properties and led to different neurophysiological synaptic deficits. R419X and A365E mutations completely abrogated the kinase activity. The R67C mutation drastically decreased the binding of PAK3 to small GTPases Cdc42, which impaired its activation. Interestingly, the expression of these three mutant proteins in cultured hippocampal neurons affected spinogenesis differently. Both kinase-dead mutants slightly decreased the number of spines but profoundly altered spine morphology, whereas R67C mutant drastically decreased spine density (Kreis et al., 2007). PAK3 si-RNA in hippocampal neurons led to dendritic spine alteration, PAK3 knockout mice showed no alteration in the structure or density of spines but exhibited cognitive impairment in conditioned aversion test (Boda et
al., 2004; Meng et al., 2005). Both models, however, showed LTP deficits. One hypothesis to explain the difference between knockout and si-RNA knockdown is that during development PAK1 and PAK2 may compensate for the loss of PAK3 expression. Furthermore, PAK knockout differs from expression of mutated PAK in that mutant protein may act as dominant negative, resulting in non-specific inhibition of other PAK isoforms.

Clinical data indicate that PAK3 mutations result in a specific form of X-linked mental retardation with fairly constant clinical features. Neuropsychological tests in affected males and carrier females revealed a common profile of impaired spatial cognitive abilities and defects in attentional and executive functions. The affected males are present with: proportionally small head size or microcephaly, inarticulate speech, short attention span, anxiety, restlessness and aggression. EEG recording from four affected males and one carrier female showed similar posterior slow wave activity, but without epileptic discharges (Peippo et al., 2007).

1.5.2. Microcephaly

Microcephaly is clinically defined as a reduced occipital-frontal head circumference of less than –3 SD (given as a standard deviation score relative to age and sex matched controls) (Leviton et al., 2002). This measurement gives an approximation of brain size since it is the outward pressure exerted by the growing brain that causes enlargement of fetal and postnatal skull bones. Microcephaly in human is almost always associated with severe cognitive and behavioral deficits.
Microcephaly can be classified as primary or secondary. Primary microcephaly occurs prenatally, while secondary microcephaly occurs postnatally. Numerous studies have shown that the number of neurons (i.e. embryonic neurogenesis) is the main determinant of embryonic brain growth (Woods, 2004; Cox et al., 2006). Accordingly, genetic deletions of a number of genes involved in neuronal proliferation, differentiation, migration and/or survival all resulted in abnormal embryonic brain development and smaller brains at birth (Feng and Walsh, 2004; Depaepe et al., 2005). However, the mammalian brain continues to undergo rapid growth even after birth. Because neurogenesis is completed by then, it is assumed that the growth (i.e. increase in size and complexity) of individual neurons is responsible for postnatal brain enlargement. Surprisingly, experimental evidence to support this notion is very limited because few animal models exhibit clear and specific deficits in postnatal brain development. In addition, the relationship between reduced brain size and cognitive / behavioral deficits remain elusive because most the mouse models with primary microcephaly are either lethal or grossly altered in brain structure (Hirotsune et al., 1998; Cahana et al., 2001; Feng et al., 2004; Depaepe et al., 2005). Severely compromised viability in these models prevents any meaningful studies on the functional consequences of microcephaly at the cellular and behavioral level.
SECTION 2
RATIONALE & HYPOTHESIS

2.1 RATIONALE

PAKs are a family of serine/threonine proteins that can be directly activated by the active forms of Rho GTPases. There are six known mammalian PAKs, among which the group 1 PAKs (1, 2, 3) are best characterized for their biochemical and regulatory properties (Bokoch, 2003). Much evidence has suggested PAKs to play key roles in neural development and cognition. Previous studies have implicated PAKs to play a wide variety of roles in neurophysiology, including: proliferation, differentiation, migration, morphogenesis and synaptic plasticity (Kreis et al., 2009). However, most of these studies were either done in vitro, or in simpler non-mammalian systems (i.e. Drosophila, C. elegans and Xenopus) that possess fewer PAK isoforms. The studies in in vitro systems enabled relatively easy means to manipulate PAKs, and made extensive use of techniques such as siRNA, gene transfection (e.g. of dominant negative constructs), and pharmacological treatments.

Nonetheless, there were several major problems with the current literature on PAK. First, nearly all previous studies do not specifically address an individual isoform, but rather address group I PAKs in general. Dominant negative protein expression and siRNA may not be specific to one member of PAK, given that group I PAKs share a high level of sequency homology. While these PAKs may very well share common roles, evidence has shown that they do differ in their expression profiles, both spatially and temporally (Allen et al., 1998; Manser et al., 1995), suggesting each serve unique roles in
neurophysiology. In addition, neither dominant negative protein expression nor siRNA produces completely suppression of gene expression unlike knockout. Secondly, in vitro conditions may or may not be applicable to in vivo system. At the minimum, they do not allow investigators to address anatomical development or behavior, both of which can only be analyzed in animal models. Thirdly, there appears to be a huge discrepancy between the diverse and numerous roles of PAKs found in non-mammalian plus in vitro studies (Kreis et al., 2009) and the relatively few roles found in vivo in mice. The three existing studies that investigated role of PAKs in adult mammalian brain described only alterations in synaptic physiology, mainly changes in synaptic plasticity (Hayashi et al., 2004; Asrar et al., 2009; Meng et al., 2005). Yet, all three models were presented with either absence of or very mild cognitive deficits. This suggests that while PAKs do play key roles in modulation of adult synaptic function, these roles may not be paramount to cognition and behavior. Finally, there are gaps between established functions of Rho GTPases in neuronal morphogenesis and the established roles of their downstream substrates, especially PAKs. Both Cdc42 and Rac have been demonstrated to play critical roles in both axonal and dendritic growth and branching (Luo, 2000), yet no study have implicated any of their downstream substrates to tranduce signals required for those aspects of neuronal morphogenesis. PAKs have long been considered a candidate due to their ability to regulate actin cytoskeleton through LIMK and cofilin. However, only one in vitro study thus far has linked PAKs to dendritic Arborization (Hayashi et al., 2007). Although PAK homologs in Drosophila and C. elegans have been implicated in axonal guidance (Hing et al., 1999b; Ang et al., 2003; Lucanica et al., 2006), evidence from in
vivo mammalian studies regarding the roles of PAKs in axonal morphogenesis (outgrowth, guidance, branching) is completely absent.

After my initial finding of postnatal brain growth deficit in PAK 1&3 DK mice, I became interested in the cellular and molecular mechanisms underlying the post-natal brain development. Presumably, attainment of normal brain volume is critical for proper brain function. This is because pathological reduction in brain size, characteristic of a class of neurodevelopmental disorders called microcephaly, is almost always associated with severely impaired intellectual abilities and other neurological deficits. The number of neurons has been shown to be the primary determinants of pre-natal brain growth (Woods, 2004; Cox et al., 2006). Indeed, mutations in genes associated with neuronal proliferation, differentiation, migration and/or survival all led to primary microcephaly in both humans and animal models (Feng et al., 2004; Depaepe et al., 2005).

Of critical importance, the mammalian brain continues to undergo rapid growth even after birth, and because embryonic neurogenesis is already completed by then, it is assumed that the growth of individual neurons is the key driving force for post-natal brain enlargement. Surprisingly, experimental evidence to support this notion is very limited, because few animal models exhibit clear and specific deficits in post-natal brain development. In addition, the relationship between reduced brain size and cognitive/behavioral deficits remain elusive. This is because most microcephaly mice models are either lethal or suffer from severely impaired viability (Cahana et al., 2001; Hirotsune et al., 1998), thus making any meaningful study of the functional consequences of the microcephalic genes unfeasible.
2.2. Hypotheses

My hypotheses are as follow:

1. PAK1 and PAK3 share redundant, but crucial roles in neural development. Since the two proteins are both highly expressed in the mammalian CNS, developmental compensation by one for the loss of the other may mask the functional deficits in PAK1 knockout or PAK3 knockout mice.

2. Due to the postnatal nature of microcephaly in PAK1&3 DK mice, I hypothesize that it is the individual neuronal morphology, rather than the neuronal organization or number, that constitutes the main cause of the smaller anatomy.

3. PAK1&3 DK neurons fail to undergo normal morphogenesis, and consequentially develop simpler or shorter processes. This leads to a reduction in the number of synapses, and corresponding changes in synaptic properties, both structurally and functionally. So the abnormal synaptic properties in turn form the underlying basis for cognitive and behavioral deficits.

4. PAKs mediate neuronal morphogenesis through regulation of actin dynamics, probably through LIMK-cofilin pathway or MLCK pathway.
2.3. Objectives

My objectives are as follow:

A. To decipher the roles of PAKs *in vivo* in mammalian CNS development

B. Specifically address the brain dominant group I PAK isoforms, PAK1 and PAK3

C. To understand the mechanism through which loss of PAK function leads to cognitive behavioral deficits associated with XLMR

D. To further our understanding of the cellular and molecular causes of secondary microcephaly

To address objectives A and B, I took a genetic approach by generating PAK1&3 DK mice. These global knockout mice lack expression of both PAK1 and PAK3 throughout all developmental stages and in all tissues. To address the objectives C and D, I first investigated the anatomical changes using magnetic resonance imaging (MRI) (with help from Dr. Mark Henkelman), immunohistochemistry and various histological methods (e.g. Nissl staining, Golgi impregnation, stereology). Next, I employed primary hippocampal neuron culture and immunocytochemistry to study the different aspects and stages of neuronal morphogenesis in greater detail. To assess the functional consequence at the synaptic level, I conducted fEPSP recordings of CA3-CA1 synapse and whole cell recordings of CA1 neurons (with help from my colleague Zikai Zhou). Finally, I used an array of behavioral tests, including Morris water maze and fear memory acquisition, to assess cognition and behavior.
CHAPTER 3
MATERIALS & METHODS

3.1. Animal subjects

3.1.1 Generation of PAK1&3 double knockout mice

The generation of PAK1 knockout mice (Asrar et al., 2009) and PAK3 knockout mice (Meng et al., 2005) were previously described. PAK1 heterozygous line was crossbred with PAK3 heterozygous line to generate PAK1&3 DK mice. For all experiments, heterozygous littermates (PAK1+/k PAK3+/+ or PAK1+/k PAK3 +/-) were used as wild-type controls. All experiments were gender and age-matched. Double mutant mice had normal viability and life span. No home-cage behavioral abnormalities or consistent differences in body weight were observed in the DK mice. While the female DK mice were infertile, the male DK mice were fertile (albeit reduced fertility).

3.1.2 Genotyping

The DNA was first isolated from the tail samples. The tip of tail (~0.5 cm) was biopsied and digested in 0.5 ml of Proteinase K in a lysis buffer solution (20 mg/ml) (MP Biomedicals, LLC) overnight in 55 C incubator. Afterwards, the samples were chilled on ice and 200 µl of 5M NaCl was added to each tube. Then, 600 µl Chloroform was added to the aqueous sample, enabling biphasic mixture to form. The samples were gently mixed by inverting the tubes 5 times, followed by 10 min centrifugation at 10,000 rpm at 4 C. This step allows for phase separation, with DNA retained in the top aqueous phase and cell debris trapped in the interphase (a compacted band between the top aqueous and the bottom organic phases). 400 µl of the aqueous phase is carefully transferred to a clean
tube, and equal volume of 100% ethanol is added. The tubes inverted several times to allow mixture, then centrifuged at 14,000 rpm for 5 min at 4°C to pellet DNA. Since DNAs are insoluble in ethanol, they aggregate to form a white pellet. 100% ethanol is discarded, and the DNA pellet is washed with 200 µl of 70% ethanol. After centrifugation, ethanol is carefully removed with p200 pipette and the pellet is left to dry in room temperature for 30 min. Afterwards, 100 µl of water was added to redissolve the pellet.

The genotype of DNA sample was determined by allele-specific amplification by means of Polymerase Chain Reaction (PCR). The primers used for PAK1 and PAK3 (ACGT Corp.) are as follow:

PAK1+/+   Primer 1: 5’-CTGAGGAAGAGACTGAGAG-3’
Primer 2: 5’-AGGCAGAGGTGTGGAGCCGTG-3’
PAK1-/−  Primer 1: 5’-CTGAGGAAGAGACTGAGAG-3’
Primer 2: 5’-GGGGGGAACTTCCTGACTAGG-3’
PAK3+/+   Primer 1: 5’-GAGTCAATTGCTTCACCAGCTG-3’
Primer 2: 5’-CTTCTGTGATAGTCATGACATAC-3’
PAK3-/−  Primer 1: 5’-GAGTCAATTGCTTCACCAGCTG-3’
Primer 2 (GGGGGAACCTTCCTGACTAGG)

1.5 µl of the redissolved DNA was added to 13.5 µl of PCR Master Mix (containing 0.4 µM primer1, 0.4 µM primer 2, 2.67x10⁻³ u/µl Taq polymerase, 133 µM dNTPs, 1x PCR buffer with HCl, and 1.67 mM MgCl²). PCR amplification was
conducted on an MJ Research thermocycler using a program of 35 cycles of denaturation at 94 C for 45 sec, annealing at 60 C for 60 sec, and extending at 72 C for 50 sec. The PCR products (plus 1x loading buffer) were loaded onto 1.2% agarose gel containing ethidium bromide and subjected to electrophoresis at 120 mV for 45 min. The gel was then visualized under UV to detect DNA bands. Sizes of wildtype and knockout PCR products for PAK1 are 350 bps and 250 bps, respectively. Sizes of wildtype and knockout PCR products for PAK3 are 450 bps and 300 bps, respectively.

3.2. Electrophysiology

3.2.1. Preparation of acute hippocampal slices

The preparation of acute brain slices has been previously described (Asrar, 2009). Mice were sacrificed via spinal dislocation. The brain was quickly removed using a curved spatula, and chilled by submerged for 1~2min in ice-cold ACSF (artificial cerebrospinal fluid) containing (in mM): 120 NaCl, 2.5 KCl, 1.3 MgSO\(_4\), 1.0 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 2.5 CaCl\(_2\) and 11 D-glucose, pre-saturated with 95% O\(_2\) and 5% CO\(_2\). 400µm thick acute hippocampal slices were prepared using a vibratome (Vibratome 1000 Plus – Tissue Sectioning System, Intracel) in the presence of of ice-cold perfused ASCF. The slices were then allowed to recover in submerged holding chamber perfused with carbogen (95% O\(_2\), 5% CO\(_2\)) for at least 2 hours at room temperature.

3.2.2. Field excitatory post-synaptic potential (fEPSP)

fEPSP recording entails stimulating of a group of neuron extracellularly and recording the evoked population response. This response comprises of superimposed
post-synaptic potentials from dendrites (and if recorded too close to cell body layer, action potentials from axon or cell body referred to as population spikes). fEPSP is often the technique of choice for studying synaptic plasticity (i.e. LTP and LTD). I used the rising slope of fEPSP to quantify its size, since it is most representative of AMPA-induced excitatory glutamate current. There are two separate peaks that precede fEPSP. The first is called the stimulus artifact, which is caused by the current applied from the stimulating electrode. The second is the prefiber volley, which immediately precedes and sometimes partially overlaps fEPSP onset. The prefiber volley slope reflects the density and excitability of axon fibers stimulated. However, it also varies depending on the electrode placement (i.e. increased distance between stimulating and recording electrode decreases prefiber volley). Fig 8 shows an illustration of typical fEPSP traces with and without interference from population spike.
Fig 8. CA3-CA1 fEPSP. (A) shows the relative positions of stimulating and recording electrode on the hippocampal slice on the left, and a typical fEPSP trace on the left. The prefiber volley is labelled, with stimulus artifact preceding it and fEPSP following it. (B) shows fEPSP traces with and without interference from population spikes, action potentials generated at the cell body. Modified from (Bortolotto et al., 2001).
3.2.3. fEPSP protocol

Following recovery, a single sagittal slice with distinct hippocampus is placed on a piece of lens paper and transferred to submerge-style recording chamber perfused with 95% O₂ 5% CO₂ ASCF (perfusion rate 2~2.5 ml/min). The glass recording pipette (3 MΩ) was filled with ACSF. Field EPSP recordings were conducted at CA3-CA1 Schaffer Collateral pathway in the hippocampus (Meng et al., 2002, 2003b). Synaptic responses were evoked by bipolar tungsten electrodes placed 200-400 μm from cells in the CA1 area (see Fig 8). fEPSPs were measured by taking the slope of the rising phase between 5% and 60% of the peak response. All data acquisition and analysis were done using pClamp 9 software (Molecular Devices, Union City, CA).

For all experiments, a stable baseline was obtained for 20 min. For basal synaptic response experiments (input-output curves and paired-pulse facilitation) and LTP experiments, mature mice of 8 weeks to 3 months of age were used. Early-phase LTP was induced by high-frequency stimulation (HFS) of 2 trains of 100Hz at 10 sec intervals with each train lasting 1 s, while late-phase LTP was induced by 4 trains of 100 Hz at 20 sec intervals with each train lasting 1 s. For paired-pulse facilitation, the ratio of the slope of second response to the slope of first response was calculated for each interpulse interval. For low frequency stimulation-induced (1 Hz, over 15 min period) LTD experiments, 13-15 days old mice were used. Finally, for DHPG-induced (50 μM, 5 min) LTD, 2-3 weeks old mice were used.
In all fEPSP figures, n represents the number of hippocampal slices, and up to two slices were used from each animal for any particular experiment. All data was statistically evaluated by Student’s t-test. Error bars represented SEM.

3.3. Primary hippocampal neuron culture

Cultured hippocampal neurons were prepared and maintained according to the procedure recommended for Neurobasal-A medium (Invitrogen, San Diego, CA). Prior to the dissection, a round glass coverslip was placed in each well of 24-well culture plates. The wells were then coated with poly-D lysine for 2 – 24 hrs in 37°C. After coating, the poly-D lysine was removed and the wells were washed thoroughly (6x 5min) with 1x PBS. The culture medium was prepared by supplementing 200 mL of plain Neurobasal-A medium (GIBCO) with 4 mL B27 (GIBCO) and 25 µM Glutamax.

Postnatal day 1 (P1) mice were sterilized using 70% ethanol and then decapitated. The tails were saved for genotyping. The brain was removed and placed in ice-cold PBS containing 1x anti-biotics. The hippocampus was isolated under a light microscope using a pair of sharp forceps and a thin spatula. Using a fine scapel, a single hippocampus was diced into 5-6 pieces. It was then transferred using forceps into 500µL pre-warmed papain solution (1mg/mL Neurobasal-A medium) and incubated at 37°C for 25 minutes. Afterwards, it was triturated gently after 5 min (10 times), 15 min (10 times), and 25 min (20 times). Before the last trituration, a minute amount of DNAase was added to prevent cell clumping. The undigested debris was allowed to settle for about 30 sec, and the supernatant was transferred into a new tube and centrifuged at 700 rpm at room temperature for 5 min to pellet cells. The supernatant was removed, and the pellet
containing mostly neurons was resuspended in 6 mL of culture medium. The dissociated neurons were then plated onto poly-D-lysine-coated glass coverslips at the density of approximately 60,000 cells/ml. For 24-well plate, 0.5 mL was added to each well. 2 – 4 hours after the initial plating, the old medium is replaced with fresh medium. From this point on, half the medium was replaced weekly.

3.3.1. Immunocytochemistry and imaging

For immunostaining, cells were fixed using ice cold 4% paraformaldehyde in PBS at different developmental stages. After 20 min fixation, the cells were washed 3 times in PBS. They were then permeabilized with 0.1% Triton X-100/PBS for 30 minutes, and incubated in 5% fetal bovine serum (FBS) for 1 hour to block non-specific antibody binding. Next, the cells were incubated in appropriate primary antibody solution overnight at 4 C. For co-immunostaining, a cocktail mixture of two or more primary antibodies from different sources applied. The next day, the primary antibody solution was removed and the cells were washed 3 times 5 min in PBS. They were then incubated for 1 hr in fluorophore-tagged secondary antibody solution. This incubation was done in the dark to prevent photobleaching of the fluorophore. For F-actin labeling, after blocking with FBS, cells were stained for 1 hr with 1 ug/ml tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma, St. Louis, MO). A thorough final wash in PBS (6x 10 min) was done following the secondary antibody incubation. Finally, the coverslips picked up using sharp forceps and mounted onto glass microscope slide using DAKO fluorescence mounting medium. The slides were allowed to dry for at least 2 hrs before imaging.
Images were collected using Zeiss LSM 510 confocal microscope (63x objective, for dendrites and synapses), or on Nikon TE2000 histology microscope equipped for fluorescence imaging (20x objective, for axons). During image acquisition, identical laser power, gain and contrast settings were used for all immunostained samples within an experiment. For each genotype, a total of 30 neurons from three independent hippocampal cultures were used for quantification. All measurements were quantified using either Zeiss LSM Image Brower or ImageJ software. Primary antibodies used in immunocytochemistry were against phospho-neurofilament (Sternberg Monoclonal Inc.), MAP2 (Upstate Biotechnology), synaptophysin (Cell Signaling), GluR2/3 (Chemicon), coflin (Cytoskeleton) and GFAP (DAKO).

3.4. Histology methods

Histology refers to the microscopic study of tissue. The visualization of the brain’s cytoarchitecture is an important complement to the studies of function and gross anatomy. Histology also refers to the techniques used to prepare tissue for microscopic studies. This includes staining tissues for light and electron microscopy, tracing fiber tracts, and mapping the distribution of certain gene expression to name a few. For the study of PAK1&3 DK brains, I prepared sections of brain tissue for viewing under light, electron and fluorescence microscope. In order to be visualized, the tissue must first be fixed and stained. Fixing prevents enzymatic degradation (and other post-mortem) of the tissue. The most common method of fixing is via perfusion. The animal is sacrificed and perfused with a fixative – that is, the blood is drained from the body and a fixation solution (e.g. paraformaldehyde) is pumped into the vascular system. Removing the
blood improves the results of staining, and the fixative preserves and hardens the jelly-like brain so that it can be smoothly sectioned. The sections are cut sufficiently thin (usually 5-20 µm) to enable viewing under microscope, and are stained to bring out cellular details that would otherwise be impossible to observe.

3.4.1. Nissl method

The Nissl substance, described by Franz Nissl more than a hundred years ago, is found abundantly in neuronal bodies. It is composed of ribosomal RNA associated with rough endoplasmic reticulum in neurons. It is especially enriched in neuronal cell body, reflecting the usually high protein synthesis capacity of neurons. The Nissl method refers to staining of the cell body, and in particular endoplasmic reticulum. This can be achieved using various basic dyes (e.g. cresyl violet, methylene blue, safranin-O and toluidine blue-O) to stain the negatively charged RNA. In injured or regenerating neurons, the Nissl substance breaks apart and redistributes around the periphery of the cell body (a histological change known as chromatolysis). In this way, Nissl stain can be used as markers for physiological state of neurons, particularly in dorsal root ganglion cells and primary motor neurons.

In this study, I used Cresyl Volet to stain the cell bodies of neurons (and nucleus of glia) in the brain. Cresyl violet is an organic compound (C₉H₁₈ClN₃O). The fresh brain was cut into 20 µm sections (see immunohistochemistry method 2, section 4.4.4) using a cryostat, and adhered onto microscope slide. The sections were fixed first, then washed (3x 2min) in distilled water (dH₂O). They were then incubated in warm Cresyl violet solution for 2 min, followed by wash in dH₂O. The sections were often
overstained, in which case the excess dye was stripped from the sections by immersing
the sections in 70% ethanol containing hydrochloric acid (2 drops per 500 ml). The
staining and de-staining process is repeated until the staining appeared optimal under a
light microscope (i.e. only cell bodies stained). They were next washed in water 3x 2 min
to remove residual dye and acid. Afterwards, the sections were sequentially dehydrated
via immersion in 95% ethanol, 100% ethanol and a second 100% ethanol, for 2 min each.
Finally, the sections were cleared in xylene for 2 min, and coverslipped using Permount
mounting medium (Daigger) and left to dry overnight. The following day, the images
were taken using Nikon TE2000 histology microscope.

3.4.1.1. Stereology: optical fractionator

To estimate the total cell count in the cerebral cortex, perfusion fixed brains were
crystated into 40 µm sections from the anterior most to the posterior most end of the
cerebral cortex. Sections were collected every 280 µm (i.e. one in every seven sections).
Four WT brains and four DK brains were used, yielding an average of 23.5 ± 0.29 and
21.0 ± 0.41 sections per brain respectively. To estimate the total cortical neurons,
sections were floating stained with NeuroTrace 500/525 green fluorescent Nissl stain
(Invitrogen). The stained neuronal cell bodies were counted using the standard optical
fractionator technique (Harding et al., 1994), on the Nikon TE2000 histology microscope.
150 µm by 150 µm sample frames were used for counting. Six randomly selected frames
were counted for each sample: two from layer I~III, two from layer IV/V, and two from
layer VI. Based on the cortical volume (calculated as surface area multiplied by 40 µm
section thickness) and neuronal density, the number of cortical neurons for each given
section was then estimated. The sum of neuronal count in all sections throughout the forebrain (multiplied by a correction factor of seven) provided an estimate of the total neurons in one cortical hemisphere.

Similar approach was used to estimate the number of hippocampal glia. A glia was identified as co-localization of DAPI (nucleus) with GFAP (glial processes). The total glia in the hippocampus was estimated based on glial densities in the CA1 region and its occupied volume.

3.4.2. Golgi-Cux Impregnation

In neuroscience, perhaps the most familiar technique is the Golgi staining. Golgi’s method is a nervous tissue staining technique discovered by the reknowned Italian physician and scientist Camillo Golgi. Historically, it was famously used by Santiago Ramon y Cajal to prove the neuron doctrine, which states that the nervous system is made up of discrete individual cells. The neurons in the brain are densely packed and little information on their structure can be obtained if all the cells are stained. Golgi’s method randomly stains only 1 – 3% of total neurons in the tissue. Staining of those neurons enables detailed visualization of its dendritic morphology and even spines. Golgi’s staining is achieved by impregnating fixed nervous tissue with potassium dichromate and silver nitrate, causing selected neurons to be filled by microcrystallization of silver chromate. The exact mechanism by which this occurs is still largely unknown.

In my study, I utilized a rapid Golgi stain kit. The staining was performed according to the specifications of the manufacturer (FD NeuroTechnologies, Germantown, MD) with minor modifications. Adult (2–4 months old) PAK1&3 DK or
wildtype mice were sacrificed by cervical dislocation and their brains were quickly washed in 1x PBS. Briefly, whole brains were treated for silver impregnation for 1 week, cryoprotected for 48 hours, and sectioned at 100 µm (for spine analysis) and 200 µm (for dendrite analysis) on a cryostat. After sectioning and mounting on gelatin-coated slides, sections were developed, clarified, and mounted in resinous medium (Permount Mounting Medium). Whole section images were captured using Zeiss Mirax Scan. Individual neurons were visualized using Nikon TE2000 histology microscope. Camera lucida drawing was constructed by tracing the entire dendritic tree of a single impregnated neuron, using a series of images taken at a range of focal planes containing its dendrites. The tracing was done on Photoshop 7.0 (Adobe Systems Incorporated). Dendritic spines were analyzed using Volocity 4.4 software (PerkinElmer).

3.4.3. Immunohistochemistry (IHC)

For IHC, two different methods were used. In the first method, the mice were perfused transcardially with 4% paraformaldehyde (PFA), then the brains were rapidly isolated and incubated overnight in 4% PFA. After fixation, the brains were cryoprotected in 30% sucrose solution until they submerged (about 36 hrs). They were then snap frozen in isopentane cooled to −80°C on dry ice, and cryostated into 20 µm (immunocytochemistry) or 40 µm (FluoroNissl staining and stereology) sections. Cryostat sections were transferred to PBS and rehydrated. Rehydrated brain sections were either stained right away, or kept in 4°C for later use (for no more than a week). Immunostaining was carried out in 24-well culture plates. Sections were first permeabilized using 2% Triton X-100 in PBS for 30 min. They were then incubated in
10% FBS for 1 hour to block non-specific antibody binding. After blocking, sections were placed in primary antibody solution overnight at 4 C, followed by 3x 5 min washes in PBS. They were then incubated in fluorescence-conjugated secondary antibody solution for 1 hr while protected from light. Afterwards, slices were washed thoroughly in PBS (6x 10min) and transferred onto a microscope slide and allowed to dried for 10 min in room temperature in dark. Finally, the coverslips were mounted onto section using DAKO fluorescence mounting medium, and the mounted sections were left to dry for at least 2 hours before imaging.

The second method I used for immunohistochemistry is as follows: The fresh brains were rapidly isolated and immediately snap frozen isopentane chilled to –80 C (after brief wash in chilled PBS to remove surface blood). The frozen fresh brain is then cryostated into 20 µm sections, which were adhered directly onto coated microscope slide and allowed to dry at room temperature for 1 hr. The slides containing the fresh brain sections were either stored at –80 C or used immediately for staining. For staining, the area of slide containing the specimen was encircled using a wax pen. Subsequently, the staining was done direct within the wax circle. The brain slices are fixed using 4% paraformaldehyde for 30 min. The remainder of the procedure (blocking, primary and secondary antibody incubation, mounting) was identical to that described in the first method. Prior to coverslip mounting, the wax was carefully removed with a sharp razor.

Lower magnification (whole section) images were collected on Zeiss Mirax Scan and Leica Fluorescence Stereomicroscope, and higher magnification (region or cell specific) images were collected on Nikon TE2000 histology microscope and Zeiss confocal microscope. The confocal z-stack imaging allowed reconstruction of GFAP-
stained glial cells. Primary antibodies against cortical layer specific proteins used for immunohistochemistry were anti-Tbr1 (Millipore) and anti-Cux1 (Santa Cruz). Myelinated tracts were stained using FluroMyelin Green (Invitrogen) fluorescent myelin stain, based on protocol suggested by the manufacturer.

3.5. Western blot protein assay and primary antibodies

Western blot is a technique used to detect the level of a specific protein in a given sample of tissue homogenate or extract. Briefly, it uses gel electrophoresis to separate denatured proteins by length of the polypeptide. These segregated proteins are transferred onto a membrane (e.g. nitrocellulose) where they are probed using antibodies specific to a target protein (e.g. cofilin) or a specific post-translationally modified form of the target protein (e.g. phosphorylated cofilin). This method enables easy quantification of the overall protein level, but does not provide any information on the localization or distribution of the protein within the tissue.

3.5.1. Whole brain protein lysate

The whole brain protein lysate was utilized for Western blotting. The fresh brain was quickly homogenized in ice-cold cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, 1 mM PMSF, and 0.5% protease inhibitor cocktail). The homogenate was incubated on ice for 1 hr, then centrifuged at 125,000 rpm at 4 C to pellet undigested tissue debris. Supernatants containing proteins were then transferred to new tubes, to which gel loading buffer and
1mM dithiothreitol (DDT) were added to make the loading samples. The loading buffer contains 30% glyceral, which weighs the sample down in the well, and 0.25% bromophenol blue. Bromophenol blue, a negatively charged dye, is used as a colored marker to monitor the progress of electrophoresis. DDT is a strong reducing agent that helps to break down the tertiary structure of the proteins by reducing the disulfide bonds. Before loading, the sample was thoroughly mixed and boiled for 5 min to fully denature the protein.

3.5.2. SDS-PAGE and transfer

The proteins were then separated using gel electrophoresis. The speed at which the proteins migrate from anode to cathode depends on the molecular weight and charge; the smaller the size and more negative the protein, the further it migrates. I employed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels contained 10% polyacrylamide. SDS-PAGE maintains polypeptide in a denatured state and SDS, an anionic surfactant, surrounds the protein with negative charges. Together, this enabled separation of proteins solely based on molecular weight. The samples are loaded into wells in duplicates, with the first well been reserved for a colored molecular marker (New England Biomed). The electrophoresis was conducted at 20 mA per gel.

In order to make proteins accessible to antibody detection, they were transferred from the gel onto a membrane made of nitrocellulose in a process called blotting. The membrane was placed on top of the gel, and the duo was sandwiched with filter papers. The entire stack was placed in a transfer buffer solution, and the transfer was done on ice at 120 mV for 1.5 ~ 2 hrs depending on the size of protein of interest (longer duration for
larger proteins). The success of transfer procedure was sometimes evaluated through use of dyes, such as Ponceau S.

3.5.3. Immunoblotting and detection

After transfer, the membrane was washed briefly in Tris Buffered Saline with Tween 20 (TBST). Any non-specific secondary antibody binding was blocked using 10% non-fat dry milk (in TBST) for 1 hr. This step is essential for reducing the background of the final Western blot product. Next, the blocked membrane was washed 3 times 5 min in TBST, then incubated overnight in 4°C in 1:1000 dilution of the primary antibody raised against the protein of interest. The primary antibody recognizes and binds directly to the protein of interest. The following day, primary antibody solution was removed (saved for reused, up to 3 times) and the membrane was washed 3 times 5 min before adding 1:5000 dilution of horse radish peroxidase-conjugated secondary antibody from the appropriate source (e.g. donkey anti-rabbit, if primary is raised in rabbit), and incubating for 2 hrs at room temperature. The secondary antibody binds to the primary antibody and is itself coupled to chemiluminescence signaling. Following secondary antibody incubation, the membrane was washed 3x 10 min to thoroughly remove any unbound antibodies. The membrane was then incubated in detection reagent mixture (Denville Scientific Inc., USA) for one minute to trigger chemiluminescence. The excess detection solution was immediately drained off and the membrane was visualized using FluorChem 2 (Alpha Innotech) acquisition system. The band intensity / size was quantified using SpotDenso analysis which measures the optical density against a nearby background.
3.5.4. Primary antibodies used

The primary antibodies used for this study were specific for: MAP2 (Upstate Biotechnology), PAK3 (Santa Cruz Biotechnology), PAK3 (Santa Cruz Biotechnology), Cofilin (Cytoskeleton), CREB (06-863; Upstate Biotechnology), and Rho-associated kinase 2 (ROCK2; santa Cruz Biotechnology), MAPK p44/42 (Cell Signaling Technology), and MLCK (Santa Cruz Technology). Primary antibodies used to detect phosphorylated forms of proteins of interests included those specific for: phospho-Cofilin (Santa Cruz, CA), phospho-CREB (s.c.-7978; Santa Cruz Biotechnology), phospho-P44/42 (Cell Signaling Technology), and phospho-MLCK (Santa Cruz Biotechnology).

3.6. Behavioral tests

Only male mice between ages of 2~4 months old were used for behavioral tests. All behavioral experiments were performed during the light phase of the cycle (between 9:00 A.M. and 6:00 P.M.) in accordance with institutional and Canadian Council on Animal Care guidelines. Before behavioral experiments commenced, all mice were handled 3 times daily for 2 days.

3.6.1. Open field test

TruScan2.0 (Coulbourn Instrument, Whitehall, PA) was used to acquire and analyze open field behavior. The setup consisted of a E63-10 Mouse Arena (25.40 cm wide by 25.40 cm deep by 40.64 cm high plexiglass cage with grid floor). The arena was enclosed by sensor rings, which emitted photo beams to track the movement of the mouse. The beams were spaced 1.52 cm apart providing a 0.76 cm spatial resolution. The
mice were given 5 minutes to explore the arena, during which raw data were recorded for analysis.

3.6.2. Fear memory learning

For fear memory training, an aversive unconditioned stimulus (US), an electric shock, was paired with conditioned stimuli (CS), cue (tone) and context (experimental chamber) to elicit a freezing response, a reliable measure of fear in rodents. On the first day, the pre-handled animals were placed in the testing chamber (22.5 cm wide x 32.5 cm long x 33.3 cm high Plexiglass cage with a grid floor, encased in an isolation cubicle) and were allowed to explore for 5 min. For fear memory training, mice were allowed to explore the cage for the first 120 s, after which a 30 s white noise tone was delivered followed by a 2 s, 0.7mA footshock. Three such US-CS pairs were delivered in succession, with 30 s intervals in between. After training, the mice were tested 1~2 hours later for short-term fear memory and 24 hours later for long-term fear memory. For contextual fear memory test, mice were returned to the same chambers in which they were trained and their freezing response was analyzed over the course of 240 s. For cued fear memory test, the mice were placed in a separate chamber with different context, allowed to explore for 120 s, and then exposed to 120 s of white noise tone. All data are analyzed using FreezeView2 (Coulbourn Instrument, Whitehall, PA). Freezing was defined as Motion Index of 25 or less.
3.6.3. Morris water maze

The water maze apparatus and general methodology were described in detail previously (Meng et al., 2005). Briefly, it consisted of a circular pool (2.2 m in diameter, 1 m in height) made of black plastic. The pool was filled to a depth of 70 cm with water (room temperature) that was made opaque by the addition of a nontoxic white paint. The pool was enclosed by white curtains, on which spatial cues (solid shapes) were attached. An escape platform (13 cm in diameter) made of transparent plastic with grooved surface was submerged 1.0 cm under the water level, and placed mid-point between the center of the pool and the wall. The swim path of a mouse was recorded using a camera connected to a video-tracking system (Noldus Information Technology) and a personal computer running the EthoVision software (Noldus). For the visible platform test, a blue pole 15 cm in height was erected in the center of hidden platform, in such a way that the mice can identify the location of the platform by the sight of the pole.

For both the visible platform and the hidden platform tests, mice were lowered into the water facing the wall of the pool, and given 1 min to locate the platform. After locating and climbing onto the platform, they were allowed 15 s to observe the surrounding spatial cues. If a mouse failed to find the platform within 1 min, it was guided by the experimenter to the platform, where it was also given 15 s to observe the surrounding. Visible platform training was given over the course of 2 days, with two 1 min trials each day. Hidden platform training was given over the course of 5 days, with three 1 min trials each day. All mice were tested in squads, rotating between WT and mutant, with inter-trial interval of 30 ± 10 min. Swim distance and latency to reach the platform were used as indicators of spatial memory acquisition. To assess spatial
memory retention, probe trials were given after training. During probe trials, the platform was removed from the pool. The probe trials were given 1 hour after the last training session to assess short-term memory, then 24 hours afterwards to assess long-term memory. The spatial memory for the platform location was evaluated by the analysis of the dwelling time in the zone (a circular area 20 cm in diameter) where platform was previously located.

3.6.4. Elevated plus maze

The design of elevated plus maze was based on that described in by Walf and Frye (Walf and Frye, 2007), with slight modification. The maze was made of plastic, painted black, and consisted of four arms (two open arms without walls and two close arms enclosed by 15.25 cm high walls). The arms were 30 cm long and 5 cm wide. Each arm of the maze was attached to sturdy legs such that the maze was elevated 50 cm off the ground surface. The mice were first placed on the edge of an open arm. Once they reached the maze center, the data recording was initiated and mice were given 5 min to explore the maze. An observer was always present in the testing room and recorded the frequency of open and closed arm entries, as well as the time spent in each arm and in the maze center. The frequency and duration of open versus closed arm entries were used as indices of anxiety. While mice have natural tendency to explore open arms which present greater sight of the surrounding landscape, the more anxious mice will prefer the protected close arms which offer greater sense of safety.
6.9. **Statistical analysis**

Statistical analysis was performed using non-paired, two-tailed student’s *t*-test in SigmaPlot 10.0 software. All results were expressed as mean ± Standard Error and a probability of less than 0.05 was considered statistically significant.
4.1. Impaired postnatal brain growth

Both PAK1 and PAK3 are highly expressed in the developing brain and neurons (Nikolić, 2008), suggesting that they may play important roles in neuronal and brain development. Indeed, inhibition of PAKs using small inhibitory RNA and dominant negative constructs disrupted neurite growth and neuronal migration, implicating PAKs’ involvement in neural development. However, our previous studies showed that genetic deletion of either PAK1 or PAK3 in mice resulted in no detectable changes in the nervous system development or overall cellular organization of the brain.

Studies have demonstrated that PAK1 and PAK3 are co-expressed in the developing brain and neurons (Cobos et al., 2007; Causeret et al., 2009b; Souopgui et al., 2002). Therefore, I reasoned that their functions may be redundant, allowing one to developmentally compensate for the loss of the other in the single knockout mice. To address this hypothesis, I crossbred heterozygous PAK1 KO line (PAK1+/−) with heterozygous PAK3 KO line (PAK3+/−) to generate PAK1&3 DK mice. The DK pups were born healthy, and their prevalence in the litter followed the classic mendelian ratio. To assess the size of brain at birth, I isolated and fixed brains from post-natal day 1 (P1) pups in 4% paraformaldehyde, then measured their mass. At birth, the DK mice were healthy with normal brain mass (WT = 93.0 ± 1.6 mg, DK = 91.1 ± 1.7 mg, p = 0.45) as shown in Fig 9. Nissl stained equivalent coronal sections showed comparable anatomy between P1 WT and DK brains. However, the mutant brain failed to grow normally, resulting in drastically smaller brain by 12 weeks of maturity (WT = 413 ± 6 mg, DK =
255 ± 8 mg [61.7% WT], p < 0.0001). Yet, the average mutant body weight was similar to that of the controls, indicating that the growth deficit was specific to the brain.

To better determine which brain regions were affected in the mutant, I performed magnetic resonance imaging of perfusion fixed brains in the skull with help from Dr. Mark Henkelman. The analysis of MRI images was done using a 3D surgical atlas developed by Dr. Jeff Henderson’s lab. Table 1 summarizes the regional absolute volume reductions throughout all regions of the brain. The frontal lobe of the cerebral cortex (WT = 36.02 ± 3.05 mm$^3$, DK = 23.23 ± 1.00 mm$^3$, p < 0.0001) and hippocampus (WT = 19.43 ± 1.05 mm$^3$, DK = 12.72 ± 0.51 mm$^3$, p < 0.0001), two regions of interest in this study, were reduced by 35.5% and 34.5% in volume, respectively (Fig 10). Coronal MRI sections of frontal cortices (Fig 10) depicted not only reduced cortical thickness in the mutant, but also notably thinner corpus callosum (Fig 10) (WT = 19.23 ± 1.35 mm$^3$, DK = 11.73 ± 0.81 mm$^3$, 39.0% reduction). A particularly small medulla and spinal cord (Fig 1 i, arrow) is highlighted in the coronal MRI section of hindbrain. These results indicate that PAK1&3 DK mice can serve as a robust model for secondary microcephaly. Table 1 lists all the brain regions (> 3mm$^3$ in volume) for which significant differences were found between WT and DK mice.
**Fig 9. Impaired postnatal brain growth.** Mutant brains were of comparable size to WT brains at birth (A) (whole brain mass at P1: WT = 93.0 ± 1.6 mg, DK = 91.1 ± 1.7 mg, p = 0.45), but failed to mature properly (B). At 12 weeks (C), WT brains weighed an average of 413 ± 6 mg, whereas DK brains weighed 255 ± 8 mg (61.7% of WT mass). FluoroNissl staining of equivalent WT and DK coronal sections further illustrated reduced brain size at maturity (D), but normal brain size at birth (E).
Fig 10. MRI atlases of WT and DK brains. (A) Equivalent sagittal planes of averaged MRI atlases of 7 WT (top) and 8 DK (bottom) adult brains depicted reduced size but normal overall anatomical organization. All identifiable brain regions, including cortex and hippocampus, were reduced in volume (Table 1) in the DK mice. (B) Coronal MRI sections of frontal cortical region depicted not only reduced cortical thickness, but also thinner corpus callosum (arrow). (C) Coronal MRI images showed particularly small medulla and spinal cord (arrow) in the DK brain. (I) Averaged volumes for selected brain regions. N represents the number of animals. Scale bar: 2 mm.
Table 2. Differences in regional volume between WT and DK brains. Only the structures greater than 3mm$^3$ in volume were analyzed. False Discovery Rate was used to control for the number of false positives. All results shown are significant at a 0.1% FDR.

n = 8 mice for each genotype.

<table>
<thead>
<tr>
<th>Structure</th>
<th>WT Mean ± SE (mm$^3$)</th>
<th>DK Mean ± SE (mm$^3$)</th>
<th>t-statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>276.6 ± 2.8</td>
<td>432.3 ± 7.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>5.6 ± 0.1</td>
<td>9.8 ± 0.1</td>
<td>27.3</td>
</tr>
<tr>
<td>Midbrain</td>
<td>6.8 ± 0.1</td>
<td>14.1 ± 0.2</td>
<td>25.3</td>
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<tr>
<td>Pons</td>
<td>8.2 ± 0.1</td>
<td>16.8 ± 0.3</td>
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<tr>
<td>Superior colliculus</td>
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<td>Olfactory bulbs</td>
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<td>25.4 ± 0.4</td>
<td>15.3</td>
</tr>
<tr>
<td>Arbor vitae of cerebellum</td>
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<td>11.7 ± 0.3</td>
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</tr>
<tr>
<td>Corpus callosum</td>
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<td>19.2 ± 0.4</td>
<td>13.3</td>
</tr>
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<td>36.0 ± 1.0</td>
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<td>20.9 ± 0.4</td>
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</tbody>
</table>
4.2. Normal brain anatomy and neuronal organization

To search for the underlying causes for the reduced brain volume in the DK mice, I first addressed whether the number and/or organization of the brain cells were altered. Previous studies using cultured neurons or other cell lines suggested that PAKs may be involved in cell proliferation (Vadlamudi et al., 2005; Souopgui et al., 2002), survival (Kreis et al., 2009; McPhie et al., 2003) and/or migration (Causeret et al., 2009b; Boda et al., 2008; Sakakibara and Horwitz, 2006). Therefore, it is possible that knockout of PAK1&3 led to defects in any of these processes, resulting in reduced neuronal number and/or abnormal cellular organization.

To study neuronal organization, I first performed a cresel violet staining of parafirm-embedded 5 µm brain sections where the cell bodies of neurons were stained blue (Fig 11A). Although cortical thickness was clearly reduced, all the cortical layers were identifiable in the DK mice in similar relative thickness, suggesting normal cortical laminization. This was further confirmed by immunostaining experiments using cerebral cortical layer-specific markers Cux1 (Fig 11B, upper layers) and Tbr1 (Fig 11C, deep layers), where no differences in the layer-specific distribution of these marker proteins were observed between the WT and DK mice. Furthermore, Golgi-stained brain sections showed that cortical pyramidal neuron polarity was preserved in the DK brains (Fig 11D). These results suggested that cortical neuronal migration was not affected in the DK mice.

However, magnified view of Cresel Violet staining (Fig 11E, equivalent regions in Layer IV of frontal cortex) revealed that neurons in the DK mice were more densely packed and had notably smaller cell body (neuronal cell body cross-sectional area: WT =
219.6 ± 3.8 µm², DK = 131.1 ± 2.1 µm², p < 0.0001). Increased neuronal density with reduced soma was observed throughout hippocampal CA1, CA3 and dentate gyrus regions. These results suggested that the total cell number was not affected in the DK mice.
Fig 11. Normal neuronal organization in PAK1&3 DK brain. (A) Cresel Violet staining of the frontal cortices showed normal cortical layers, identified based on neuronal cell body morphology. (B, C) Immunostaining of cortical sections using cortical layer-specific markers Cux1 (specific for upper layers, B) and Tbr1 (specific for deep layers, C) also showed normal cortical lamination. (D) Golgi-cux impregnation showed normal pyramidal neuron polarity in the frontal cortex. (E) Magnified view of equivalent Cresel Violet stained regions (Layer IV, frontal cortex) revealed smaller and more densely packed neurons in the DK cortex. Scale bar: 50 µm.
4.3. Stereology: total neuronal and glial counts minimally changed

To determine the change in neuronal and glial cell number, I performed stereological counting using the optical fractionator approach on a series of 40 µm coronal brain sections (1 section every 320 µm interval) from beginning to the end of cortex (Fig 12). As expected based on results from section 4.2, the neuronal density was significantly higher (by 32%) in the DK mice (WT = 1.71 x 10^5 ± 1.03 x 10^3 per mm^3, DK = 2.25 x 10^5 ± 1.19 x 10^3 per mm^3, p < 0.0001). Importantly, the cell density was similarly increased across all cortical layers (Fig 12A), consistent with the idea that neuronal migration was unaffected in the DK mice. Total cortical neuron count was only slightly reduced in the DK cortex (WT = 1.83 ± 0.06 x 10^7, DK = 1.64 ± 0.05 x 10^7), and the difference, although statistically significant, only accounts for about a quarter of the reduction in the cortical volume (Fig 12B). Using the same approach to count cortical neurons, glial cells in the hippocampus were also estimated using glial fibrillary acidic protein (GFAP)-DAPI co-stained brain sections (Fig 12C). Similar to neurons, the density of glia was also significantly increased in the DK mice (WT = 124.4 ± 4.2 per mm^3, DK = 188.0 ± 5.5 per mm^3, p < 0.0001), but the total hippocampal glial counts were comparable between the genotypes (WT = 2417 ± 154, DK = 2391 ± 119, p = 0.90) (Fig 12D). Thus, I concluded that the vast majority of the volume reduction could not accounted for by the reduction in cell number. Together, these results indicated that neither the anatomical nor the cellular organization of the brain was significantly altered by the absence of PAK1&3.
Fig 12. Total neuronal and glial counts minimally changed. (A) Summary graphs showing evenly and significantly increased neuronal density in each of the cortical layers. (B) Stereological measurements reveal that, despite drastic cortical volume reduction, the total neuronal count was minimally changed due to a proportionate increase in cell density. (C) GFAP(green)-DAPI(blue) co-stained hippocampal CA1 areas showed increased glial cell density in DK (left). (D) Summary graph showing significantly reduced hippocampal volume and proportionately increased glial cell density, resulting in equal total glia count between WT and DK mice. A total of 5 WT and 4 age-matched DK male brains were used for stereological optical fractionator measurements.
4.4. Reduced dendritic complexity

DK brain contained nearly normal total cell number (10% reduction in neuron, normal glial count), yet had drastically reduced volume (36%). This led me to believe that neurogenesis was not the main cause for reduced brain growth in DK mice. Rather, I hypothesized that the morphology of individual neurons must be the primary cause of the reduced brain volume in DK mice. This hypothesis was consistent with the observed secondary or postnatal nature of the microcephaly in these mice. Indeed, both PAK1 and PAK3 were shown to be highly expressed in post-mitotic neurons, suggesting that they may be particularly important for neuronal morphogenesis and maturation (Nikolić, 2008; Ong et al., 2002; Zhong et al., 2003). Indeed, a number of studies using cultured neurons have shown that PAK1 is important for neurite formation and outgrowth (Hayashi et al., 2004), but in vivo evidence is lacking. To address the in vivo roles of PAK in neuronal morphogenesis, I first characterized the dendritic growth and morphology in the mutant mice.

4.4.1. Reduced dendritic arborization in vivo contributed to reduced brain volume

Staining for a dendritic marker microtubule-associated protein 2 (MAP2) showed a clear reduction in the size of the dendritic field in the hippocampus (Fig 13A, B; basolateral CA1 width: WT = 164.1 ± 1.3 µm, DK = 90.3 ± 1.5 µm, p < 0.0001; apical CA1 width: WT = 298.4 ± 1.7 µm, DK = 241.3 ± 2.5 µm, p < 0.0001). In addition, the fluorescence intensity of MAP2 staining per dendritic area was also significantly reduced in the DK mice (Fig 13D, DK = 77.6 ± 3.2% of WT, p < 0.0001). Interestingly, despite the reduction in MAP2 staining, the fluorescence intensity for glutamate receptor
GluR2/3 immunoreactivity was significantly increased (Fig 13C, D; ratio of GluR2/3 to MAP2: DK = 130.2 ± 5.51 %WT, p < 0.0001). These results suggested that either the dendritic length and/or arbor complexity was reduced in the DK mice. To directly examine this possibility, I performed Golgi-impregnation on fixed brain sections and found that the dendritic complexity of both cortical and hippocampal pyramidal neurons was greatly reduced in the DK mice (Fig 5E shows a Golgi-impregnated hippocampal CA1 neuron). Camera lucida drawings were made from selected stained CA1 neurons from WT and DK brains that met the following criteria: 1) neuronal body at or near the center of the section, 2) apical dendrite parallel to the plane of the section, and 3) intact basolateral dendritic tree. Using Volocity 7.4 software (PerkinElmer), these neurons were traced across different focal planes in order to capture the entire dendritic trees. Sholl analysis of camera lucida drawings confirmed that the dendritic arborization was significantly and drastically reduced in the DK mice (Fig 13F). While the average lengths of individual dendrites were similar, the number of intersections and total dendritic length were vastly reduced in the DK neurons, indicating that PAK1&3 is particularly important for dendritic branching in vivo.
Fig 13. Reduced dendritic arborization contributes to reduced hippocampal volume.

(A, B) Hippocampal sections stained for the dendritic marker MAP2 (A) and summary data (B) showing reduced width for both basolateral and apical dendritic fields in CA1 areas. Scale bar: 300 µm. (C) Hippocampal sections stained for AMPA glutamate receptor subunits GluR2/3. (D) Summary data showing significantly reduced fluorescence intensity of MAP2, but not GluR2/3. (E) Golgi-impregnated CA1 pyramidal neurons (left) and their camera lucida drawings (right) depicting reduced dendritic complexity in the DK mice. Scale bar: 100 µm. (F) Sholl analysis of lucida drawings of intact CA1 neurons showing profoundly reduced number of intersections across all radii.
4.4.2. Deficit specific to arborization, average length identical

To test whether the dendritic defect was an intrinsic property of the DK neurons rather than altered environment in the brain leading to a failure to thrive, I analyzed cultured hippocampal neurons, which also allowed me to characterize their developmental profile. Sholl analysis confirmed profound deficits in dendritic arborization in mature (21 \text{ div}) DK neurons (Fig 14B), reminiscent of findings \textit{in vivo} in Golgi stained sections. In the early stages of development however, both the growth cone morphology and dendritic growth appeared to be normal in DK neurons (Fig 14A-C). To quantify dendritic growth, I measured two parameters: 1) the total dendritic length (TDL), and 2) the total dendritic branch tip number (TDBTP), an indicator of the degree of arborization. Up to 7 \text{ div}, both TDL and TDBTP are similar between WT and mutant neurons (Fig 14C, D). Both TDL and TDBTP became drastically different at later stages of development by 7-21 \text{ div} (TDL \textit{at div 21}: WT = 1894 \pm 62 \text{ um}, DK = 1054 \pm 30 \text{ um}, p < 0.0001; TDBTN: WT = 26.0 \pm 1.62, DK = 15.2 \pm 1.76, p<0.0001). Remarkably, when I calculated the average length of dendritic segments by dividing TDL by TDBTN (Fig 14E), I found that the average length was identical throughout all stages of development (TDL/TDBTN \textit{at div 21}: WT = 72.9 \pm 5.1 \text{ um}, DK = 69.5 \pm 8.5 \text{ um}). Together, these results indicated that PAK1&3 affected dendritic morphogenesis specifically through regulation of dendritic arborization and not neurite extentions.
Fig 14. Dendritic deficit specific to arborization, not average length. (A) Cultured hippocampal neurons stained for MAP2 (green) and F-actin (red; phalloidin) showing drastically reduced dendritic complexity at $\text{div} 15$ and 21, but not at $\text{div} 3$ and 7 in the DK mice. (B) Sholl analysis of hippocampal neurons at $\text{div} 21$ showing profound deficits in dendritic arborization. (C, D) Summary data of cultured hippocampal neurons showing significant reduction in the total dendritic length (C) and total dendritic brach tip number (TDBTN) (D) in the DK mice. (E) The average dendritic length (total dendritic length / TDBTN) was identical between the WT and DK neurons throughout all stages. Scale bar: 50 µm.
4.4.3. **PAK3 inhibition in PAK1 KO neurons reduced dendritic arborization**

To verify the role of PAKs in dendritic morphogenesis, I took a different independent approach by transfecting cultured hippocampal neurons with either a dominant negative form of GFP tagged PAK3 (DN-GFP-PAK3) or control GFP. The transfection was achieved through adenovirus-mediated delivery in cultured hippocampal neurons. Consistent with my previous data, I found that DN-GFP-PAK3, but not GFP, significantly reduced dendritic arbors in neurons cultured from PAK1 KO mice (Fig 15). Therefore, the reduced dendritic arborisation in the DK mice is likely a direct consequence of PAK1&3 deletion and not a secondary chronic effect. These results indicate that PAK1&3 are directly and specifically involved in the regulation of late stages of dendritic development during which rapid addition and extension of dendritic branches occur.

4.4.4. **Normal glial morphology.**

To determine whether PAKs are also essential for morphogenesis of other brain cell types, I examined the morphology of hippocampal CA1 glial cells both *in vivo* and in culture. The glial cells were identified by co-staining of glial fibrillary acidic protein (GFAP) process and DAPI in the mature brains (Fig 16A, z-stack reconstruction of 20 μm sections) and in primary hippocampal neuron culture (Fig 16B). Three parameters were measured: cell body cross-sectional area, the number of main processes, and the total length of all processes. None of the three indicators of glial size were significantly different between WT and DK glia (Fig 16C) (cell body area: WT = 55.6 ± 2.54 μm², DK = 56.7 ± 3.55 μm², p > 0.05; number of primary processes: WT = 5.7 ± 0.21, DK = 5.4 ±
0.36, p > 0.05; total process length: WT = 121 ± 1.95 μm, DK = 124 ± 2.23 μm, p >
0.05). The results indicated that PAKs 1 and 3 do not regulate morphogenesis in all brain
cell types and suggested that its effect on cellular morphogenesis may be neuron
specific.
Fig 15. Effect of dominant negative PAK3 expression on PAK1 KO neuron complexity. Cultured hippocampal PAK1 knockout neurons were transfected with viruses expressing either control GFP or dominant negative GFP-PAK3 (DN). (A) Dendritic arbors were reduced in DN transfected neurons, but not in GFP transfected neurons. Scale bar: 50 µm. Vehicle indicates non-transfected neurons. (B) Summary graph showing significant reduction in TDBTN in DN transfected neurons compared to non-transfected or GFP transfected neurons. (C) Higher magnifications of cultured neurons in (A) showing abnormal dendritic spine in DN-transfected neurons, as was shown in a previous study. Scale bar: 5 µm.
Fig 16. Normal glial morphology. Hippocampal glia (A; z-stack reconstruction of GFAP-stained glia using confocal microscope) from PAK1&3 DK brain showing cell body size and morphological complexity comparable to that of WT glia. Similar results were obtained from glia in primary hippocampal culture (B). (C) shows a summary table for (A). Scale bar: 10 µm.
4.5. Reduced white matter and axonal collaterals

Axons are the major structural components of the neurons that relay synaptic output. They travel long distances and are often bundled together to form nerves and tracts within the nervous system. Myelinated axons form tracts called white matters in the CNS. The relationship between axon abundance and brain volume is, to my knowledge, unestablished. Previous studies in invertebrates have implicated PAKs in the regulation of axon guidance and growth (Hing et al., 1999b; Ang et al., 2003; Fan et al., 2003; Hu et al., 2001), but little data regarding the role of PAKs in axon morphogenesis (Jacobs et al., 2007) is available in vertebrates and none in vivo in mammalian system. Of particular interest to me is the finding from MRI data (Fig 10, Table 1) that showed PAK1&3 DK mice were drastically reduced in white matter tracts, suggesting that axonal properties are altered in these mice.

4.5.1. In vivo: thinner white matter tracts and fewer Schaffer collateral filaments

To address the possible role of PAKs in axon outgrowth and guidance in mice, I first performed myelin staining of brain sections using FluoroMyelin Green (Invitrogen), a marker for myelinated axons. I found that the myelinated axonal tracts were significantly reduced in thickness throughout the DK brain (Fig 17A), consistent with earlier findings from MRI. Analysis of myelin fluorescence (on 20 µm thick coronal sections, 1mm interval throughout the cortex) showed that total white matter volume was drastically reduced in the DK brain (total fluoromyelin intensity across cortex: DK = 57.3 ± 5.1 %WT, p < 0.0001). However, the staining patterns were indistinguishable between equivalent WT and DK sections, suggesting that axonal guidance/connectivity was not
affected in the DK mice. To specifically examine the hippocampal regions, I performed immunostaining using the axon-specific marker phospho-neurofilament (pNF) and found a striking reduction in the density of Schaffer Collateral axon fragments in the DK mice (Fig 17B, C and E; normalized pNF immunofluorescence intensity: DK = 42.9 ± 3.2 % of WT, p < 0.0001). This raises two possibilities that are not mutually exclusively. First, some neurons might have failed to specify an axon. In support of this hypothesis, Jacob and collaborators showed in hippocampal neuron culture that acute manipulation of PAK1 disrupts axon specification (Jacobs et al., 2007). Second, Schaffer collateral axons might have fewer branching in DK, leading to reduced number of axonal collaterals in the CA1 region. Indeed, it is well established that hippocampal neuron axons, especially those from CA3 neurons, branch extensively (Finch et al., 1983). To distinguish the two possibilities, I employed lower density hippocampal neuron culture, in which single neurons and their axonal projections can be fully tracked up to 7 div.

Despite the drastic reduction in pNF staining fluorescence, the fluorescence intensity of the presynaptic marker synaptophysin was significantly increased in the DK brain (Fig 17D, E; ratio of synaptophysin to pNF fluorescence intensity: DK = 214 ± 7.5 %WT, p < 0.0001). The increased synaptophysin expression in the DK mice was similar to the earlier observation that the level of postsynaptic glutamate receptors was enhanced despite the drastically reduced dendritic arbors (Fig 13A-D), suggesting possibly enhanced synaptic properties in these mice (see section 4.7 and 4.8).
4.5.2. Deficit specific to branching; no significant difference in average length

To determine the exact nature of axonal deficit, I co-stained low-density cultured hippocampal neurons for pNF (axonal marker) and F-actin to study the course of axonal morphogenesis (Fig 18 A-D). All WT and DK neurons sprouted a single primary axon, enabling me to rule out defects in axon specification as the cause of reduced white matter and Schaffer collateral filaments in vivo. However, at 3 and 7 div (at which periods the entire axons of isolated neurons can be traced and analyzed), the DK neurons showed a drastic reduction in both total axonal length growth (Fig 18 B; 3 div: WT = 1426 ± 75 µm, DK = 451 ± 40 µm, p < 0.0001; 7 div: WT = 3230 ± 131 µm, 1340 ± 52 µm, p < 0.0001) and in the number of branch points (Fig 18 C; 3 div: WT = 4.12 ± 0.33, DK = 1.48 ± 0.20, p < 0.0001; 7 div: WT = 7.50 ± 0.40, DK = 3.77 ± 0.26, p < 0.0001) (Fig 18E, F). Using these two parameters, I determined the average length for axons by dividing the total length (TAL) by the number of branch points (BP) (Fig 18 D), and found no statistically significant differences (TAL/BP at 3 div: WT = 346.1 ± 33.2 µm, DK = 304.7 ± 49.4 µm, p = 0.489; at 7 div: WT = 430.7 ± 28.9 µm, DK = 355.4 ± 28.2 µm, p = 0.067). This led me to conclude that PAKs are critical for axonal branching but not for specification or extension, a finding that is reminiscent of its role in dendritic morphogenesis. That both dendrites and axons exhibit drastic arborization deficits in PAK1&3 DK neurons indicate that these proteins serve a essential general role in the regulation neurite branching.
**Fig 17. Thinner myelinated tracts and fewer Schaffer collateral axons.** (A) Brain sections stained for myelin (green) showed thinner white matter tracts throughout the forebrain of the DK mice. (B) Hippocampal sections stained for the axon-specific cytoskeletal protein phospho-neurofilament (pNF) (green, with blue DAPI counterstain) revealed drastically reduced pNF immunofluorescence intensity in the CA1 stratum radiatum (arrow) in the DK mice. (C) Higher magnification image showing reduced number of axonal fragments in the DK section. (D) Hippocampal sections stained for the presynaptic marker synapsin showing comparable immunofluorescence intensity in the DK section. (E) Summary graph shows significantly reduced immunofluorescence intensity for myelin and pNF, but not for synapsin, in the DK mice.
Fig 18. Axonal deficit specific to branching, not average length. (A) Cultured hippocampal neurons stained at div 3, 7 and 21 for pNF (green) and F-actin (red) showing reduced axonal growth in DK neurons. (B, C) Summary graphs show a significant reduction in both total axon length (B) and number of branching points (C) in the DK neurons. (D) However, no significant difference (i.e. p > 0.05) was found in average axonal length, for both 3 and 7 div. Scale bar: 20 µm.
4.6. Electrophysiological properties of DK neurons

4.6.1. fEPSP: enhanced synaptic efficacy

To assess the functional consequence of reduced neuronal complexity at both dendritic and axonal level, I employed field excitatory synaptic potential (fEPSP) electrophysiology recordings at the CA3-CA1 Schaffer collateral synapse. The result was unexpected; despite blatant reduction neuronal morphology, the basal synaptic transmission was nearly doubled. The input-out curves of fEPSPs showed enhanced evoked responses throughout all stimulus intensities in the mutant (Fig 19B). Yet, plot of prefiber volley versus stimulus intensity was identical between the WT and DK mice (Fig 19C). Enhanced overall synaptic efficacy was confirmed by plotting fEPSP against prefiber volley. The results indicated that enhanced transmission was not due to the presence of greater number of fibers in DK, but rather reflected functional changes at the synaptic level. These findings were consistent with increased immunoreactivity of post-synaptic and pre-synaptic proteins in CA1 synaptic region of DK mice.

4.6.2. Heightened presynaptic function

In addition, I recorded changes in the presynaptic function in PAK1&3 DK. Paired-pulse facilitations were significantly diminished in the DK slices (Fig 20A). The ratio of amplitude of second fEPSP to that of first was smaller throughout all interpulse intervals. In collaboration with Zikai Zhou, I further examined the presynaptic function by recording miniature EPSP (mEPSP) using whole cell recording. Analysis of mEPSPs in mutant mice revealed a mild increase in their average amplitude and a notable increase
in their frequencies (Fig 20 B). Together, these results indicated heightened presynaptic release mechanism. To understand how overall synaptic efficacy and presynaptic release function were elevated, morphological studies at the level of synapses were required (see section 5.7).

4.6.3. Whole cell recording: passive membrane properties

Using whole cell recording, the passive membrane properties of CA1 neurons were assessed. The access resistance was identical between WT and DK neurons (Fig 21A), reflective of the identical recording condition. DK neurons exhibited increased input resistance (Fig 21 B) and required smaller current injection to elicit an action potential (Fig 21 D). The two changes are consistent with the simpler and smaller neuronal morphology found in PAK1&3 DK brain. On the other hand, resting membrane potential (Fig 21C) and the threshold membrane potential to fire an action potential were unaffected (Fig 21E). Furthermore, the amplitude of action potential was identical between WT and DK (date not shown). These results suggested that while the neurons in DK brain were indeed smaller, their basal membrane properties (including channel properties and distribution) remained unchanged.

4.6.4. Impaired bi-directional CA3-CA1 synaptic plasticity

Because the mutant mice were severely impaired in learning and memory, I hypothesized related changes in synaptic plasticity – the established basis of learning and memory at the level of the synapse. I found that both long-term potentiation (LTP) and depression (LTD) were significantly attenuated in DK mice compared to the WT
controls (Fig 22) at the hippocampal CA3-CA1 synapse. While early-phase NMDA receptor-dependent LTP (induced by 2 trains of 100 Hz stimulation) was comparable between WT and DK slices (Fig 22A) for up to 60 minutes after induction, late-phase NMDA receptor-dependent LTP (induced by 4 trains of 100 Hz stimulation) was severely impaired (Fig 22B). The data for the latter was provided by Suhail Asrar. Both NMDA receptor-dependent LTD (Fig 22 C; WT = 72.8 ± 4.6 %, DK = 88.8 ± 3.9 %, p<0.024) and metabotropic glutamate receptor-dependent LTD (Fig 22D, WT = 79.3 ± 1.7%, DK = 94.5 ± 2.9 %, p < 0.001) were also impaired in the mutant mice. Because both LTP and LTD are thought to be important cellular mechanism underlying learning and memory, their deficits may be responsible (at least partially) for cognitive deficits associated with the PAK1&3 DK mice.
Fig 19. Input-output curves: enhanced synaptic excitability. (A-D) fEPSPs and their corresponding prefiber volleys were recorded at CA1 synapse at various stimulation intensities (A). Summary graphs show enhanced synaptic responses (B) in DK mice despite normal presynaptic volleys (C). (D) Plot of fEPSP against prefiber volley shows heightened synaptic efficacy in DK. n indicates the number of slices from at least 3 different mice for each genotype.
Fig 20. Altered presynaptic properties. (A) CA1 paired-pulse facilitations (PPFs) were significantly diminished, from 20 to 500 ms interpulse intervals. (B, C) Whole-cell recording of CA1 pyramidal neurons revealed increased amplitude and frequency of mEPSPs. n indicates the number of slices (A) or the number of neurons (B) from at least 3 different mice for each genotype. Data in (B) courtesy of Zikai Zhou. Scale bar for (B): x = 0.5s, y = 10 pA.
Fig 21. Passive membrane properties of CA1 neurons. Membrane access resistance was comparable between WT and DK neurons (A). However, DK neurons exhibited increased input resistance (B) and required less current injection to trigger action potential (D). The resting membrane potential (C) and threshold membrane potential for action potential (E) remained unchanged. Data courtesy of Zikai Zhou.
Fig 22. Diminished CA3-CA1 late-phase LTP and LTDs. fEPSP recordings in mature mice showed normal early-phase NMDA receptor-dependent LTP induced by high frequency stimulation (A) (2 trains of 100 Hz lasting 1 sec each, arrow) in DK mice, but severely reduced late-phase NMDA receptor-dependent LTP (B) (induced by 4 trains of 100 Hz, arrow). NMDA-dependent LTD induced by low frequency stimulation (1 Hz, 15 min, arrow) was diminished in 13-15 day old DK mice (C). Metabotropic glutamate receptor (mGluR)-dependent LTD induced by a brief (5 min, gray bar) application of 50 μM DHPG was nearly abolished in 2-3 week old DK mice (D). n indicates the number of slices from at least 3 different mice for each genotype.
4.7. Synapse morphology, number and architecture

4.7.1. Abnormal dendritic spines

To determine how reduced number of dendritic arbors and axonal branches can lead to enhanced basal synaptic transmission, I first analyzed the number and morphology of the dendritic spine, the major site of excitatory synaptic input. Analyses of Golgi-impregnated cortical and hippocampal pyramidal neurons revealed that the DK mice were profoundly altered in spine morphology (Fig 23). Spines from DK brain were characterized by: less than half the number of mushroom shaped spines (Fig 23C, WT = 48.7 ± 3.2%, DK = 20.0 ± 1.5%, p < 0.0001), more than double the number of filopodia (Fig 23C, WT = 8.4 ± 1.0%, DK = 27.3 ± 2.1%, p < 0.0001), reduced head/neck ratios (Fig 23D), smaller head areas (Fig 23E) and increased spine length (Fig 23F). In particular, the DK neurons displayed over 10-fold increase in the number of uncommon spine shapes namely spine head protrusions and bifurcated spines (Fig 23B, C; WT = 1.31 ± 0.05 %, DK = 15.33 ± 1.12 %, p < 0.0001). The density of the total dendritic protrusions, however, was not significantly altered in DK mice (number per 10 µm dendritic segment: WT = 7.02 ± 0.25, DK = 6.60 ± 0.22, p = 0.24). Similar quantifiable changes in spine morphology were obtained from the analysis of cultured hippocampal neurons (Fig 24) co-stained with MAP2 (dendrite specific) and phalloidin-labelled F-actin (enriched in spines). These results indicated that PAKs 1&3 are essential for normal spine morphogenesis.
4.7.2. EM: fewer synapses and increased PSD width

To investigate the effect of PAK1&3 deletion on synaptic properties, I analyzed both synapse density and the structure of individual synapses *in vivo* using electron microscopy (EM). Earlier immunostaining experiments showed that the levels of both postsynaptic glutamate receptors (Fig 13) and presynaptic marker synaptophysin (Fig 17) were elevated in the DK hippocampal area, suggesting that either synapse density or the synaptic properties of individual synapses must be enhanced. However, the former possibility is unlikely given the reduced dendritic arbors and unaltered spine density in the DK mice. To directly confirm this, EM thin sections of hippocampal CA1 area were obtained and analyzed. As shown in Fig 25, the density of asymmetric (i.e. excitatory) synapses (Fig 25A, arrows, identified by the presence of post synaptic density [PSD] apposed to presynaptic vesicles) was significantly reduced in the DK sections (Fig 25A, B, WT = 34.63 ± 0.94/100 μm², DK = 26.28 ± 0.71/100 μm², p < 0.001). However, the frequency distribution plot of PSD width revealed that the size of PSD was significantly enlarged in the DK mice (Fig 25C). For example, only 17.0 ± 0.3% of the synapses in WT had PSD greater than 250 nm in width, whereas 66 ± 0.8% of synapses in the mutant fell within this range (p < 0.001). Therefore, gross enlargement of individual synapses most likely contributed elevated fEPSP amplitude in spite of simpler neuronal morphology in PAK1&3 DK.
4.7.3. *In vitro*: enlarged presynaptic release sites, altered synaptic architecture

To further assess the distribution and architecture of individual synapses, I analyzed *div* 21 cultured neurons co-stained for various combinations of: presynaptic marker synaptophysin, postsynaptic marker GluR2/3, axonal marker phospho-neurofilament, and phalloidin which stains for F-actin. Neurons cultured from WT mice were characterized by elaborate axonal network interposed with elaborate dendritic trees, with large number of fine synaptophysin puncta (Fig 26D, right) representing the extensive distribution and relatively small size of WT synapses. In contrast, neurons cultured from DK mice developed simpler axonal network interposed with simpler dendritic trees, with fewer number of larger synaptophysin puncta (Fig 26D, left). Analysis of randomly selected dendritic segments showed that the number of synapses (defined as synaptophysin-stained presynaptic puncta apposed to GluR2/3-stained dendrite) per dendritic length was actually identical between WT and DK (Fig 26C, F; per 10 µm dendrite, WT = 6.90 ± 0.28 synapses, DK = 6.93 ± 0.20 synapses, p = 0.94). Therefore, I concluded that the reduced total synapse number must be as a direct consequence of reduced dendritic arborisation.

Consistent with EM findings, the cross-sectional area of individual presynaptic puncta was significantly enlarged in DK neurons (Fig 26B, D). The mean synaptophysin puncta area in mutant was more than twice as large as that in wild type (WT = 0.396 ± 0.021 µm², DK = 0.819 ± 0.042 µm², p < 0.0001). A frequency distribution of the puncta size shown in Fig 26D depicts a notable right shift in DK. Furthermore, the location of the synapses was strikingly different between the two genotypes in culture. While most of the synapses in WT neurons were found on spine heads, a large proportion of the DK
synapses were formed on the main dendritic shaft (proportion of spine head synapses: WT = 84.0 ± 2.4 %, DK = 43.7 ± 1.6%, p < 0.0001) (Fig 26B, E). Many of DK dendritic protrusions, especially filopodia / immature spines, in fact failed to form synapses. These spines appeared to be non-functional, as they contained little to no detectable levels of postsynaptic glutamate receptors (Fig 26B).

These morphological data altogether indicated that the total number of synapses was reduced in the DK mice as a key consequence of reduced dendritic-axonal branching. Interestingly, individual synapses were grossly enlarged and their architecture was remarkably altered in the DK mice, likely accounting for the increased synaptic transmission and enhanced presynaptic function previously described (see section 4.6). An interesting question that remains to be addressed is whether the alterations in synapse size and architecture represents A) a compensatory developmental response to fewer synapse number, or B) a distinct role of PAKs.
Fig 23. Abnormal spine morphology in vivo. (A, B) Dendritic segments of Golgi-impregnated CA1 neurons showing changes in DK spine morphology (A). One such change entails especially high prevalence of uncommon spines (B) – bifurcated necks (black arrows) and spine head protrusions (red arrows). (C-F) Summary graphs show significant abnormalities in spine properties, including reduced mushroom-shaped spines, increased filopodia and uncommon spines (C), reduced head-to-neck ratios (D), reduced spine head size, and increased spine length (F) in the DK mice. No significant differences in the density of total dendritic protrusions were found. Scale bar: 4 µm. n denotes the number of spines from at least 3 separate mice.
Fig 24. Spine deficits in cultured hippocampal neurons. (A, B) Dendritic segments of MAP2 (green) and phalloidin (red) co-stained neurons showing changes in spine morphology. (B-F) Summary graphs show significant abnormalities in spine properties, including reduced mushroom-shaped spines, increased filopodia and uncommon spines (B), reduced head-to-neck rations (C), reduced spine head size (D), and increased spine length (E) in the DK mice. n denotes the number of spines from at least 3 independent cultures.
Fig 25. Reduced CA1 synapse number with increased PSD width. (A) Electron micrographs of hippocampal CA1 areas with excitatory synapses marked by red arrows. (B) Frequency distribution plot of PSD width shows a remarkable right shift in DK. (C) Summary graph shows significant reduction of synapse density (count per unit area) in the DK mice. Scale bar: 500 nm.
Fig 26. Differences in synapse size and location in vitro. (A) Cultured hippocampal neurons triple stained for F-actin (red), pNF (blue) and synaptophysin (green) show reduced dendritic (red) and axonal (blue) complexity, accompanied by enlarged synapses (green) in DK compared to WT. (B) High magnifications image depicting dendritic segments triple stained for F-actin (red), synaptophysin (green) and GluR2/3 (blue). White arrows denote one of many examples of immature spine (i.e. filopodia). Such spines were commonly found along DK dendrites, and failed to form synapse and/or accumulate postsynaptic receptors. (C) Summary graph of cultured hippocampal neurons shows similar synapse density between the WT and DK mice. (D) Synaptophysin puncta size distribution plot depicts a significant left shift in the DK mice. (E) Summary graph of synapse location shows decreased proportion of DK synapses on the spine heads and increased proportion on the dendritic shaft. Scale bar: 10 µm.
4.8. PAK3-associated XLMR patient-like behaviors

A recent clinical study reported that PAK3-linked mental retardation patients display a common set of behavioral patterns that include hyperactivity, excessive anxiety, restlessness and impaired spatial memory (Peippo et al., 2007). Therefore, to assess whether PAK1&3 DK mice possess human clinical features, I carried out a number of behavioural tests as described below.

4.8.1. Hyperactivity and increased anxiety

First, I used open-field test to evaluate the spontaneous exploration of new environment and locomotor activity (Fig 27A). The DK mice traveled longer distance (WT = 3.4 ± 0.4 m, DK = 6.8 ± 0.4 m, p < 0.0001), exhibited greater movement velocity (WT = 68 ± 8 cm/min, DK = 136 ± 7 cm/min, p < 0.0001), and showed reduced rest time (WT = 16.5 ± 2.0 s, DK = 6.5 ± 1.9 s, p < 0.005). These results strongly indicated hyperactivity and restlessness in these mice.

Second, I evaluated anxiety level of the mice by using a custom designed elevated plus maze. In WT mice, open arm entries constituted 55.8 ± 3.7 % of total arm entries, whereas in DK mice, open arm entries were greatly reduced and constituted only 16.5 ± 3.6 % of total arm entries (Fig 27B, p < 0.001). While both WT and DK mice spent comparable amount time in the maze center (Fig 27C, WT = 29.2 ± 1.2, DK = 26.7 ± 2.3, p > 0.05), DK mice spent significantly less time exploring the open arms and more time exploring the closed arms (Fig 27C, time spent in open arms: WT = 39.2 ± 1.2, DK = 15.4 ± 4.3, p < 0.001). These results indicated that the DK mice had heightened anxiety compare to the WT control.
4.8.2. Abolished fear memory retention

To evaluate learning and memory performance, I employed two independent learning paradigms: fear conditioning and Morris water maze. In fear conditioning, both WT and DK mice showed low levels of basal freezing prior to foot shock (Fig 28A, WT = 13.8 ± 3.4 %, DK = 11.8 ± 3.2 %, p > 0.5) and comparable elevated levels of freezing after the foot shock (Fig 28A, WT = 40.2 ± 9.4 %, DK = 34.2 ± 7.0 %, p > 0.5), indicating that the DK mice displayed normal pain processing and freezing response to pain. However, memory tests carried out 1 hour after the training showed profound memory deficits in the DK mice. They displayed greatly reduced freezing compared to the WT control in both cued (Fig 28B, WT = 46.8 ± 8.3 %, DK = 10.2 ± 2.2 %, p < 0.001) and contextual test (Fig 28C, WT = 49.9 ± 8.4 %, DK = 11.8 ± 2.2 %, p< 0.001). In fact, the level of freezing in DK mice before and after tone (or during contextual test) was not statistically different, indicating a complete abolishment of fear memory retention.

In contrast, mice lacking either PAK1 or PAK3 alone showed completely normal fear memory retention indistinguishable from those of age-match WT control mice (Fig 28D-F). One week after fear memory conditioning, PAK1 KO and PAK3 KO mice exhibited levels of freezing response to conditioned stimuli equal to those of WT controls, in both cue and contextual tests. Thus, the cognitive deficits appeared to manifest only with simultaneous knockout of both PAK1 and PAK3, supporting the hypothesis that the two brain-dominant isoforms of the group I PAK family share redundant roles in regulating this cognitive behavior.
4.8.3. Impaired spatial memory acquisition and retention.

I used the Morris water maze to specifically assess spatial learning and memory. WT and DK mice performed equally well in acquisition of the visible platform (Fig 29 A, B), suggesting the DK mice retained normal motor function, vision and associative learning ability. On the other hand, both the average swim distance (Fig 29C, on 5th day: WT = 290 ± 30 cm, DK = 720 ± 86 cm, p < 0.005) and latency (data not shown) to reach the hidden platform were significantly greater for the DK mice than for the WT control. After the initial training day, the swim speed was not significantly different between the WT and DK mice (Fig 29D, on 5th day, WT = 24.26 ± 0.63 cm/s, DK = 22.89 ± 0.64 cm/s, p > 0.05). Probe tests carried out 1 and 24 hours after the 5th day of training both showed that the DK mice spent significantly less time exploring the target zone than the WT mice (Fig 29E, F; 1 hour: WT = 6.89 ± 1.27 s, DK = 2.61 ± 0.65 s, p < 0.01; 24 hours: WT = 5.48 ± 0.83 s, DK = 1.94 ± 0.27 s, p < 0.001). These results indicated that PAK1&3 DK mice were profoundly impaired in learning and retention of spatial memories.

Together with presence of microcephaly, human patient-like cognitive and behavioral deficits (hyperactivity, restlessness, elevated anxiety, severe learning & memory deficits) demonstrated in these behavioural tests strengthen PAK1&3 DK mice as a clinically relevant models for PAK3-associated XLMR.
Fig 27. Increased locomotor activity and anxiety in DK mice. (A) Open field exploratory behavior revealed increased travel distance and speed, and reduced rest time in the DK mice. (B, C) In elevated plus maze test, DK mice showed fewer open arm entries (B) and less time spent in the open arms (C) compared to WT controls. n denotes the number of mice used for each experiment.
Fig 28. Abolished fear memory learning. (A) Fear memory training phase showed that WT and DK mice behaved equally before and after US-CS presentation. (B, C) One hour after the training, the WT controls responded strongly by freezing when either cue (B) or context (C) was presented. However, the DK mice displayed a complete lack of freezing in both cue and contextual tests. In contrast, fear memory acquisition and retention were completely preserved in PAK 1 or 3 single knockout mice (D-F). 1 week after training, the levels of freezing were comparable among WT, PAK1 KO and PAK3 KO mice in both contextual (E) and and cue (F) test. n denotes the number of mice used for each experiment.
**Fig 29. Severely impaired spatial memory acquisition.** Learning acquisition phase for visible plateform test (A) as well as the swim velocity (B) were identical between WT and DK. Learning acquisition phase for the hidden platform test (C) of the Morris water maze test was greatly impaired in DK mice, as indicated by longer swim distances to plateform. No significant difference in swim speed was observed after the initial training day (D). Probe test carried out 1 hr (E) and 24 hrs (F) after the 5th day of training showed that DK mice spent significantly less time exploring the target zone than WT mice, but equal amount of time in the non-target zones. n denotes the number of mice.
4.9. Perturbed cofilin activity and actin dynamics

To elucidate the molecular mechanisms underlying the structural and functional deficits in PAK1&3 DK mice, I analyzed the activities and the expression of a wide range of proteins that might be affected by PAK1&3 (some are shown in Fig 30A). In cultured cell lines, PAK1&3 have been shown to interact with and regulate the activity of numerous effector proteins, but whether such regulations exist in the brain is largely unknown. Western blot analysis of whole brain and hippocampal lysate showed that despite brain volume reduction, the protein level of any of the tested molecules was not altered in DK mice (Fig 30A). Also, no significant differences were found in the basal phosphorylation level (used as an indicator of active form) of MLCK and ERK1/2, two major downstream targets of PAK1&3. However, basal level of phosphorylated (inactive) cofilin was drastically reduced (p-cofilin/cofilin: DK = 23.5 ± 3.3 %WT, p < 0.0001 compared to WT), indicating that cofilin activity was enhanced in the DK brain. Yet, the total cofilin levels in the brain (total cofilin: DK = 98.5 ± 2.1 % WT) were indistinguishable between WT and DK brain lysates. Our lab has previously shown that the basal level of p-cofilin in PAK1 or PAK3 single KO mice was indistinguishable from that of the WT control (Asrar et al., 2009; Meng et al., 2005). Together these data indicated that PAK1 and PAK3 are functionally redundant in regulating cofilin phosphorylation. Immunohistochemical analysis of brain sections also revealed that the level of p-cofilin, but not of the total protein was drastically reduced throughout DK brain sections (Fig 30B). It is interesting to note that cofilin expression in the WT animals was detectable throughout the brain, but was particularly enriched in white matter (Fig 30B, arrows), which was among the most reduced regions in the DK brain.
Consistent with enhanced cofilin activity, the amount of F-actin was significantly reduced in brain sections (Fig 31A; CA1 phalloidin intensity: WT = 100 ± 1.6, DK = 69.7 ± 1.7, p < 0.0001), suggesting that abnormal cofilin-mediated actin regulation was responsible for the brain deficits in the DK mice. To investigate this possibility, I examined the subcellular distribution of cofilin and F-actin in cultured hippocampal neurons. In both developing and mature neurons, cofilin was expressed throughout the dendritic processes, but was particularly enriched in actively growing regions, including growth cones of branching processes of young 7-days old neurons (Fig 30B) and the dendritic spines of mature 21-days old neurons (Fig 30C). Cofilin was found highly colocalizes with F-actin along dendrites and in spine heads (Fig 30C), where it presumably interacts and regulates actin dynamics. Consistent with enhanced cofilin activity, F-actin level was significantly attenuated both in DK brain sections (Fig 31A) (CA1 phalloidin intensity: WT = 100 ± 1.6, DK = 69.7 ± 1.7, p < 0.0001) and in developing processes of cultured DK neurons. In Fig 31B, C, the intensity of phalloidin staining in the DK dendrites was digitally increased to match that of the WT to enable analysis of F-actin distribution in dendrite and spine.

In WT neurons, F-actin is found highly enriched in the spine heads compared to the adjacent dendritic area where phalloidin staining was appeared low and even (Fig 31 B, C). In DK neurons, F-actin enrichments in both filopodia (div 7) and spines (div 7) were significantly attenuated (protrusion-to-dendrite F-actin ratio, div 7: WT = 2.42 ± 0.26, DK = 1.38 ± 0.12, p = 0.0004; WT = div 21: 2.28 ± 0.24, DK = 1.39 ± 0.17, p = 0.003) (Fig 31B-D). Moreover, abnormal clusters of F-actin were frequently observed along DK dendrite (Fig 31C, arrow). Thus, PAK1&3 signaling through ADF/cofilin
appeared to be essential for proper F-actin regulation that is necessary for normal filopodia and spine morphogenesis.
Fig 30. Perturbed basal cofilin phosphorylation. Western blot analysis (A) showed drastically perturbed cofilin phosphorylation (normalized ratio of P-cofilin to cofilin: WT = 100 ± 2.1, DK = 23.5 ± 3.3, p < 0.0001) but normal total cofilin level in the mutant brain. Both the phosphorylated states and total levels of the other proteins adjacent to and downstream of PAK signaling remained unchanged. No significant difference was found in the level pre- and post-synaptic proteins and tubulin. Statistically significant changes in protein levels are denoted by * in (A). (B) shows that cofilin is ubiquitously expressed in the brain, but particularly concentrated in the white matter tracts (arrow) in WT mice. Immunostaining of culture neurons (C) revealed high level of cofilin expression along dendrites and in spine heads, where it was found highly colocalized with F-actin.
Fig 31. Perturbed F-actin assembly and dendritic distribution. The intensity of phalloidin staining was reduced in both brain sections (A) (CA1 phalloidin intensity: WT = 100 ± 1.6, DK = 69.7 ± 1.7, p < 0.0001) and in neurons. For analysis of subcellular F-actin distribution, phalloidin intensity was digitally enhanced in DK dendrites in (B, C) to match that of WT. In WT dendrites, F-actin was highly concentrated in filopodia (B right) and spines (C right) compared to adjacent dendrites (comparison shown in circles), where F-actin was weakly and evenly distributed. In DK, F-actin levels were similar between filopodia/spine head and dendritic shaft (B & C left; D). High prevalence of abnormal F-actin clusters was observed along DK dendrites (C arrows).
One focus of my study is to understand the cellular and molecular mechanisms of post-natal brain development. More specifically, I am interested in a family of proteins called p-21 activated kinases (PAKs). PAK1 and PAK3 are predominantly expressed in the brain, and evidence from in vitro studies and from non-mammalian animal models have implicated their involvement in neuronal morphogenesis (Kreis et al., 2009). Moreover, majority of previous genetic studies involved acute manipulations of PAKs in culture, using approaches such as siRNA and dominant negative expression that do not discriminate one member of PAK from other. These approaches left two major unresolved questions: 1) What is role of PAK in mammalian neural development, and 2) How are the roles of each PAK member unique. Importantly, mutation of PAK3 in humans is associated with X-linked Mental Retardation and microcephaly. To study the roles of PAKs in vivo in mammalian CNS, my lab first generated PAK1 and PAK3 knockout mice. Despite the multiple roles of PAKs implicated in previous studies, the only deficit found in both PAK1 knockout and PAK3 knockout mice was impaired late-phase LTP (Asrar et al., 2009; Meng et al., 2005). Both lines of PAK single knockouts presented with normal brain anatomy, neuronal complexity, and spine and synaptic morphology. They also exhibited normal learning and memory behaviors.

To explain the discrepancy between in vitro / non-mammalian studies (it should be noted that simpler organisms such as Drosophila possess only a single PAK) and in vivo mammalian studies, I hypothesize that co-existence of multiple isoforms of PAKs in
the mammalian nervous system allowed for compensation during development. Therefore, the *in vivo* roles of PAKs in brain development would have been masked in the single knockout mice. To address this hypothesis, I crossed heterozygous PAK1 KO and PAK3 KO lines to generate PAK1&3 DK mice.

### 5.1. Normal neuroanatomy in PAK1&3 DK mice

DK mice were born healthy and indistinguishable from WT and single KO littermates in brain size, gross anatomy and body weight. However, their brains failed to undergo proper post-natal growth. At 8 weeks of maturity, the brains of the mutant mice were much smaller than those of wild type controls both in mass (38% reduction) and in volume (36% reduction), despite animals having comparable body size and mass. In addition to the microcephaly, these mice exhibited behavioral abnormalities that are strikingly similar to those found in humans with PAK3-associated XLMR. These include: anxiety, hyperactivity and severe learning and memory deficits (especially that of spatial memory).

An array of histological approaches were taken to determine the cellular cause of the microcephaly. Age-matched adult male mice were used to generate MRI atlases for each group. Comparison of the WT and DK brain atlases revealed that although all identifiable brain regions were reduced in size, all structures present in the WT brain were preserved in the mutant. In fact, with the exception of volume reduction, gross anatomy appeared identical between the two groups. Immunolabeling for cortical layer specific markers Tbr1 and Cux1 in brain sections showed normal cortical lamination, suggesting that neuronal migration was likely unaffected by PAK1&3 DK. Furthermore,
Golgi-stained brains displayed similar pattern of pyramidal neuron polarity and organization in both the cortex and the hippocampus. Taken together, these results provide strong evidence that normal cellular organization was preserved in the DK mice.

5.2. Neuronal basis of postnatal brain development and brain size regulation

In all previous mouse models of microcephaly (with the exception of one, the MeCP2 knockout mice), loss of neurons due to abnormal proliferation, neurogenesis and/or cell migration was identified as the primary cause of reduced brain volume. However in all these models, the development deficits occurred prenatally and microcephaly was already present at birth. To determine whether the same mechanisms were applicable to PAK1&3 DK mice, I used FluoroNissl stained brain sections and applied a stereological approach (optical fractionator method) to estimate total neuron numbers in the cerebral cortex. I found a nominal reduction in total neuron count (~10%). Glial numbers were unchanged in mutant mice. Examination of Cresyl Violet-stained DK neuronal cell bodies revealed they were more densely packed and that their cross-sectional area was notably reduced. Given these results, I propose that it must be individual neuronal morphology that was primarily responsible for the brain volume reduction. To investigate the cellular cause of microcephaly, I analyzed the neuronal morphology at 3 levels: dendrite, axon and synapse.

I took a particular interest in the hippocampus for a number of reasons. Its anatomy is easily distinguishable, with neuronal cell bodies forming a neat compact layer. The neuronal circuitry in the hippocampus is well-established and can be easily demarcated. This locus has thus been classically used as the region of choice for
electrophysiological studies. The hippocampus has long been established to be key for memory consolidation. Studies of synaptic plasticity (i.e. LTP and LTD), a process thought to be the cellular basis of learning and memory, are extensively done in rodent hippocampus. Lastly, hippocampal neurons can be maintained in primary culture for up to a month, making it an excellent complementary approach to study neuronal development and morphogenesis.

As hypothesized, hippocampal CA1 pyramidal neurons in the mature DK mice brain exhibited a structurally simpler morphology. In particular, dendritic arbors were fewer, despite comparable average and maximal lengths of individual dendrites. Interestingly, the total length and growth of dendrites were similar between WT and DK neurons up to 7 div, a period in which primary dendrites elongated and sprouted filopodia that have yet to stabilize into novel dendritic arbors. Phalloidin immunocytochemistry revealed that distribution of F-actin in the filopodia was abnormal. WT filopodia accumulate abundant F-actin compared to its adjacent dendritic shaft. On the other hand, F-actin concentration was found much weaker in DK filopodias, suggesting that they may be less likely to stabilize into new branches.

The consequence of reduced dendritic arborization on the brain size could be clearly seen when comparing the CA1 region of WT and DK hippocampus in the Golgi-impregnated brain sections. In the DK mice, reduced basolateral dendritic complexity contributed directly to narrower width of stratum oriens and stratum radiatum layers (Fig 13A). Similar findings were previously made in Rett Syndrome patients and MeCP2 knock mice, the best known cases of secondary microcephaly. In Rett Syndrome,
dendritic arborization deficits was also shown to underlie reduced brain size (Armstrong, 2002).

This study is the first to associated deficits in axonal branching with reduced brain volume. Both MRI and myelin staining clearly demonstrated thinner white matter tracts throughout the mutant brain. To understand the cause of reduced tract width at the level of individual neurons, I investigated the hippocampal CA3-CA1 circuitry. Immunolabelling of thin hippocampal sections with an axon-specific protein, phospho-neurofilament, revealed strikingly fewer Schaffer collateral fragments in the CA1 region of the DK brain. Importantly, the axonal thickness appeared identical between the two genotypes, ruling out any reduction in individual axon thickness as a contributing factor. However, the exactly cause of reduced number axonal filament fragments was still unclear. I reason that this may be due to reduced branching of the primary axons from CA3 neurons, leading to generation fewer axonal arbors at the CA1 stratum radiatum area. Alternatively, I reason that (some) CA3 neurons lacking PAK1 and 3 may have failed to specify an axon. In this case, reduced fragments might reflect projections from fewer CA3 neurons rather than simpler axonal morphology. The latter possibility is unprecedented in in vivo models, but deserves consideration since a recent study has shown that acute manipulation of PAK expression disrupts axonal specification in vitro (Jacobs et al., 2007). To address these two possibilities, I returned to primary hippocampal neuron culture. Growth of axons precedes that of dendrites, and axons typically cover much larger distances. Hence, I plated the neurons at low enough density such that the axonal projections of single neurons can be traced entirely. All neurons from both genotypes sprouted one and only one axon. However, reminiscent of the
deficits in dendrites, fewer branch points were seen along axons from PAK1&3 DK neurons despite comparable average axon length between the two groups. Together these data indicate that PAKs 1 and 3 share common essential roles in regulating both dendritic and axonal branching, the defect of which directly contributes to reduced brain volume. A point of interest is that glial cell size and complexity (both in vivo and in culture) were not affected by the double knockout. This suggests that the role of PAKs in cell morphogenesis is not ubiquitous, and that PAK1 and PAK3 together may play specific roles in the morphogenesis of neurons but not other cell types.

5.3. Functional synaptic properties of PAK1&3 DK brain / neurons

Based on the morphological characterization of individual neurons, I initially predicted greatly diminished synaptic transmission and abnormal electrophysiology properties in the mutant mice. Surprisingly, in spite of much simplified connectivity in CA3-CA1 circuitry, basal synaptic transmission as shown by the input-output curves was two-fold greater in the mutant mice, indicative of enhanced synaptic efficacy. Analysis of mEPSP and paired-pulse facilitation (PPF) further validated this finding. Both mEPSP amplitude and frequency were increased in the mutant. The increase in mEPSP frequency would account for the reduction in PPF, since the initial presynaptic release probability was already high. Together, these changes in basal synaptic property strongly indicate enhanced synaptic strength in DK mice. Furthermore, the synaptic plasticity was impaired bi-directionally; both LTP and LTDs were diminished in strength in DK slices compared to the wild-type slices. It was demonstrated that cofilin regulation of actin was severely disrupted as a result of loss of PAK1&3. Thus, a plausible cause of impaired bi-
Directional synaptic plasticity is that PAKs are critical for LTP and LTD through regulation of actin dynamics. Actin dynamics, in turn, is required for membrane insertion and internalization of surface receptors, as well as for spine morphology which changes during LTP / LTD. Alternatively, the cause for compromised synaptic plasticity may be associated with the enhancement of basal synaptic strength, which would interfere with the induction of both LTP and LTD.

Although I cannot rule out the possibility that PAKs 1 and 3 may also functioned at mature synapses to directly regulate synaptic plasticity, manipulation of PAK in adult animals (i.e. without causing alterations in brain size or neuronal dendrites/axons) produced no effect on basal synaptic strength and only a very mild effect on synaptic plasticity and behavioral responses (Hayashi et al., 2004). Therefore, I reason that the lack of adult PAK function at the mature synapses was unlikely to be the main cause of altered synaptic plasticity in the DK mice, and that PAK1&3 control synaptic properties predominantly through promoting dendritic and axonal elaboration. Because both LTP and LTD are considered to be the synaptic bases for at least certain forms of learning and memory, their deficits in DK mice would likely to have contributed to the cognitive deficits in these mice.

5.4. The effects of morphological complexity on synapse count and architecture

To better understand how reduced morphology leads to enhanced transmission, I analyzed the morphology and distribution of dendritic spines and synapses. Analysis of spine morphology in both Golgi-impregnated neurons and phalloidin-stained cultured neurons revealed reduced proportion of mushroom-shaped spines and increased
proportion of filopodia in PAK1&3-lacking mice. Mutant spines were characterized by elongated neck and reduced head size, features that are typically found in mental retardation patients and related animal models. Interestingly, a drastic increase in the number of uncommon spine types (bifurcated spines, spine head protrusions) was observed, further implicating disregulation of cytoskeletal dynamics in the mutant mice.

Using two separate approaches (Golgi staining and glutamate receptor-synaptophysin co-immunocytochemistry), I demonstrated that the reduced synaptic number was as direct result of reduced neurite arborization. In culture, a large fraction (~50%) of synapses in DK were formed on the main dendritic shaft; in contrast, majority ~80% of synapses on WT are formed on the spine head. Indeed, many of the spines / filopodia in the mature DK neurons failed to recruit glutamate receptor or form synaptic connections. Whether alteration of synaptic morphology (i.e. size and location) represents a compensatory response to the simpler dendritic complexity, or a separate role of PAKs in regulating synapses, remains to be addressed.

5.5. Gene candidates and models for primary and secondary microcephaly

A significant amount of study has been done to address the genetic and molecular basis of embryonic brain development and cortical mal-formations (Cox et al., 2006). As a result, a whole array of genes have been identified to be associated with primary microcephaly and pre-natal brain development. These include: microcephalin (MCPH1), cyclin-dependent kinase 5 regulatory associated protein 2 (CDK5RAP2), centromere-associated protein J (CENPJ) and abnormal spindle protein-like microcephaly-associated (ASPM), lissencephaly 1 (LIS1) and doublecortin (DCX) (Cox et al., 2006; Woods,
Consistent with established mechanisms of prenatal brain growth, these genes all code for proteins that regulate mitotic cell division, migration and/or cell death. In other words, prenatal brain growth is determined mostly by the number of neurons.

In contrast, genetic and molecular basis underlying postnatal brain growth and secondary microcephaly remains enigmatic. Several metabolic diseases are linked with secondary microcephaly. For instance, the mutation of SLC25A19 gene, a mitochondrial deoxynucleotide carrier, causes abnormal brain growth. The growth deficit is a result of an inability of mitochondria to synthesize their own DNA, which leads to crucial energy deficiency (Rosenberg et al., 2002). However, the defects are not specific to the brain, and the reduced brain growth is the result of depriving the neurons of sufficiency energy for growth (i.e. secondary to metabolic effects).

The Rett syndrome protein Methyl CpG Binding Protein 2 (MECP2) is the best-studied molecule related to secondary microcephaly. MECP2 is a transcriptional repressor that binds to methyl CpG to silence gene expression. However, exactly what target gene MECP2 regulates to achieve postnatal brain growth and through what cellular mechanism remain elusive. Furthermore, the effect of MECP2 on brain growth appears to be mild and non-specific to the brain in the knockout mice model (Luikenhuis et al., 2004).
5.6. PAK1&3 DK mice as a robust model of secondary microcephaly and XLMR

Human microcephaly is often co-morbid with mental retardation or other psychiatric and neurological disorders. However, the relationship between brain size and cognition in mice has been difficult to interpret because reduced volume is often confounded by cortical malformation. For example, genetic inactivation of a number of proteins involved in neuronal progenitor cell proliferation and/or migration resulted in gross defects in embryonic brain development. Mutations of proteins involved in mitotic spindle formation such as human ASPM led to primary microcephaly through its disruptive effect on the mitotic efficiency of neuroblasts (Ponting and Jackson, 2005). However, in many cases the animal models are either lethal or show severely compromised viability, making it impossible to assess the effects of reduced brain size on synaptic and behavioral properties in these animals. Very few animal models of microcephaly display clearly reduced brain size with normal neuronal organization and number. The only mutant mice I am aware of that display a selective defect in postnatal brain growth is the genetically altered mice lacking the expression of the Rett syndrome gene MECP2 (Shahbazian and Zoghbi, 2002).

The MECP2 mutant mice appears to be normal at birth in both brain size and structure, but by approximately 6-8 weeks of age display arrested brain growth and neurological symptoms, including uncoordinated gait, reduced spontaneous movement, gain of body weight and irregular breath, which result in death at approximately 50-60 days. Although these mice are valuable tools for studying the role of MECP2 and the pathogenic mechanisms underlying Rett Syndrome due to their phenotypic similarity to
the human patients, they are not the ideal model for studying the postnatal brain development and its relationship to cognition for a number of reasons. First, the brain size reduction in mature MECP2 mutant mice is rather mild (by about 10%) (Luikenhuis et al., 2004), which presents considerable challenge for analyzing the underlying mechanisms for the deficit. Second, growth retardation is not specific to the brain as MECP2 mutant mice have a 40% reduction in body weight by 8 weeks of age. Thus, the deficit most likely represents a general growth retardation instead of a brain-specific one. Thirdly, neurological symptoms (including microcephaly) do not initiate until later on in the development, raising the issue whether the deficit is due to impaired growth or problems with maintenance. Lastly, MECP2 knockout mice develop severe neurological symptoms that lead to pre-mature death, rendering the analysis of reduced brain size and behavior in mature animals unfeasible.

In contrast, PAK1&3 DK mice display a drastic yet specific postnatal impairment in brain size, with body size and weight been unaffected. Second, brain size deficit is manifested shorted after birth (as earl as the first week), within the developmental period that coincides with rapid postnatal brain enlargement. Third, these mice have normal life span and viability, with no symptoms of neurological or metabolic abnormalities (epilepsy or motor deficts), enabling the analysis of brain and behavior throughout adulthood. Finally, the brain structure and cellular organization are perfectly preserved in the DK mice. All these factors make PAK1&3 DK mice an unique and robust model for studying postnatal brain enlargement driven by neuronal maturation, and its relation to cognitive behaviors.
5.7. Cofilin-mediated actin regulation in neuronal and brain development

Evidence from my study suggests that PAK1&3-dependent regulation of postnatal brain growth is through ADF/cofilin-mediated regulation of actin, which stabilizes and promotes formation of new dendritic and axonal branches. It has been shown that during development, both axons and dendrites constantly extend and retract their filopodia containing F-actin. F-actin assembles and dissambles in response to external signals, leading to filopodia movements. In dendrites, only selected number of filopodia are stabilized into new dendritic shafts and become novel dendritic arbors. Similar process later in development enables filopodia to develop into dendritic spines (Luo, 2002). Axonal branching also depends heavily on actin, as treatment with latrunculin and cytochalasin result in severe reduction of axonal branching (Gallo and Letourneau, 1998).

Of the many substrates downstream of PAK signaling that I have assayed, only a specific and drastic reduction in basal cofilin phosphorylation was detected. This change translates into enhanced cofilin activity, which should facilitate F-actin disassembly. In accordance, reduction in F-actin levels was observed in the DK brain and DK primary neuron culture. At the dendritic level, the overall level of F-actin and its enrichment in dendritic protrusions (filopodia and spines) were both significantly attenuated in both developing and mature neurons lacking PAK1&3. Moreover, abnormal F-actin clusters were detected on the main dendritic shafts of these neurons, providing further evidence of misregulated actin dynamics in these mice. It is interesting to note that the total protein levels of both signaling and synaptic proteins remained unchanged, indicating that loss of PAK1&3 had negligible effect on the expression of these proteins. These findings are of
particular importance because they provide the first tangible molecular mechanism responsible for postnatal brain enlargement and consequent cognitive development.

Fig 32 summarizes the findings made in this study regarding the roles of PAK1 and PAK3 in mammalian neural development.
Fig 32. Proposed model of PAK1&3 function in mammalian brain development.

During postnatal brain development, external cues (activity, neurotrophin) lead to PAK1&3 activation through Rho GTPases. PAK1&3 regulates the form of actin dynamics in neurites specifically required for filopodia stabilization, which results in formation of new dendritic arbor / axonal branches in young neurons and spines in mature neurons. Degree of neurite branching directly contributes to synapse number and neural network complexity. Actin dynamics, neuronal complexity and spine morphology can all influence synapse morphology. Morphology and the number of synapse, in turn, determine the properties of synaptic transmission and synaptic plasticity, both of which are critical for normal cognitive function. Furthermore, this study has demonstrated that degree of neurite branching directly contributes to postnatal brain enlargement, defect of which results in secondary microcephaly.
5.8. Bridging the gap between Rho GTPases and regulation of neurite branching

PAKs are key kinase substrates of Rho family small GTPases, Cdc42 and Rac. Significantly, both Cdc42 and Rac have been established to play a wide variety of roles in neuronal morphogenesis, including axonal and dendritic growth and branching (Luo, 2000). External signals converge on these GTPases, and they in turn regulate a diverse range of downstream pathways by activating distinct effectors. Of the immediate downstream effectors of Rho GTPases (including PAKs, Rho kinases, WASP, WAVE, Toca-1, IRSp53), to date none has been linked with neurite branching in vivo. This study is the first to establish that, downstream of Rho GTPases, PAKs in particular serve as critical mediators of neurite branching in vivo. Loss of PAK1&3 function led to specific deficit in neurite branching at the cellular level, with profound functional consequences at anatomical, synaptic and behavioral levels.
5.9. A comparison of PAK1&3 DK, PAK1 KO, PAK3 KO and LIMK1 KO mice.

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Table 3. A summary of phenotype comparison between PAK1&3 DK and related KO mice.
6.1. Distinguishing acute and developmental roles of PAK1&3 through adenovirus mediated PAK3 transfection in culture.

One future aim will be to rescue the deficits in PAK1&3 DK mice and neurons lacking PAK1&3 expression. I intend to achieve this through adenovirus-mediated transfection of PAK3. Using this approach, I can address a number of questions. First, I can address whether PAK3 is directly responsible for regulating the dendritic and axonal morphology. I hypothesize that returning PAK3 expression to cultured hippocampal neurons lacking PAKs 1&3 at div 3 will at least partially rescue axonal branching deficits and, if returned at div 7, will rescue dendritic arborization deficits. If so, then it can be concluded that these deficits observed in DK mice represent a direct function of PAKs, rather than some secondary effect induced by the loss of PAK1&3 function. Second, I can address the developmental critical periods for PAK function. More specifically, will re-expression of PAK in the mature mutant neurons promote morphogenesis, or is this rescue only possible if done at an earlier stage of the development? The answer to this question will have significant consequences at the clinical level. If neuronal complexity can be rescue in mature animal, for instance, this would suggest that there exist an extended window of time to treat human patients with PAK3-associated XLMR or secondary microcephaly. In this case, molecules in PAK1&3 signaling pathway would be potential therapeutic targets. Finally, PAK3 adenovirus can be injected stereotactically into mature DK mice to address whether PAKs affects synaptic function acutely or
through its regulation of neuromorphogenesis. Likewise, this question will have important implications at the clinical level if acute expression of PAK, without rescuing microcephaly, can rescue behavior and cognition.

6.2. *In vivo* rescue of structure and behavior using an inducible transgenic system.

While adenovirus carrying PAK3 gene is relatively easy to construct compare to transgenic mice, its use is most likely limited to *in vitro* experiments. This is due to the toxic effect of adenovirus and its low transfection efficiency (at most 10% at the optimal viral titre that maximizes transfection and minimizes toxicity). Furthermore, with stereotactic injection the gene expression is limited to local regions, for instance the CA1 area, where the virus is injected. In contrast, the anatomical and neuronal structural deficits in the DK mice were present throughout the whole brain; MRI revealed that all structures are reduced in size (see section 5.1). Furthermore, stereotactic injection of virus is limited to mature animals, whereas the developmental defects in PAK1&3 DK mice initiated immediately after birth. Therefore, returning the knockout gene through transfection may rescue synaptic function locally (e.g. CA3-CA1 synaptic plasticity), but it is unlikely to rescue *in vivo* neuroanatomical deficits and behavior.

In order to rescue *in vivo* structure and behavior, one strategy is to generate an inducible transgenic mice line (using tetracycline transactivator system coupled to CaMKIIα promoter) that expresses a dominant negative PAK3 construct. This line can be crossed with PAK1 KO line to produce PAK1 KO mice whose PAK3 function can be inhibited once the animal is mature, by inducible expression of the dominant negative construct. In essence, the inducible transgenic mice would be allowed to mature with
PAK3 function intact, and thus would develop normal neuroanatomy, neuronal morphology and behavior. If shutting off both PAK1 and PAK3 function in adulthood leads to synaptic and behavioral deficits, then it can concluded that it was the acute roles of PAKs that were responsible for the functional deficits in DK mice. Even then however, developmental roles of PAKs may still be critical in addition to its acute roles. On the other hand, if inactivating PAKs in adulthood has no functional consequence, then it can be concluded that it was solely the developmental roles of PAKs that is required for normal functions at the synaptic and behavioral level. In either case, this approach will enable me to distinguish the acute roles of PAK1&3 from the developmental roles in vivo in a mammalian system.

6.3. Activity dependence of neurite branching regulation by PAKs

It has been well established that neuronal activity promotes neuromorphogenesis (Luo, 2000). However, even in the absense of (or with minimal) activity such as in the condition of low density culture, neurons still extend neurites that branch to form dendritic arbors and axon collaterals. It would be interesting to determine whether the role of PAK in neurite branching is an instrinsic property of this gene or if it is activity-dependent. This question is difficult to address in vivo, but can be addressed using various chemical agents that depolarize / excite the neurons artificially in vitro. Two such agents are glutamate and potassium chloride (KCl). Addition of 25 µM glutamate in the initial plating step of primary culture preparation from rats has been shown to increase the number of primary dendrites during the first 4 div, presumably through increased stimulation of neurons (Brewer, 1997). However, when maintaining primary culture for
longer than four days, the excess glutamate induces excitatory neurotoxicity, leading to compromised neuronal viability. Increasing the $K^+$ in the culture medium raises its extracellular concentration. The resulting disruption of ionic balance depolarizes the resting membrane potential, bringing it closer to (or above, depending on extracellular $K^+$ concentration) the threshold for action potential firing. In 7 days old rat primary neuron culture, 10 µM of KCl has been shown to increase dendritic arborization (Yu and Malenka, 2003). Both excess glutamate and high KCl in the culture medium can excite neurons and promote dendritic growth and arborization. I hypothesize that PAKs 1&3 are key mediators of activity-dependent dendritic arborization. Based on this hypothesis, I predict that neurons from WT mice will exhibit enhanced branching in response to glutamate or KCl treatment, but not neurons from PAK1&3 DK mice. I further predict that morphological response to glutamate or KCl can be rescued in PAK1&3 DK neurons through adenovirus-mediated PAK3 expression.

Although neuronal activity promotes growth, it can also simultaneously inhibit growth or lead to apoptosis through neurotoxicity. All experiments involving the effect of KCl on dendritic morphogenesis were previously done in rats, whose neurons grow more robustly in primary culture (i.e. less susceptible to excitotoxicity). Optimal level and duration of treatment with excitatory agents needs to be carefully determined in order to minimize their toxic effects.
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